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Regulated Expression of the *nifM* of *Azotobacter vinelandii* in Response to Molybdenum and Vanadium Supplements in Burk's Nitrogen-Free Growth Medium

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***Azotobacter* is a diazotrophic bacterium that harbors three genetically distinct nitrogenases referred to as *nif, vnf,* and *anf* systems. The *nifM* is an accessory gene located in the *nif* gene cluster and is transcriptionally regulated by the NifA. However, *Azotobacter* mutants that lack NifA are known to synthesize functional NifM and this accessory protein is known to be needed for the activity of nitrogenase-2 and nitrogenase-3. To determine how the transcription of *nifM* is regulated when *Azotobacter* is grown under conditions in which nitrogenase-2 or nitrogenase-3 is expressed, we generated an *Azotobacter vinelandii* strain that carries a *nifM:lacZ*-kanamycin resistance gene cassette in its chromosome. In this strain the *nifM* open reading frame was disrupted by the presence of a *lacZ*-kanamycin resistance gene cassette so that it could not produce active NifM. Moreover, the *lacZ* gene was placed under the transcriptional control elements of the *nifM* gene so that the *lacZ* expression could be used as a marker to determine the extent of expression of the *nifM* gene under different growth conditions. Our results show that this strain was unable to grow in Burk's nitrogen-free medium supplemented with either molybdenum or vanadium or lacking both metals suggesting that in the absence of functional NifM none of the nitrogenases were active. It was also found that the *nifM* expression was differentially regulated when the *A. vinelandii* cells were grown under conditions that activate nitrogenase-2 and nitrogenase-3, as determined by liquid β-galactosidase activity measurements. These results suggest that the transcriptional activators, VnfA and AnfA, may regulate the *nifM* expression.** © 1999 Academic Press

Azotobacter is a gram-negative, non-symbiotic, diazotrophic soil bacterium that possesses three genetically distinct nitrogenases [1]. The best characterized of the three nitrogenase complexes is the nitrogenase-1, or the molybdenum (Mo)-containing nitrogenase encoded by the genes in the *nif*-system [2, 3]. This complex metalloenzyme is composed of two components, the dinitrogenase reductase-1 and the dinitrogenase-1. The dinitrogenase reductase-1, encoded by the gene *nifH* is a dimer of two identical subunits with a molecular weight of approximately 60, 000 daltons [4]. The dinitrogenase-1 is a tetramer made up of two pairs of non-identical subunits, α and β, encoded by the *nifD* and *nifK* genes, respectively [5]. Apart from these structural genes, a number of *nif*-accessory genes were identified whose products are important for the synthesis and assembly of active nitrogenase [6]. The genes involved in this system are coordinately regulated by two *nif*-regulatory genes, *nifA* and *nifL* [7]. Activation of the genes involved in the biosynthesis and assembly of the Mo-containing nitrogenase occurs only when the cells are grown in the medium containing molybdenum [7–9].

The nitrogenase-2 is the vanadium (V)-containing nitrogenase that is encoded by the genes of *vnf*-system. As in the *nif*-system, this enzyme complex also is consisted of two components, the dinitrogenase reductase-2 and the dinitrogenase-2. The dinitrogenase reductase-2, encoded by the *vnfH* gene, is a dimer and has a molecular weight of about 62,000 daltons. The dinitrogenase-2 is thought to be a hexamer. Like dinitrogenase-1 of the *nif*-system, this protein is also made of non-identical subunits, α, β and γ encoded by the *vnfD*, *vnfK* and *vnfG* respectively [10–12]. Biosynthesis and assembly of the vanadium-containing nitrogenase also need the help of several accessory proteins. Both the structural and accessory genes of the *vnf*-system are expressed only when the cells are grown in the Burk's nitrogen-free medium supplemented with vanadium. The nitrogenase-3 does not contain either molybdenum or vanadium and is synthesized and assembled in the cells only when the cells are grown in Burk's nitrogen free medium that does not contain either Mo or V. As in the other two systems, the

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TABLE 1

Bacterial Strains and Plasmids Used in This Study

Strain/plasmid	Relevant characteristics and description	Source or reference
E. coli TG1	K12 Δ(*lac-pro*) *supE thi hsd5/F′ traD36 proA*$^+$*B*$^+$ *lacIq lacZΔM15*	Amersham Life Sciences Inc. IL
Azotobacter vinelandii OP	Wild Type, Nitrogen Fixing, Soil Bacterium	Laboratory stock
A. vinelandii BG599	*nifM:lacZ*, kanamycin resistant, Nif$^-$ strain, generated by integration of *lacZ*-kan via *nifM* homology using the plasmid pBG599.	This work
pKOK6	plasmid carrying the *lacZ*-kanamycin-resistance cassette, colE1 origin, ampicillin and kanamycin resistant markers.	[26]
PUC18	*E. coli* cloning vector, colE1 origin, Ampicillin resistant marker	New England Biolabs, Beverly, MA
pCR 2.1	Amp^{r}Kan^r; Size: 3.9 kb; Used for direct cloning of PCR products	Invitrogen Corp., Carlsbad, CA
pBG119	Derivative of pCR 2.1 in which 821 bp DNA fragment encoding the *nifM* gene was cloned. This fragment was generated by PCR amplification using oligo nucleotide primers as described in Materials and Methods. This fragment could be released by digesting with *Eco*RI partial and *Hind*III.	This work
pBG598	pUC18 derivative in which the *Eco*RI and *Hind*III fragment carrying the *nifM* (obtained by partial *Eco*RI digestion and a complete cleavage with *Hind*III of pBG119) was cloned to generate a plasmid that harbors *nifM* and has no polycloning sites.	This work
pBG599	pBG598 derivative in which the *nifM* was partially deleted by cleavage with internal *Pst*I sites and the 4.7 kb DNA fragment corresponding to the promoterless *lacZ* and a kanamycin resistance gene was ligated to generate the *nifM:lacZ*-kanamycin fusion.	This work

nitrogenase-3 is also composed of dinitrogenase reductase-3 and dinitrogenase-3. Dinitrogenase reductase-3 is a dimer encoded by *anfH* and dinitrogenase-3 a tetramer encoded by two dissimilar pairs of subunits (α and β) encoded by *anfD* and *anfK* genes, respectively. Dinitrogenase-3 lacks significant amounts Mo or V atoms and can be isolated in two active configurations ($\alpha_1\beta_2$ or $\alpha_2\beta_2$) [13, 14].

Although each of the three alternate nitrogenases is encoded by their own structural genes and modified by their own accessory gene products, all the three nif-systems require several common accessory *nif* gene products for full activation [6, 15]. For example, it was shown that five *nif* gene products are essential for the activity of all three nitrogenase systems. These include NifB, NifU, NifS, NifV, and NifM [16–19]. The genes encoding these proteins are located in the molybdenum-nitrogenase gene cluster and they are preceded by NifA binding sites suggesting that they are activated by the transcriptional activator NifA [20]. However, nitrogenase-2 and nitrogenase-3 are synthesized in *nifA* mutants as well, when the cells are grown in Mo-deficient medium. Therefore, it is reasonable to assume that transcriptional regulatory element of *vnf* and *anf* systems must play a role in activating these common ancillary genes although they are located in the molybdenum-nitrogenase gene cluster [16, 21]. It was observed that one of the common ancillary genes, the *nifB*, is indeed activated by NifA, VnfA or AnfA transcriptional activators [22]. In this study we report, that expression of the *nifM* is activated when the cells are grown in Burk's nitrogen free medium supplemented with appropriate metal for the activation of any of the three alternate nitrogenase systems. Our analyses support the idea that the *nifM* is regulated not only by NifA but also by VnfA and AnfA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains TG1 was grown at 37°C in Luria broth or 2YT [23]. The ampicillin was used to a final concentration of 50 μg/ml wherever the selection was made. *Azotobacter vinelandii* strains were grown at 30°C in modified Burk's nitrogen-free (BN$^-$) medium (Table 1) [24]. When it was necessary to include fixed nitrogen in the medium, ammonium acetate ($NH_4OAc.\ H_2O$) was added to a final concentration of 400 μg/ml.

General molecular biology techniques. DNA sub-cloning, plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformations were carried out as described in the laboratory manuals [23]or as suggested in the manufacturer's instructions. Restriction enzymes were purchased either from Boehringer Mannheim (Indianapolis, IN) or from Promega (Madison, WI). Oligonucleotides used for PCR amplification were purchased from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD). Radiolabeled material for sequencing (]^{35}S]dATP) was obtained from Dupont NEN (Boston, MA). The nucleotide sequencing was performed using T_7 sequencing kit purchased from USB-Amersham Inc. (Cleveland, OH).

β-Galactosidase activity assays. To determine the extent of expression of the *lacZ* gene present in *A. vinelandii* strain carrying the *nifM:lacZ*-kanamycin resistance gene cassette under different growth conditions, the cells were grown to mid-log phase in Burk's medium supplemented with nitrogen. The cells were then trans-

ferred to Burk's nitrogen-free medium containing appropriate metal and were incubated for 5 hours before proceeding to determine the β-galactosidase activity. Cells from 1.5 ml culture were harvested by centrifugation and resuspended in 300 μl of Z-buffer [23]. A 100-μl aliquot of the resuspended cells were subjected to quick freeze-thaw-lysing by liquid nitrogen treatment followed by heating at 37°C. The β-galactosidase activity in the cell lysate was measured by adding a 0.7 ml of Z buffer/β-mercaptoethanol solution (27 μl of/β-mercaptoethanol per 10 ml of Z-buffer) to each sample followed by 0.16 ml of Z-buffer/ONPG (4 mg of ONPG per 1 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 minutes at 10,000×g to remove cellular debris and the OD_{420} was recorded. The β-galactosidase units were defined as the amount of enzyme which hydrolyses 1 μmole of ONPG to 0-nitrophenol and D-galactose per minute.

RESULTS AND DISCUSSION

Construction and Growth Characteristics of A. vinelandii strain harboring a nifM::lacZ-kanamycin gene cassett. To construct an *A. vinelandii* strain that carries transcriptional fusion of *nifM* and *lacZ* genes, the DNA fragment encoding the open reading frame (ORF) of the *nifM* gene was isolated by PCR amplification using *A. vinelandii* chromosome as the template and olignonucleotide primers corresponding to the 5′ and 3′ regions of the *nifM* ORF. The 821 bp DNA fragment encoding the NifM obtained by PCR amplification [25] was cloned into the pCR2.1 vector (Invitrogen Corp., San Diego, CA) to generate pBG119. An *Eco*RI-*Hind*III fragment containing the *nifM* ORF was released from pBG119 and was ligated into the *Eco*RI-*Hind*III digested pUC18 to generate the plasmid pBG598. Since pBG598 contained two *Pst*I sites in the DNA fragment encoding the NifM, the plasmid was digested with *Pst*I to remove small internal DNA fragment encoding part of the NifM. The plasmid pKOK6 [26] carries a 4.7 kb *Pst*I fragment that encodes a promoterless *lacZ* gene and a kanamycin resistance gene with its own promoter. This 4.7 kb DNA fragment was isolated by *Pst*I restriction digestion of pKOK6 and was ligated with the *Pst*I digested pBG598. This resulted in generating the plasmid pBG599 (Fig. 1) that carries the promoterless *lacZ* gene and a kanamycin resistance gene (with its own promoter) located within the *nifM* open reading frame. The *BamH*I site located asymmetrically in the 4.7 kb fragment was used to identify the direction of trascription of the *lacZ* in the pBG599. *A. vinelandii* strain was transformed with pBG599 using methods described previously [27]. Since pBG599 has a colE1 replicon, it is unable to replicate in *A. vinelandii*. However, since *A. vinelandii* cells have a high efficiency recombination system and the plasmid pBG599 as well as *A. vinelandii* chromosome carries the *nifM*, homologous recombination resulting in the rescue of the *nifM:lacZ*-kanamycin resistance gene cassett into the *A. vinelandii* chromosome could occur (Fig. 1). Such

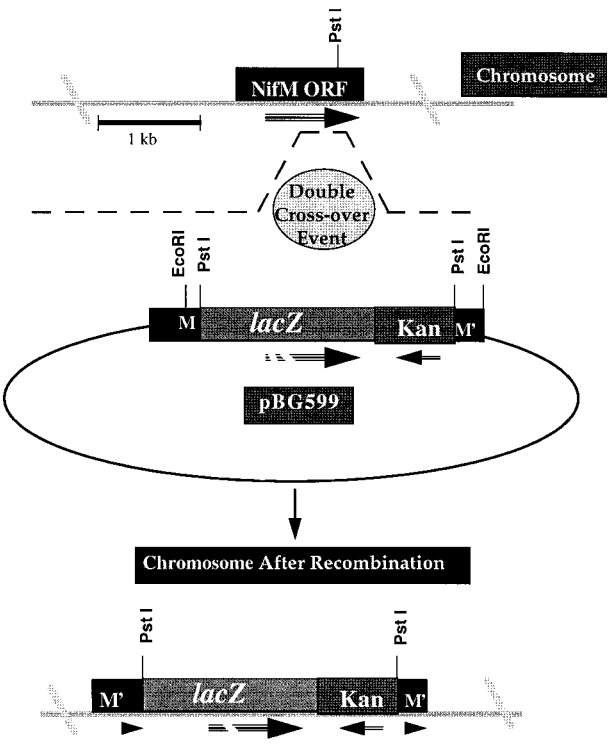

FIG. 1. Strategy for the construction of *A. vinelandii* strain BG599 harboring *nifM:lacZ*-kanamycin resistance gene cassett in the chromosome. Organization of the *nifM:lacZ*-kanamycin resistance gene cassett and the appropriate restriction sites used for the construction of pBG599 are shown. In this experiment the double crossover occurred within the *nifM* ORF, thus placing the *lacZ* gene under the transcription regulatory elements of the *nifM*. Arrows indicate the direction of translation.

recombinants were scored by growing the transformants on BN^+ plates containing 5 μg/ml of kanamycin. Since the recombination occurred within the NifM ORF, the regulatory elements of *nifM* present on the chromosome were undisturbed. Thus, the recombination resulted in placing the *lacZ* gene under the transcription regulation of the *nifM* promoter(s). The *A. vinelandii* strain carrying the *nifM:lacZ*-kanamycin resistance gene cassett in the chromosome was designated *A. vinelandii* BG599.

The insertion of *lacZ*-kanamycin resistance gene into the *nifM* resulted in disrupting the *nifM* ORF in *A. vinelandii* BG599. Since it was predicted that the *NifM* is needed for the production of functional nitrogenases by all three alternate *nif* systems, we analyzed if the *A. vinelandii* BG599 could grow in Burk's nitrogen free medium supplemented with or lacking of either Mo or V. As shown in the Table 2, the *A. vinelandii* BG599 could grow only when the Burk's medium was supplemented with nitrogen. This result was in agreement with the prediction that the NifM is essential for the production of functional nitrogenases by all three *nif* systems.

TABLE 2

Results of the Liquid β-Galactosidase Activity Assay with ONPG as Substrate to Demonstrate the Regulated Expression of *nifM-lacZ* in *Azotobacter vinelandii*BG599

Growth medium	β-Galactosidase activity (MU)[1]	β-Galactosidase activity Percentage	Growth characteristics[2]
Burk's Nitrogen Free medium with Molybdenum but no Vanadium added	257.42 ± 5.18	100	−
Burk's Nitrogen Free medium with Vanadium but no Molybdenum added	212.81 ± 3.46	83	−
Burk's Nitrogen Free medium with no Vanadium or Molybdenum added	184.43 ± 4.05	72	−
Burk's medium supplemented with fixed nitrogen	29.02 ± 1.07	11	++

[1] Miller Units were defined as the amount of enzyme which hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minute.
[2] Growth characteristics of strain in indicated medium. ++ indicates positive growth, − indicates negative growth.

Expression of nifM-lacZ in Burk's nitrogen-free medium supplemented with or lacking of Mo or V. Since the NifM is predicted to be essential for the activity of all three alternate nitrogenases, we analyzed if the *nifM* transcription is activated when the cells were grown in Burk's nitrogen free medium supplemented with or lacking of either Mo or V. To do this we used *A. vinelandii* BG599. As mentioned above these cells were unable to grow in the absence of nitrogen. Therefore, initially we grew *A. vinelandii* BG599 cells to mid-log phase in Burk's medium containing nitrogen. Then the cells were harvested, washed with Burk's nitrogen free medium and resuspended in Burk's nitrogen free medium appropriate for the induction of different alternate nitrogenase systems as shown in Table 2. The cells were incubated under these conditions for five hours and the *lacZ* expression (that located within in the *nifM*) was monitored using liquid β-galactosidase assay as described in Materials and Methods. When the cells were incubated in Burk's Nitrogen free medium supplemented with Mo, robust *lacZ* expression was observed (Table 2). In the Burk's Nitrogen free medium supplemented with only V or that lacked Mo and V, also the *A. vinelandii* BG599 cells expressed *lacZ* (Table 2). However, the extent of β-galactosidase expression under different growth conditions varied. The maximum expression of *lacZ* was observed under the growth conditions that were favorable for nitrogenase-1 expression. In the growth conditions favorable for nitrogenase-2 expression, the extent of *lacZ* expression corresponded to 82.5% of the *lacZ* expression observed under conditions favorable for the expression of nitrogenase-1 (Table 2). Similarly, under growth conditions favorable for nitrogenase-3 expression, the extent of *lacZ* expression corresponded to only 71.6% of the *lacZ* expression observed under conditions favorable for the expression of nitrogenase-1 (Table 2). These results suggested that the *nifM* expression is under the regulatory control of different *nif* transcriptional activators such as NifA, VnfA or AnfA.

Identification of putative vnfA binding site upstream to the NifM ORF. It was predicted previously that the expression of *nifM* under growth conditions favorable for nitrogenase-1 expression is regulated by the NifA transcription activator. The binding site for NifA was localized upstream to the ORF8 suggesting that under growth conditions favorable for nitrogenase-1 expression, the *nifM* is transcribed as a polycistronic message. However, a *cis*-acting regulatory element that is responsible for the transcription regulation of *nifM* by VnfA or AnfA is not identified to date. Woodley *et al.* have identified a consensus sequence for VnfA binding by comparing the nucleotide sequences of the known VnfA-regulated promoters [28]. Our analysis of the upstream nucleotide sequence of *nifM* shows the existence of a putative VnfA-binding sequence located

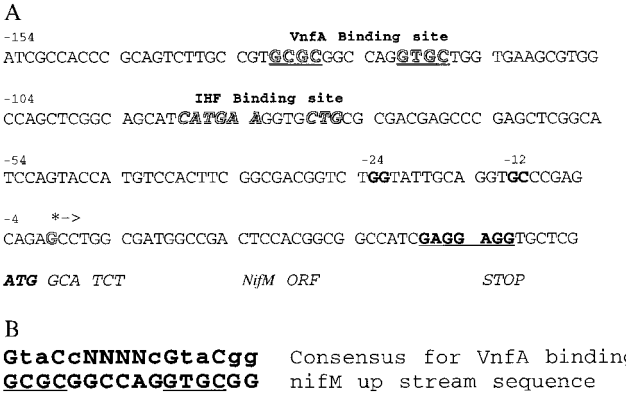

FIG. 2. Identification of a putative VnfA-activated promoter located upstream to the *nifM* ORF. (A) The nucleotide sequence upstream to the predicted *nifM* initiation codon is shown. The sequence corresponding to the ribosome binding site is shown in bold with underlining. Putative transcription start site is marked with *. The predicted canonical sequence of sigma 54 promoter, IHF binding site and VnfA binding site are marked. (B) Consensus sequence for VnfA binding identified previously [28] by comparing different VnfA regulated promoters is shown. Uppercase letters show invariable nucleotides in this consensus sequence. N represents any of the four nucleotides and characteristic −12, −24 nucleotide sequence of *nif*-promoters is marked in bold.

within the NifZ coding region (Figs. 2A and 2B). This region is consisted of a sigma 54-regulated promoter sequence, an Integration Host Factor (IHF) binding sequence [20], and a VnfA binding sequence (Fig. 2A).

In summary, we have shown that the *nifM* expression is differentially regulated under different growth conditions known to activate the expression of different alternate nitrogenases. Furthermore, the presence of a putative VnfA binding site, IHF binding site and the sigma 54 promoter sequence just upstream to the NifM ORF suggests the transcription of *nifM* could be regulated by VnfA transcriptional activator from this site, even though the authenticity of this site for trancription of *nifM* needs to be tested.

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