JOURNAL OF BACTERIOLOGY, Oct. 1994, p. 6139–6142
0021-9193/94/$04.00+0
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Vol. 176, No. 19

Effect of Amino Acid Substitutions in a Potential Metal-Binding Site of AnfA on Expression from the *anfH* Promoter in *Azotobacter vinelandii*†

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Received 11 April 1994/Accepted 27 July 1994

AnfA, an activator required for transcription of the structural genes encoding nitrogenase 3 (*anfHDGK*) in *Azotobacter vinelandii*, has a potential metal-binding site [(S19)H(C21)FTGE(C26)R] in its N terminus. Growth studies and expression of an *anfH-lacZ* fusion in mutants containing amino acid substitutions in this site indicate that Ser-19 is not required for AnfA activity whereas Cys-21 and Cys-26 are required. Residual expression of the *anfH-lacZ* fusion in AnfA$^-$ mutants was found to be due to activation by VnfA, the activator required for expression of genes encoding nitrogenase 2.

The expression of the three genetically distinct nitrogenases in *Azotobacter vinelandii* is regulated by the presence or absence of ammonium (NH_4^+), molybdenum (Mo), and vanadium (V) in the growth medium (5, 10). All three nitrogenases are repressed by NH_4^+. Diazotrophic growth in the presence of Mo is nitrogenase 1 dependent, diazotrophic growth in Mo-deficient medium containing V is nitrogenase 2 dependent, and nitrogenase 3 is synthesized only in the absence of both Mo and V (5, 10). The structural genes for nitrogenase 1 and nitrogenase 3 are each arranged in a single transcriptional unit, *nifHDK* and *anfHDGK*, respectively (14, 16), whereas those for nitrogenase 2 are split into two units (17). This enables the independent expression of dinitrogenase reductase 2 (*vnfH* gene products) 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 in which nitrogenase 3 is present (4, 8, 26). Dinitrogenase reductase 2 has since been shown to be required for the expression of nitrogenase 3 (18).

The products of the regulatory genes *nifA*, *vnfA*, and *anfA* are required for the transcriptional activation of *nif*, *vnf*, and *anf* operons, respectively (1, 15). VnfA is required for the synthesis of nitrogenase 2 and directly or indirectly represses the formation of nitrogenase 1 in cells growing diazotrophically in Mo-deficient medium with or without V (15). VnfA also seems to be involved, albeit indirectly, in mediating the repression of nitrogenase 3 by V (15, 20). The activator proteins AnfA, VnfA, and NifA have a three-domain structure (15): an N-terminal domain thought to interact with a transmitter of environmental signals (9); a central domain proposed to interact with RNA polymerase, the sigma factor, or both; and a C-terminal DNA-binding domain. The N-terminal domains of AnfA and VnfA differ from the N terminus of NifA in that they contain the motifs SXC(X4)C in AnfA and CXC(X4)C in VnfA. These motifs have been considered as possible sites for interaction with metals such as Mo and V (15). However, these sites could also be involved in redox sensing. As pointed out by Pau (25), it is interesting that AnfA and VnfA resemble the FNR family of redox-sensitive regulatory proteins (12) in having an N-terminal cluster of cysteine residues.

In view of the potential importance of the serine and cysteine residues in the motif found in AnfA, we investigated the effect of replacing these amino acids with others on the expression of nitrogenase 3 and its regulation by NH_4^+, Mo, and V.

(Preliminary results from this study were presented at the 9th International Congress on Nitrogen Fixation [28].)

Bacterial strains and media. The bacterial strains and plasmids used in this study are given in Table 1. The manipulations of *A. vinelandii* strains were done as described previously (3, 18, 28). Mo- and V-deficient medium, designated −Mo,−V medium, was prepared by extraction with 8-hydroxyquinoline as described previously (2). When required, Na_2MoO_4 or V_2O_5 was added to a final concentration of 1 μM to −Mo,−V medium. Fixed N was added as ammonium acetate (final concentration, 28 mM). *Escherichia coli* K-12 71-18 and GM33 were maintained and grown as described previously (18).

Site-directed mutagenesis. The sense strand of the 3.8-kbp *Eco*RI insert, containing *anfA* and most of *anfH*, from plasmid pMJH3 (15) was cloned into M13mp18. Site-directed mutagenesis was carried out with this as a template by the method of Kunkel et al. (19) according to the instruction manual for the Muta-gene M13 in vitro mutagenesis kit purchased from Bio-Rad Laboratories, Richmond, Calif. The altered codons in the oligonucleotide primers employed for site-directed mutagenesis are as follows (the number indicates the position of the amino acid according to the numbering in reference 15, and the existing amino acid on the left is changed to the amino acid on the right of the number; the altered bases are shown in bold): S19A, AGC→**G**CC; S19C, AGC→**T**GC; C21N, TGC→**AA**C; C21A, TGC→**G**CC; C21S, TGC→**A**GC; C26L, TGC→**CT**C; C26A, TGC→**G**CC; C26S, TGC→**A**GC; E25Q, GAG→**C**AG; R27A, CGC→**G**CC; F22A, TTC→**G**CC; C143A, TGT→**GC**T; and C143S, TGT→**A**GT. The sequencing primer used for confirmation of the base changes was TAC TCG AGA CCA ATG GAT. Each mutant *anfA* derivative was subcloned into pUC9 (*Eco*RI site) and then transformed into *E. coli* K-12 71-18 or GM33.

Construction of *A. vinelandii* AnfA mutant strains. The

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† Cooperative study between the Agricultural Research Service, U.S. Department of Agriculture, and the North Carolina Agricultural Research Service.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics or genotype	Source or Reference
E. coli strains		
GM33	*dam-3* mutation in prototrophic *E. coli* W3110	21
K-12 71-18	*supE thi* Δ(*lac-proAB*) F′(*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	23
Plasmids		
pUC9	Amp^r	31
pDB303	Amp^r, 1.7-kbp *Eco*RI fragment containing *rpoB* in pUC8	Dennis Dean
pMJH3	Amp^r, 3.8-kbp *Eco*RI fragment containing *anfA* and most of *anfH* cloned into the *Eco*RI site of pUC9	15
pTMPH3	Amp^r Spc^r, same as pMJH3 except the *Xho*I fragment containing *anfA* replaced by 2-kbp Spc^r cartridge from pHP45Ω	This study
A. vinelandii strains		
CA	Wild type	7
CA46.73	*vnfA46*::Tn*5* *anfH73*::Tn*5*-B21 Tet^r	18
CA46.113.73	*vnfA46*::Tn*5* Δ*anfA113*::*spc* *anfH73*::Tn*5*-B21 Tet^r	This study
CA66	Δ*anfA66*::*kan*	15
CA66.73	Δ*anfA66*::*kan* *anfH73*::Tn*5*-B21 Tet^r	18
CA73	*anfH73*::Tn*5*-B21 Tet^r	18
CA100	*anfA*(S19A)	This study
CA100.73	*anfA*(S19A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA101	*anfA*(S19C)	This study
CA101.73	*anfA*(S19C) *anfH73*::Tn*5*-B21 Tet^r	This study
CA102	*anfA*(C21N)	This study
CA102.73	*anfA*(C21N) *anfH73*::Tn*5*-B21 Tet^r	This study
CA103	*anfA*(C21A)	This study
CA103.73	*anfA*(C21A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA103.46.73	*anfA*(C21A) *vnfA46*::Tn*5* *anfH73*::Tn*5*-B21 Tet^r	This study
CA104	*anfA*(C21S)	This study
CA104.73	*anfA*(C21S) *anfH73*::Tn*5*-B21 Tet^r	This study
CA105	*anfA*(C26L)	This study
CA105.73	*anfA*(C26L) *anfH73*::Tn*5*-B21 Tet^r	This study
CA106	*anfA*(C26A)	This study
CA106.73	*anfA*(C26A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA106.46.73	*anfA*(C26A) *vnfA46*::Tn*5* *anfH73*::Tn*5*-B21 Tet^r	This study
CA107	*anfA*(C26S)	This study
CA107.73	*anfA*(C26S) *anfH73*::Tn*5*-B21 Tet^r	This study
CA108	*anfA*(F22A)	This study
CA108.73	*anfA*(F22A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA109	*anfA*(E25Q)	This study
CA109.73	*anfA*(E25Q) *anfH73*::Tn*5*-B21 Tet^r	This study
CA110	*anfA*(R27A)	This study
CA110.73	*anfA*(R27A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA111.73	*anfA*(C143A) *anfH73*::Tn*5*-B21 Tet^r	This study
CA112.73	*anfA*(C143S) *anfH73*::Tn*5*-B21 Tet^r	This study
CA113	Δ*anfA113*::*spc*; 1.7-kbp fragment containing most of *anfA* replaced with a 2-kbp Spc^r cartridge	This study
CA113.73	Δ*anfA113*::*spc* *anfH73*::Tn*5*-B21 Tet^r	This study

transformation procedures employed for *A. vinelandii* were those described by Page and von Tigerstrom (24). Plasmid DNA containing the *anfA* mutation was prepared from *E. coli* GM33. A 3.2-kbp *Eco*RI-*Bcl*I fragment isolated from this plasmid DNA was used in congression crosses (6) with CA66 or CA66.73 (Table 1). The selection marker used in the congression cross was a 1.7-kbp *Eco*RI fragment from pDB303 containing an *rpoB* mutation conferring rifampin resistance (Rif^r). In order to favor transformation of mutagenized *anfA* DNA, a ratio of at least 50 to 100 to 1 of mutant *anfA* DNA to the DNA fragment having the *rpoB* mutation was used. Rif^r transformants were selected on ammonium-supplemented Burk medium containing rifampin (10 μg/ml) and subsequently screened for the loss of kanamycin resistance (Kan^r). Loss of kanamycin resistance indicated that the *anfH* deletion plus interposon was replaced by the DNA containing the *anfA* mutation through a double crossover event. Loss of the interposon was also verified by Southern hybridization analysis. The *anfH-lacZ* fusion strain carrying a deletion and a spectinomycin resistance (Spc^r) cartridge insertion in *anfA* (CA113.73 in Table 1) was constructed as follows. Plasmid pMJH3 (Table 1) was cleaved with *Xho*I, and the 4.8-kbp fragment (consisting of the pUC9 vector plus the insert containing a 1.7-kbp *Xho*I deletion) was isolated and blunt ended by filling in the *Xho*I site with the Klenow fragment of DNA polymerase I (U.S. Biochemicals, Cleveland, Ohio). This was ligated to a 2.0-kbp Spc^r cartridge isolated from pHP45Ω after cleavage with *Sma*I (29). *E. coli* GM33 was transformed with the ligation mixture, and Spc^r Amp^r (ampicillin-resistant) transformants were tested for the presence of a plasmid containing the Spc^r cartridge in place of the deleted *Xho*I fragment. A 3.5-kbp *Eco*RI-*Bcl*I fragment was isolated from

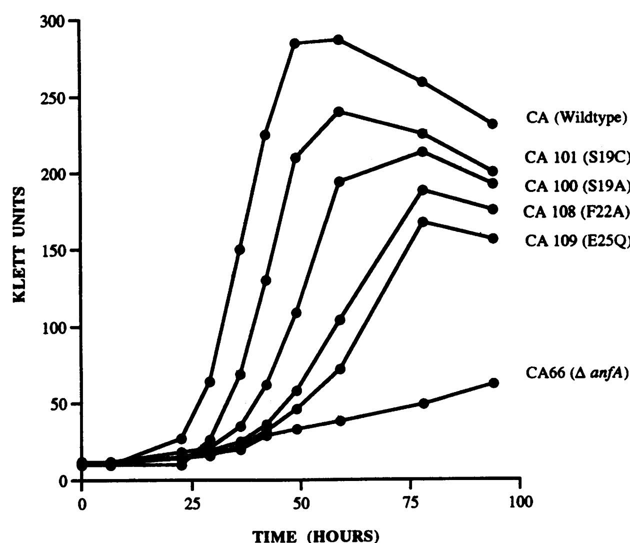

FIG. 1. Diazotrophic growth by AnfA mutant strains of *A. vinelandii* in −Mo,−V medium.

the resulting plasmid (pTMPH3) and was used to transform *A. vinelandii* CA73. A Tet^r (tetracycline-resistant) Spc^r transformant was selected and designated CA113.73. Strain CA46.113.73 (*vnfA46*::Tn*5* Δ*anfA113*::spc *anfH73*::Tn*5*-B21) was constructed by transforming CA113.73 genomic DNA into CA46 competent cells and then selecting for Tet^r Spc^r Kan^r transformants.

Growth of AnfA mutant strains in N-free, −Mo,−V medium. We previously hypothesized that reduced AnfA is required for transcriptional activation of the genes that encode nitrogenase 3 (*anfHDGK*) and that dinitrogenase reductase 2 acts as the reductant (28). This hypothesis requires the presence of a center in AnfA which can undergo oxidation-reduction. Since a potential metal-binding site [(S19)H(C21)FTGE(C26)R] in the N terminus of AnfA could be involved with this redox center, we used site-directed mutagenesis to make individual amino acid substitutions in this site. The following amino acid substitutions were made: S19A (Ala is in the corresponding position in NifA [15]); S19C (Cys is in the corresponding amino acid in VnfA); C21A, C21S, and C21N (Gln is in the corresponding position in NifA, but this change required three base changes compared with two for Asn, and therefore Asn was substituted); C26A, C26S, and C26L (Leu is the corresponding residue in NifA); F22A (Phe is not conserved in AnfA and VnfA); and E25Q and R27A (both Glu and Arg are conserved in AnfA and VnfA). Inoculum cultures of *anfA* mutant strains with the substitutions were prepared by transferring three or more times on −Mo,−V agar medium containing ammonium acetate and then allowing growth to mid-log phase in liquid −Mo,−V medium supplemented with ammonium acetate. These cultures were used to inoculate 35 ml of N-free, −Mo,−V medium (in 300-ml sidearm flasks) to a density of approximately 10 Klett units (1 Klett unit equals 5 × 10^6 CFU/ml). Growth at 30°C, with vigorous shaking, was monitored with a Klett-Summerson colorimeter equipped with a no. 66 (red) filter. Figure 1 shows the diazotrophic growth profile of the strains in −Mo,−V medium. Mutant strains CA100 (S19A), CA101 (S19C), CA108 (F22A), and CA109 (E25Q) grew faster than the *anfA* deletion strain CA66 but more slowly than the wild type (Fig. 1). For these strains, the substitutions appear to impair AnfA function only to a limited extent. The remaining mutants, CA102 (C21N), CA103 (C21A), CA104 (C21S), CA105 (C26L), CA106 (C26A), CA107 (C26S), and CA110 (R27A), like strain CA66, showed only minimal diazotrophic growth. Thus, on the basis of growth studies, residues Cys-21, Cys-26, and Arg-27 seem to be critical for AnfA function.

TABLE 2. Expression of the *anfH-lacZ* fusion (*anfH73*::Tn*5*-B21) in *A. vinelandii* strains with different AnfA mutant backgrounds

Strain	Phenotype	β-Galactosidase activity (%)[a]			
		No addition	Mo	V	NH_4^+−Mo
CA73	Wild-type	100[b]	0.1	0.4	0.4
CA66.73	AnfA$^-$(Kan^r)	9.4	4.7	6.4	6.2
CA100.73	AnfA(S19A)	72.4	0.1	0.4	0.4
CA101.73	AnfA(S19C)	82.8	0.1	0.5	0.5
CA102.73	AnfA(C21N)	3.8	0.1	0.4	0.4
CA103.73	AnfA(C21A)	6.1	0.2	0.4	0.3
CA103.46.73	AnfA(C21A) VnfA$^-$	0.1	ND[c]	ND	ND
CA104.73	AnfA(C21S)	4.6	0.2	0.5	0.4
CA105.73	AnfA(C26L)	4.5	0.2	0.5	0.4
CA106.73	AnfA(C26A)	4.3	0.1	0.4	0.3
CA106.46.73	AnfA(C26A) VnfA$^-$	0.1	ND	ND	ND
CA107.73	AnfA(C26S)	2.2	0.1	0.5	0.3
CA108.73	AnfA(F22A)	64.5	0.1	0.4	0.5
CA109.73	AnfA(E25Q)	11.9	0.1	0.4	0.4
CA110.73	AnfA(R27A)	4.6	0.1	0.5	0.4
CA111.73	AnfA(C143A)	64.5	0.1	0.3	0.3
CA112.73	AnfA(C143S)	84.8	0.1	0.4	0.5
CA46.73	VnfA$^-$	59.7	0.1	50.9	0.1
CA113.73	AnfA$^-$(Spc^r)	3.2	ND	ND	ND
CA46.113.73	VnfA$^-$ AnfA$^-$	0.3	0.2	0.5	0.4

[a] Cells were grown in Mo-free Burk medium containing 28 mM ammonium acetate and later derepressed for 16 h in Mo-free Burk medium in the presence of 1 μM Na_2MoO_4 (Mo), 1 μM V_2O_5 (V), 28 mM ammonium acetate (NH_4^+-Mo), or no metals (No addition). Each value represents an average value from three experiments.

[b] 100% β-galactosidase activity of the wild-type CA73 in N-free, −Mo,−V medium was 7,406 ± 539 Miller units.

[c] ND, not determined.

Expression of an *anfH-lacZ* transcriptional fusion in AnfA mutant strains. In order to study the effect of the amino acid substitutions in AnfA on expression from the *anfH* promoter, an *anfH-lacZ* fusion was placed in the AnfA mutant backgrounds. β-Galactosidase activities of the strains were assayed as described before (18). The accumulation of β-galactosidase by the strains containing the *anfH-lacZ* fusion (Table 2) generally paralleled rates of growth by the same strain lacking the *lacZ* fusion (Fig. 1). The fusion strains with substitutions for Ser-19 (CA100.73 and CA101.73) and for Phe-22 (CA108.73) gave β-galactosidase activities ranging from 65 to 83% of the activity observed with the wild type (CA73), while strain CA109.73 with an E25Q substitution showed only 12% of the activity found for CA73 (Table 2). All strains with substitutions for Cys-21, Cys-26, and Arg-27 in AnfA gave low β-galactosidase activities (2.2 to 6.1% of the activity found for CA73). The presence of Mo, V, or NH_4^+ essentially eliminated β-galactosidase accumulation in all of the mutants containing amino acid substitutions in AnfA (Table 2). To determine whether the residual activities observed with strains containing substitutions for Cys-21 and Cys-26 were due to partially active AnfA or to activation of the *anfH* promoter by VnfA (directly or indirectly), a *vnfA* mutation (*vnfA46*::Tn*5*) was introduced into strains CA103.73 (C21A) and CA106.73 (C26A). The expression of the *anfA-lacZ* fusion in these strains was only 0.1% of that of the wild type (Table 2); thus, it appears that VnfA was responsible for the residual activities.

In agreement with previous results (18), the *anfH-lacZ* fusion was constitutively expressed at a low level in the *anfA* deletion strain CA66.73 (Table 2). Although the values reported here for CA66.73 are lower than the ones we previously reported, the pattern of incomplete repression of the *anfH-lacZ* fusion by Mo, V, and NH_4^+ is reproducible in this strain. In order to see if the low level of expression by the *anfH-lacZ* fusion in strain CA66.73 was due to activation by VnfA, we constructed a $VnfA^-$ $AnfA^-$ strain. To facilitate construction of this strain, a Spc^r cassette replaced the Kan^r cassette in CA66.73. This strain (CA113.73) accumulated less β-galactosidase than CA66.73 (3.2% versus 9.4% for CA66.73). The reason for the lower β-galactosidase activity in CA113.73 is not known. The $VnfA^-$ $AnfA^-$ strain (CA46.113.73) accumulated 10-fold less β-galactosidase (0.3%) than CA113.73 (3.2%). This indicates (as with the altered AnfA strains) that the low level of expression observed in CA113.73 is due to VnfA. Jacob and Drummond (13) also showed that *anfH* promoter can be activated by VnfA.

In addition to the cysteines in the amino-terminal domain of AnfA and VnfA, Cys-143 in AnfA and Cys-134 in VnfA are conserved (15). Since this conserved cysteine is analogous to the essential Cys-122 in the FNR protein (11, 22, 30), it was of interest to see if Cys-143 was essential for AnfA function. As shown in Table 2, strains CA111.73 (C143A) and CA112.73 (C143S) accumulated 64.5% and 84.8%, respectively, of the level of β-galactosidase accumulated by CA73. This indicates that Cys-143 is not essential for AnfA activity.

In summary, it is clear that Cys-21 and Cys-26 are required for active AnfA and that these residues are not essential for the repressor function of this protein. It is also apparent that the residual activity observed in Anf mutants is due, either directly or indirectly, to VnfA.

We thank Dennis Dean and Richard Pau for providing strains.

This work was supported by U.S. Department of Agriculture Competitive Grant 92-37305-7722.

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