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The role of regulatory genes *nifA*, *vnfA*, *anfA*, *nfrX*, *ntrC*, and *rpoN* in expression of genes encoding the three nitrogenases of *Azotobacter vinelandii*

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Abstract Several regulatory gene mutants of *Azotobacter vinelandii* were tested for ability to synthesize functional nitrogenase-1 (Nif phenotype), nitrogenase-2 (Vnf), or nitrogenase-3 (Anf). While *nifA* mutants were Nif$^-$, Vnf$^+$, and Anf$^{+/-}$, and *ntrC* mutants were Nif$^+$, Vnf$^+$, and Anf$^+$, *nifA ntrC* double mutants were Nif$^-$, Vnf$^-$, and Anf$^-$. A *vnfA* mutant was Nif$^+$, Vnf$^{+\backslash-}$, and Anf$^{+\backslash-}$, and an *anfA* strain was Nif$^+$, Vnf$^+$, and Anf$^-$. *lacZ* fusions in the *nifH*, *vnfH*, *vnfD*, *anfH*, and *nifM* genes of *Azotobacter vinelandii* were constructed and introduced into wild-type and regulatory mutants of *A. vinelandii*. Expression of these operons correlated with the growth phenotype of the regulatory mutants. Apparently either NifA or NtrC can activate expression of *nifM*. Also, expression of the *anf* operon required the NifA transcriptional activator, although there are no NifA binding sites at appropriate locations upstream of *anfH* (or *anfA*). The results confirm previous reports that VnfA and AnfA are required for expression of *vnf* and *anf* genes, respectively, and that VnfA is involved in repression of the *nifHDK* operon in the absence of molybdenum and of the *anfHDGK* operon in the presence of vanadium.

Key words Nitrogen fixation · Vanadium · *anf* *Azotobacter* · *nif* · Sigma(σ)^{54} · Sigma(σ)^N Molybdenum · *vnf* · *ntrC* · *rpoN*

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Abbreviations *ORF* Open reading frame · *NIL* Metal-extracted Burks medium containing $Fe(SO_4)$ *NIL+Mo* NIL containing Na_2MoO_4 · *NIL+V* NIL containing V_2O_5 · *UAS* Upstream activator-binding site

Introduction

Azotobacter vinelandii has the potential to synthesize three different nitrogenase enzymes (for review, see Bishop and Premakumar 1992). The first nitrogenase is the conventional molybdenum- and iron-containing enzyme corresponding to the nitrogenase found in all other diazotrophs so far characterized (the Nif system; Mo nitrogenase or nitrogenase-1). The second is a vanadium- and iron-containing nitrogenase also present in the related organisms *Azotobacter chroococcum*, and *Azotobacter paspali* (the Vnf system; V nitrogenase or nitrogenase-2; Robson et al. 1986; Fallik et al. 1993). The third nitrogenase apparently contains only iron (the Anf system; Fe nitrogenase or nitrogenase-3). A nitrogenase similar to the *A. vinelandii* nitrogenase-3 was recently characterized in *Rhodobacter capsulatus* (Schneider et al. 1991). Hybridization experiments suggest that *A. paspali* also contains genes for nitrogenase-3, while *A. chroococcum* does not (Fallik et al. 1991). Under normal laboratory conditions at 30° C, the composition of the growth medium determines which nitrogenase is present in *A. vinelandii*: Mo nitrogenase is synthesized when molybdenum is present, V nitrogenase is made in cultures with vanadium, and Fe nitrogenase in the absence of both metals (Bishop and Joerger 1990).

The structural proteins of the three nitrogenases are encoded by three different sets of unlinked genes arranged in the four operons *nifHDK*, *vnfHFd-vnfDGK*, and *anfHDGK* (Jacobson et al. 1989; Joerger et al. 1989b, 1990; Fig. 1). The *H* genes encode the subunits of the homodimeric Component 2 protein (also named Fe protein or dinitrogenase reductase), which is similar in structure and function in all three enzymes. The two subunits α and β, of the tetrameric Component 1 proteins (also named

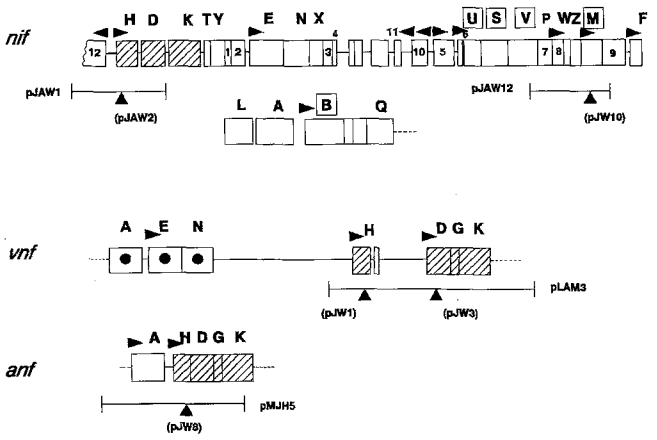

Fig. 1 The nitrogen fixation genes of *Azotobacter vinelandii*. The map is based on the published sequences of the regions shown (Bennett et al. 1988; Joerger and Bishop 1988; Jacobson et al. 1989; Joerger et al. 1989b,1990). *Cross-hatched HD(G)K regions* indicate the encoded structural subunits of the three nitrogenases. *Numbers* in the major *nif* cluster refer to ORFs apparent from the sequence. *Filled arrows* mark the locations of potential σ^{54} recognition sites deduced from the sequence. The *nif* genes appearing in *boxes* are those required for activity of all three nitrogenase systems (Kennedy et al. 1986; Joerger and Bishop 1988; Kennedy and Dean 1992). The *vnfA* and *vnfEN* genes, marked with *filled circles*, are required for synthesis or function of components of nitrogenase-2 and nitrogenase-3. *Lines* under the genes indicate the region cloned for construction of the *lacZ* fusions as described in Materials and methods. *Filled triangles* indicate the sites of fusions and plasmids carrying the fusions are named in parentheses below the map

dinitrogenase) of all three enzymes, are encoded by the *D* and *K* genes, respectively. Nitrogenases-2 and -3 also contain a third subunit, γ, encoded by *vnfG* and *anfG*. Northern blot analysis of transcripts from these genes or measurement of β-galactosidase activity in strains carrying *lacZ* transcriptional fusions has shown that molybdenum prevents transcription of the *vnfH, vnfDGK*, and *anfHDGK* operons, but is required for expression of the *nifHDK* operon; in cultures containing vanadium, transcription of *anfHDGK* is repressed (Luque and Pau 1991; Walmsley and Kennedy 1991; Premakumar et al. 1992). Molybdenum repression of *vnf* and *anf* genes and vanadium repression of *anf* genes has been observed in cultures grown at 30° C, but not in cultures grown at lower temperatures (14–20° C; Walmsley and Kennedy 1991).

The *nifHDK* operon is located in a major cluster of *nif* genes that also includes *nifTYENXUSVWZMF* and several other open reading frames (ORFs) (Jacobson et al. 1989), one of which is now known to be *nifP*, lying between *nifV* and *nifW* (Evans et al. 1991). Among these, the products of the *nifU, nifS, nifV*, and *nifM* genes are required for the function of all three nitrogenases since mutations in any one of these genes result in a Nif⁻, Vnf⁻, and Anf⁻ phenotype (Kennedy et al. 1986; Kennedy and Dean 1992). Another cluster of genes, *nifLABQ*, is unlinked to the former genes (Bennett et al. 1988; Joerger and Bishop 1988; Bali et al. 1992;). Here, only the *nifB* gene product, involved in biosynthesis of nitrogenase cofactors, is required for activity of all three enzymes (Joerger and Bishop 1988). Homologs to *nifEN*, designated *vnfEN*, were found adjacent to *vnfA*, which encodes a transcriptional activator of the *vnf* operons (Wolfinger and Bishop 1991). The *vnfEN* gene products are required for formation of cofactor associated with vanadium nitrogenase, designated VFeco, and also possibly in formation of an uncharacterized cofactor presumably associated with Component I of nitrogenase-3 (Wolfinger and Bishop 1991). Thus, there are genes necessary for activity of one, two, or all three nitrogenases in *A. vinelandii*.

Six *A. vinelandii* genes with demonstrated or potential involvement in the regulation of nitrogenase gene expression have been identified by complementation and mutant analysis and/or by gene sequencing: *rpoN (ntrA), nifA, nifL, nfrX, vnfA*, and *anfA*. The *rpoN* gene encodes the RNA polymerase factor, σ^{54}, which recognizes genes with characteristic promoter sequences located at –12 (invariant GC residues) and at –24 (nearly invariant GG) bp upstream from the transcription start site (for review, see Merrick 1992). *rpoN* mutants of *A. vinelandii* are Nif⁻, Vnf⁻, and Anf⁻ (Toukdarian and Kennedy 1986; Kennedy and Dean 1992), as expected from the presence of "–12, –24" promoter sequences upstream of all *nif, vnf*, and *anf* genes. Also as expected for σ^{54}-dependent promoters, genes encoding transcriptional activators of these promoters have been identified.

A *nifA* gene product is required for expression of other *nif* genes containing a characteristic upstream activator-binding site (UAS), lying at –80 to –180 bp, with the sequence TGT-N10-ACA. Typical UAS sequences are present at distances appropriate for functionality upstream of the *nifH, nifEN, nifU, nifW*, and *nifF* genes in *A. vinelandii* (Jacobson et al. 1989). As expected, *nifA* mutants are Nif⁻ and fail to express *nifH-lacZ* fusions (Bennett et al. 1988; Santero et al. 1988). A *nifL* gene is located upstream of *nifA* as in *K. pneumoniae* and mediates ammonium and oxygen repression of the *nifHDK* operon (Bali et al. 1992; Blanco et al. 1993). Another regulatory gene, *nfrX*, first identified in Tn*5* regulatory Nif⁻ mutants (Santero et al. 1988), is structurally and functionally homologous to *glnD* in enteric bacteria (Contreras et al. 1991). Mutant analysis suggested that the function of NfrX is to convert, either directly or indirectly, the "active" form of NifL to the "inactive" form in low fixed N. In *nfrX* mutants, NifL is always "active" and therefore inhibits NifA function as a transcriptional activator under all conditions. Thus, in *A. vinelandii*, NifA-dependent promoters also require NfrX for expression.

Two other *nifA*-like genes were identified: *vnfA* and *anfA* are associated with synthesis of nitrogenase-2 and nitrogenase-3, respectively (Joerger et al. 1989a). A consensus sequence, GTAC-N7-GTAC, which is required for VnfA recognition, lying upstream of all *vnf* promoters in *A. vinelandii*, was recently identified (unpublished results). Sequences recognized by AnfA have not been reported. The *ntrC* gene of *A. vinelandii* had been isolated by its ability to complement *ntrC* mutants of both *Escherichia coli* and *Klebsiella pneumoniae* (Toukdarian

Table 1 Plasmids

Plasmids	Vector and insert DNA	Reference or source
pUC4:KIXX	pUC4 with Km^r cartridge (*aph*) derived from Tn*5*	Pharmacia
pLAM3	pTZ19 with *Bam*HI fragment of *vnfHFd vnfDGK*	Pau et al. 1989
pJAW1	pACYC184 with 5.3 kb *Xho*I/*Hin*dIII fragment from pDB10 containing *nifH* and most of *nifD*	Walmsley and Kennedy 1991
pJAW2	pJAW1 with *Bam*HI-cut *lacZ*-KSS cloned in *Bgl*II site in *nifH*	Walmsley and Kennedy 1991
pJAW12	pTZ18 with 3.9 kb *Sal*I fragment containing *nifM*	This work
pJW1	vnfH7::Tn*5-B21* in pLAM3	Walmsley and Kennedy 1991
pJW3	vnfD1::Tn*5-B21* in pLAM3	This work
pJW10	nifM1::Tn*5-B21* in pJAW12	This work
Phage		
λ::Tn*5-B21*	λb221 c1857 Pam80::Tn*5-B21*	Simon et al. 1989

and Kennedy 1986). Unlike *ntrC* mutants of *K. pneumoniae*, which are Nif$^-$ because NtrC protein is not available for activating expression of the *nifLA* operon, *A. vinelandii ntrC* mutants are Nif$^+$. Expression of *nifLA* in *A. vinelandii* requires neither σ^{54} nor NtrC (Blanco et al. 1993; Raina et al. 1993).

This work presents results that define the Nif, Vnf, and Anf phenotype of several regulatory mutants of *A. vinelandii* and indicate roles for the various regulatory gene products in the expression of the operons controlled by promoters adjacent to the *nifH*, *vnfH*, *vnfD*, *anfH*, and *nifM* genes. Fusions to the *Escherichia coli lacZ* gene encoding β-galactosidase were constructed in these operons and introduced into *A. vinelandii* strains with mutations in the several regulatory genes described above.

Materials and methods

Strains, plasmids, and bacterial growth

The plasmids and bacteriophage used in this work and their origins are listed in Table 1. The *Azotobacter vinelandii* strains are listed in Tables 2–7; a description and origin of the individual alleles is available on request. The media, general growth conditions, and transformation procedure for *A. vinelandii* were as described previously (Toukdarian and Kennedy 1986; Walmsley and Kennedy 1991; Bali et al. 1992).

A. vinelandii strains were grown in metal-extracted Burks medium (Eady and Robson 1984) containing 30 μM puratronic grade $Fe(SO_4)$ (NIL medium). NIL + Mo medium also contained 1 μM Na_2MoO_4 (ultrapure grade), while NIL + V also contained 100 nM ultrapure V_2O_5. When appropriate, aristar grade urea was supplied as a non-repressive N source (at 2 or 10 mM) or ammonium acetate (15 mM) in repression experiments.

Construction of *lacZ* fusions

The fusions of *nifH* with a *lacZ* cassette, and of *vnfH* and *anfH* with *lacZ* carried on Tn*5-B21* have been described previously (Walmsley and Kennedy 1991). Tn*5-B21* was inserted into the *vnfD* and *nifM* genes carried on plasmids pLAM3 and pJAW12, respectively, in *E. coli* strain JC5466 by infection with λ::Tn*5-B21* as described by Walmsley and Kennedy (1991) and originally by Simon et al. (1989). Insertions into the two genes in the correct orientation for expression were identified by restriction mapping of the Tn*5-B21*-carrying plasmids. The resulting plasmids pJW3 (*vnfD*::Tn*5-B21*) and pJW10 (*nifM*::Tn*5-B21*) (Fig. 1) were transformed into *A. vinelandii* wild-type and various mutant background strains. Transformants in which the Tn*5-B21*-carrying DNA had replaced the wild-type chromosomal regions were isolated as described previously (Walmsley and Kennedy 1991). Gene replacement was verified by Southern hybridization analysis of DNA from each transformant (data not shown).

Construction of strains with multiple markers by transformation

It was possible to combine different regulatory mutations in a single strain along with a reporter gene fusion by strategic successive transformation of alleles into the appropriate starting strain. Thus, for example, strain MV166 (*nifA*$^-$ *ntrC*$^-$ *vnfH-lacZ*) was constructed by transforming strain UW1 (carrying a *nifA* point mutation) with genomic DNA prepared from MV511 (*ntrC*::Tn*5*). Kanamycin-resistant transformants were further transformed with pJW1 DNA or with DNA prepared from MV107 (or some other strain carrying the *vnfH-lacZ* mutation), followed by selection for tetracycline resistance. Retention of the original *nifA* point mutation was verified by finding the strain to be Nif$^-$; a strain which is *ntrC*$^-$ *vnfH*$^-$, is Nif$^+$.

Assay of β-galactosidase activity

Cultures were grown at 30° C for 14–17 h (OD_{600} nm = 1 – 1.5) in NIL, NIL+Mo, or NIL+V, as appropriate. Cells were centrifuged and resuspended in metal-extracted N-free medium with Fe and with or without Mo or V salts. After 6 h further incubation, the cultures were assayed for β-galactosidase activity according to Miller (1972).

Results

Nif, Vnf, and Anf phenotype of regulatory mutants

Previous publications have compared the growth phenotypes, either on plates or in liquid medium, of only one or two strains with mutations in one of the several regulatory genes so far identified in *A. vinelandii*. These genes include *rpoN*, *nifA*, *vnfA*, *anfA*, *nfrX*, and *ntrC*. In order to compare effects of all these mutations directly, both singly and in certain combinations, on the Nif, Vnf, and Anf phenotypes, growth experiments were performed with all strains using the same standardized conditions on agar plates with metal-extracted medium containing molybde-

Table 2 Growth of *Azotobacter vinelandii* regulatory mutants. Doubling times are indicated in parentheses, where appropriate

Strain	Relevant genotype	Metal supplementation of NIL medium		
		Mo	V	None
UW136	*rif*1 (Nif$^+$)	+ (2.5 h)	+ (9 h)	+ (12 h)
MV700	*rpoN*1::Tn*5*	– (> 100 h)	–	–
UW1	*nifA*1	–	+	+/– (18 h)
MV3	*nifA*3::Tn*5*	–	+	+/–
MV200	*nifA*4:KIXX	–	+	+/–
MV17	*nfrX*16::Tn*5*	–	+	+/–
MV451	*vnfA*3:KIXX	+	+/–	+/–
CA66	*anfA*66:KIXX	+	+	–
MV511	*ntrC*51::Tn*5*	+	+	+
MV520	*ntrC*11:KIXX	+	+	+
MV416	*nifA*1 *ntrC*51::Tn*5*	–	–	–
MV521	*nifA*1 *ntrC*11:KIXX	–	–	–
AS32	*ntrC*32 *nifD*	–	+	+
MV524	Δ*nifHDK*11 Δ*vnfDKG*114:Ω *ntrC*11:KIXX	–	–	+
MV523	Δ*nifHDK*11 Δ*anfDGK*70:KISS *ntrC*11:KIXX	–	+	–

num (NIL + Mo), vanadium (NIL + V), or with neither metal added (NIL). Extent of growth on these three media indicates, respectively, the Nif, Vnf, or Anf phenotype (Table 2). The visual assessment of growth of mutant strains in both liquid and on agar correlated well with growth rates (doubling times) in liquid medium (included where appropriate in Table 2; see also Kennedy and Dean 1992). The results show that, as expected, an *rpoN* mutant was completely Nif$^-$, Vnf$^-$, and Anf$^-$. The *nifA* and *nfrX* mutants were Nif$^-$, Vnf$^+$, and Anf$^{+/-}$ (grew much slower on NIL than the wild-type). The *anfA* mutant was, as expected, Nif$^+$, Vnf$^+$, and Anf$^-$. The *vnfA* mutant was Nif$^+$, Vnf$^{+/-}$, and Anf$^{+/-}$; its growth on NIL + V and NIL medium was probably due to low level activity of nitrogenase-1 in the *vnfA* mutant (see Discussion). Although *ntrC* mutants were Nif$^+$, Vnf$^+$, and Anf$^+$, two independently constructed *nifA ntrC* double mutants were completely Nif$^-$, Vnf$^-$, and Anf$^-$, suggesting that NtrC might after all have some function in regulating expression of nitrogen fixation genes.

In order to help elucidate the roles of the regulatory products in controlling expression of *nif*, *vnf*, and *anf* genes, and hence to explain the Nif, Vnf, and Anf phenotypes observed in the various regulatory mutants, transcriptional *lacZ* fusions were constructed. The *nifH*, *vnfD*, *vnfH*, *anfH*, and *nifM* genes carried on plasmids were mutagenized by insertion of a *lacZ* gene cartridge (from pGS100) or by the transposon Tn*5-B21* (*lacZ* Tc) (see Materials and methods and Table 1). These reporter fusions were transformed into the various *A. vinelandii* regulatory mutants and β-galactosidase activity was measured in the resulting strains grown in metal-extracted medium containing molybdenum (NIL + Mo) or vanadium (NIL + V) or neither metal (NIL). It was not possible to determine whether genes in any of the *nif*, *vnf*, or *anf* operons that carried *lacZ* fusions were involved in autoregulation of their respective operons. Thus, for example, if the *nifH* gene product were necessary for full expression of the *nifHDK* operon, such an effect would not be apparent using strain MV101 (*nifH:lacZ*/KSS). In merodiploid strains of *A. vinelandii* that carry a recombinant wide host range plasmid with cloned *A. vinelandii* genomic DNA, the plasmid apparently recombined with the chromosome after sub-culturing such strains on selective medium with antibiotic (unpublished results). Therefore, the construction of stable *lacZ* fusion strains with the wild-type gene in *trans*, or of stable wild-type strains with the *lacZ* fusion gene carried in *trans* on wide host range plasmids, could not be achieved.

Expression of *nifH-lacZ*

As expected, expression of *nifH* absolutely required the *rpoN*, *nifA*, and *nfrX* gene products (Table 3). Furthermore, expression did not occur in NIL or NIL + V medium except in the *vnfA* mutant. This is consistent with previous results showing that nitrogenase-1 subunits are synthesized in the absence of Mo only in a *vnfA* mutant strain (Joerger et al. 1989a). Unexpectedly, although the

Table 3 Expression of *nifH:lacZ*-KSS fusion in wild-type and regulatory mutants of *Azotobacter vinelandii*. Data are the mean percentages from between four and twelve independent assays. Standard error was never more than 10% of the mean value. 100% was set for the β-galactosidase activity obtained for MV101 grown with molybdenum, 27452 Miller units min^{-1} OD_{560}^{-1}; (*nt* not tested)

Strain	Background	Percentage of β-galactosidase activity in cultures grown in				
		NIL	+V	+Mo	+(Mo+V)	+(Mo,NH_4^+)
MV101	Wild-type	6.4	4.0	100	104	2.9
MV100	Wild-type (wrong orientation for expression)	1.0	0.7	0.8	0.8	0.7
MV104	*rpoN*1::Tn*5*	0.7	0.6	0.7	0.7	nt
MV165	*nifA*3::Tn*5*	2.1	2.7	3.7	2.5	2.8
MV106	*nfrX*16::Tn*5*	0.7	0.9	1.6	1.5	nt
MV192	*vnfA*3:KIXX	71	64	30	27	2.3
MV187	*anfA*1::Tn*5*	7.9	1.7	83	86	1.0
MV103	*ntrC*51::Tn*5*	4.4	3.1	117	110	1.0

vnfA⁻ strain MV192 grew as vigorously as wild-type on NIL + Mo, expression of the *nifH-lacZ* fusion was less in the *vnfA* mutant grown with molybdenum than in the wild-type strain or in the *anfA* or *ntrC* mutants. The reason for this is unknown. Although expression of the *anfHDGK* operon requires a functional *vnfH* (or *nifH*) gene product (Joerger et al. 1991), there is no evidence that this is also true for *nifH* expression. Nevertheless, it is possible that VnfA itself or some VnfA-activated gene product exerts some degree of positive regulation at the *nifH* promoter. Transcription from the *nifH* promoter was repressed by ammonium in all mutant backgrounds [except in a *nifL* mutant, see Bali et al. (1992)].

Expression of *vnfH-lacZ* and *vnfD-lacZ*

Transcription from both the *vnfH* and *vnfD* promoters required *rpoN* and *vnfA* gene products and was repressed by molybdenum regardless of whether vanadium was also present (Tables 4 and 5). Expression was not completely repressed by ammonium: levels of β-galactosidase were consistently 25–50% of that in the absence of ammonium, similar to results reported by Jacobitz and Bishop (1992). Full expression of *vnfD* required vanadium and was about half as much in the absence of vanadium (and molybdenum), whereas *vnfH-lacZ* expression was about the same in the presence or absence of vanadium. In contrast to *nifH* and as expected, the *nfrX* gene product was not required for *vnfH* or *vnfD* transcription. Expression of both *vnfH* and *vnfD* was similar to wild-type levels in the *nifA* and *ntrC* single mutant strains and also in the *nifA ntrC* double mutant.

Expression of *anfH-lacZ*

Transcription from the *anfH* promoter in the wild-type background was repressed by either molybdenum or vanadium and also by ammonium. Expression required the *rpoN* and *nfrX* gene products under all conditions (Table 6). As expected, expression was greatly decreased

Table 4 Expression of *vnfH* fusion in wild-type and regulatory mutants of *Azotobacter vinelandii*. Data are the mean percentages from between four and twelve independent assays. Standard error was never more than 10% of the mean value. 100% was set for the β-galactosidase activity obtained for MV107 grown with vanadium, 9197 Miller units min^{-1} OD_{560}^{-1}; (*nt* not tested)

Strain	Background	Percentage of β-galactosidase activity in cultures grown in				
		NIL	+V	+Mo	+(Mo+V)	+(V,NH_4^+)
MV107	Wild-type	101	100	2.7	4.6	21
MV133	*rpoN*1::Tn*5*	0.4	0.5	0.2	0.3	0.4
MV190	*nifA*3::Tn*5*	118	93	0.2	2.5	nt
MV159	*nfrX*16::Tn*5*	83	131	0.9	0.8	10
MV178	*vnfA*3:KIXX	0.3	0.3	0.05	0.08	nt
MV188	*anfA*1::Tn*5*	96	103	1.3	1.3	nt
MV117	*ntrC*51::Tn*5*	76	111	1.5	nt	50
MV166	*nifA*1 *ntrC*::Tn*5*	111	113	4.0	4.2	nt

Table 5 Expression of *vnfD-lacZ* fusion in wild-type and regulatory mutants of *Azotobacter vinelandii*. Data are the mean percentages from between four and twelve independent assays. Standard error was never more than 10% of the mean value. 100% was set for the β-galactosidase activity obtained for MV108 grown with vanadium, 11866 Miller units min^{-1} OD_{560}^{-1}; (*nt* not tested)

Strain	Background	Percentage of β-galactosidase activity in cultures grown in				
		NIL	+V	+Mo	+(Mo+V)	+(V,NH_4^+)
MV108	Wild-type	49	100	1.4	1.3	24
MV134	*rpoN*1::Tn*5*	0.2	0.2	0.1	0.1	nt
MV191	*nifA*3::Tn*5*	56	69	1.3	1.4	nt
MV160	*nfrX*16::Tn*5*	23	127	0.3	0.4	44
MV179	*vnfA*3:KIXX	0.3	0.3	0.1	0.2	nt
MV189	*anfA*1::Tn*5*	21	88	0.3	0.3	nt
MV118	*ntrC*51::Tn*5*	39	91	1.1	1.5	46
MV167	*nifA*1 *ntrC*51::Tn*5*	40	88	1.6	1.4	19

Table 6 Expression of *anfH-lacZ* fusions in wild-type and regulatory mutants of *Azotobacter vinelandii*. Data are the mean percentages from between four and twelve independent assays. Standard error was never more than 10% of the mean value. 100% was set for the β-galactosidase activity obtained for MV143 grown in NIL medium, 11294 Miller units min^{-1} OD_{560}^{-1}

Strain	Background	Percentage of β-galactosidase activity in cultures grown in				
		NIL	+V	+Mo	+(Mo+V)	+NH_4^+
MV143	Wild-type	100	1.7	0.6	0.3	0.6
MV152	*rpoN*1::Tn*5*	0.2	< 0.1	< 0.1	< 0.1	0.1
MV172	*nifA*4:KIXX	3.8	0.3	< 0.1	< 0.1	0.8
MV155	*nfrX*16::Tn*5*	0.4	0.3	0.2	0.2	0.2
MV169	*vnfA*3:KIXX	21	39	0.1	0.1	0.8
MV184	*anfA*1::Tn*5*	6.7	4.1	3.5	3.2	5.5
MV149	*ntrC*	127	2.3	0.7	0.9	0.7
MV170	*nifA*1 *ntrC*51::Tn*5*	< 0.1	0.2	< 0.1	< 0.1	< 0.1

Table 7 Expression of *nifM-lacZ* fusions in wild-type and regulatory mutants of *A. vinelandii*. Data are the mean percentages from between four and twelve independent assays. Standard error was never more than 10% of the mean value. 100% was set for the β-galactosidase activity obtained for MV174 grown in NIL medium, 1764 Miller units min^{-1} OD_{560}^{-1}

Strain	Background	Percentage of β-galactosidase activity in cultures grown in				
		NIL	+V	+Mo	+(Mo+V)	+(Mo,NH_4^+)
MV174	Wild-type	100	64	85	103	6.0
MV183	*rpoN*1::Tn*5*	21	20	16	15	7.9
MV180	*nifA*4:KIXX	38	42	64	61	22
MV176	*nfrX*17::Tn*5*	15	14	25	23	4.0
MV177	*vnfA*3:KIXX	118	138	100	102	5.0
MV185	*anfA*1::Tn*5*	128	70	124	106	15
MV181	*ntrC*51::Tn*5*	133	66	176	156	5.6
MV182	*nifA* ntrC51::Tn*5*	15	24	8.8	14	13

in an *anfA* mutant in NIL medium as compared to wild-type, but was much higher than wild-type in NIL + Mo, NIL + V, or NIL + ammonium. Somewhat surprisingly, *anfHDGK* expression in NIL medium was less in a *nifA* mutant than in the *anfA* mutant. Expression of *anfH-lacZ* was reduced to zero in the *nifA ntrC* double mutant (see Discussion). Expression in the *vnfA* mutant was significant, but less than in wild-type in NIL medium, was not repressed by vanadium, but, as in wild-type, was repressed by molybdenum.

Expression of *nifM-lacZ*

Levels of β-galactosidase from the *nifM* fusion were never as high as those in the *nifH-lacZ* fusion strain (about 5–10% as much) nor as low as from the other fusions in the *rpoN* mutant background (Table 7). As might be expected from its requirement in all three nitrogenase systems, expression was similar in NIL medium with or without molybdenum or vanadium. Full expression in all media required the *rpoN* and *nfrX* gene products. Levels of β-galactosidase were reduced to about 50% of wild-type in the *nifA* mutant in molybdenum-deficient medium and was unaffected in the *ntrC*, *vnfA*, and *anfA* mutants in all three media. Significantly, expression was lowest in the *nifA ntrC* double mutant, except in NIL + V, in which *nifM-lacZ* expression was even lower in the *nfrX*::Tn*5* strain (see Discussion).

Discussion

The measurement of β-galactosidase activity from *lacZ* fusions in various nitrogen fixation genes introduced into regulatory mutant backgrounds, coupled with growth experiments, has confirmed certain expected or previously reported results and also revealed some interesting new aspects of nitrogen fixation gene regulation in *A. vinelandii*. The latter include the possibility that NtrC does play a minor role in nitrogen fixation, probably through its ability to activate transcription of *nifM*, and that NifA and NfrX, are significantly involved in expression of the nitrogenase-3 operon, *anfHDGK*. These two observations may be related, as discussed below.

The inability of *nifA ntrC* double mutants to grow on N-free medium under any condition of metal supply may be best explained by their inability to express the *nifM* gene, the product of which is required for activity of all three nitrogenases in *A. vinelandii*. Although expression of the *nifM-lacZ* fusion was diminished in a *nifA* mutant and not affected in the *ntrC* mutant, β-galactosidase activity in the *nifA ntrC nifM-lacZ* strain was as low as in the *rpoN* mutant strain. The most likely explanation for this result is that transcription of the *nifM* gene can be activated by either activator, NifA or NtrC. From β-galactosidase activity measurements in the mutant strains, NifA appears to be the major activator. Nevertheless, in a *nifA* mutant, NtrC may activate expression of sufficient NifM protein to be present for the maturation step required for functional Fe component proteins of the three nitrogenases. Examination of sequences upstream of *nifM* revealed that the nearest σ^{54} "–12, –24" recognition motif begins 54 bp upstream from the translation start and lies within *nifZ*. At 55 and 107 bp upstream of the potential "–12, –24" are sequences similar to those known to be important for NtrC binding and activation at other promoters in *E. coli* and *K. pneumoniae* (see below). The potential NtrC-binding sequences upstream of *nifM* are GCAGCA-N9-TGCTGC and CGCA-N9-TGCG, while the consensus is TGCAC-N7-GTGCA. All three share GCA-N9-TGC. In order to assess the significance of the two potential NtrC binding sites, the entire 27 kb *nif* gene region was searched for sequences identical or similar to the NtrC consensus TGCAC-N7-GTGCA (allowing up to four bases of non-identity and plus or minus one base in the spacer) using the program Find in the Wisconsin Genetics Computer Group DNA analysis software(Devereux et al. 1984). Although about 40 such "hits" occurred, the two that were most like the NtrC consensus binding sequences were those identified just upstream of *nifM*, lying within *nifZ*. No NifA UAS is present in the region just upstream of *nifM*. The next nearest "–12, –24" motif is 1.3 kb further upstream within ORF7 (*nifP*) and is associated with a NifA recognition site 100 bp further uspsteam. Thus, there is a potential for at least two NifM-containing transcripts from σ^{54}-dependent promoters, one activated by NifA and including the ORF8-*nifWZM* genes and another activated by NtrC containing only *nifM*.

Another comparison of significance is between the *nifM* region of *K. pneumoniae* and that described here for

A. vinelandii. Unlike in *A. vinelandii*, which has two ORFs lying between *nifV* and *nifW* (ORFs 7 and 8), the *K. pneumoniae nif* cluster contains only *nifUSVWZMF* (Arnold et al. 1988). The *K. pneumoniae nifM* gene is included in two transcripts starting 10 bp downstream of a typical "–12, –24" σ^{54} recognition site (Beynon et al. 1983), one from the *nifU* promoter activated by NifA and another lying within *nifZ* without an associated NifA binding site. A search of sequences upstream of the "–12, –24" showed neither NifA nor NtrC binding sites. However, about 260 bp further upstream within *nifW* is another RpoN-recognition sequence and an NtrC binding motif about 80 bp upstream (Arnold et al. 1988). Thus, in both organisms, it is possible that *nifM* expression can be activated by either NifA or NtrC.

The inability of *nifA* and *nfrX* mutants to grow well on NIL medium correlates with very low expression of the Fe nitrogenase (nitrogenase-3) structural genes, *anfHDK*, in these mutants. Because activity of the Fe nitrogenase requires the products of the *nifM*, *nifV*, *nifS*, *nifU*, and *nifB* genes, all of which are probably activated by NifA, it initially seemed likely that the inability of the *nifA* and *nfrX* mutants to grow on metal-free medium would be due to insufficient expression of one of these other *nif* genes, rather than of the *anf* genes. How NifA (and hence NfrX, see Introduction) are required for *anfHDGK* expression is not clear. There are no consensus NifA binding sites present in the region upstream of the *anfH* promoter. Also, an *anfA* insertion mutant strain expressed *anfHDGK* to some degree (7% of wild-type levels). This was reduced to zero in an *anfA nifA* double mutant, suggesting that NifA is not simply required for expression of the *anfA* gene (which was unlikely because of the absence of σ^{54} and NifA binding regions upstream of *anfA*; Joerger et al. 1989a). It is possible that NifA activates *anfHDGK* without binding to upstream sequences, as shown for expression of *nifH* in certain *K. pneumoniae* mutants lacking the *nifH* UAS. In that case, however, overexpression of *nifA* was required to activate *nifH* expression. Because the *nifLA* operon of *A. vinelandii* is constitutively expressed, NifA is available under all conditions of metal supply (Blanco et al. 1993) It will be of interest to determine whether NifA can activate expression of the *anfH* promoter in an *E. coli* background.

The complete lack of expression of the *anfH-lacZ* fusion in the *nifA ntrC* double mutant background was difficult to interpret. Data from Joerger et al. (1991), coupled with the observation reported here concerning *nifM* expression, may provide the answer. Expression of the *anfHDGK* operon requires either NifH or VnfH to be present and active. Since NifM is required for activity of both of these nitrogenase proteins, then the lack of expression of *nifM* in the *nifA ntrC* double mutant means that while the VnfH protein is present, it is inactive. It has been shown recently that the *anf* operon is not expressed in a *nifM* mutant (Joerger et al. 1991). The requirement of NifH or VnfH for activity of the AnfA transcriptional activator is not known but may involve a reduction by electron transfer from VnfH (or NifH) to AnfA.

Although NifA (and NfrX) are required for full *anfHDGK* expression, ammonium still represses *anfHDGK* expression in a *nifL* mutant strain (G. Blanco, personal communication). This is in contrast to *nifHDK* expression, which is fully derepressed in ammonium-containing medium in a *nifL* mutant. Thus, ammonium repression of the *anfHDGK* operon occurs by a mechanism that does not involve NifL. Molybdenum and vanadium also repress expression of the *anf* genes, in both wild-type and *nifL* mutants (A. Bali, personal communication). However, expression of the *anfH-lacZ* fusion is higher in the presence of either metal or ammonium in an *anfA* mutant than in wild-type. This result was also reported by Joerger et al. (1991), who examined *anfHDGK* expression in an AnfA mutant. The AnfA protein may therefore have both activator and repressor functions, depending on the presence of Mo and/or V.

The experiments presented here confirm the expected requirement for σ^{54} for expression of all five of the nitrogen fixation promoters examined since all of them are preceded by typical "–12, –24" concensus sequences. Also confirmed is that *nifHDK* expression requires NifA and NfrX, but not VnfA or AnfA. In addition, expression from the *nifH* promoter is greatly decreased in medium without molybdenum, except in a *vnfA* mutant. This is another case in which an activator might also have a repressor role, as suggested above for AnfA. This is not an uncommon occurrence in prokaryotes; the NtrC gene product has both activator and repressor functions through binding to DNA sequences upstream of *glnA* in *E. coli* and other enteric bacteria (Reitzer and Magasanik 1985). VnfA as an activator is only required for expression of the three *vnf* operons, the two examined here, and also *vnfE* (Walmsley et al. 1990). Direct binding by VnfA to the *nifH* and/or *anfH* promoter regions is a possibility; both regions contain DNA sequences that are somewhat similar to the VnfA consensus binding sequence, GTAC-N7-GTAC, identified in all three *vnf* operon promoter regions (manuscript in preparation). A goal of future work is to determine the role of VnfA (and AnfA) in preventing synthesis of other nitrogenases.

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