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Regulation of V-nitrogenase genes in *Anabaena variabilis* by RNA processing and by dual repressors

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Summary

***Anabaena variabilis* ATCC 29413 fixes nitrogen in specialized cells called heterocysts using either a Mo-nitrogenase or a V-nitrogenase. V-nitrogenase structural genes, *vnfDGK*, as well as *vnfEN* form an operon with *ava4025*, located upstream of *vnfDG* that is repressed by fixed nitrogen and by Mo. The *ava4025-vnfDGKEN* operon is under the control of a Mo-repressible promoter located nearly 600 bp upstream of *ava4025*. Levels of *vnfDG* transcript were about 500-fold higher than *ava4025*, the first gene of the operon. This may be the result of RNA processing at a site 87 bp upstream of *vnfDG* that was initially identified as the transcription start site. A strain with a deletion in the coding region of *ava4025* grew diazotrophically with Mo or with V. Two similar proteins, VnfR1 and VnfR2, whose genes are located some distance from the *ava4025-vnfDGKEN* operon, each repressed transcription from the *ava4025-vnfDGKEN* promoter and a mutant lacking both VnfR1 and VnfR2 made the V-nitrogenase in the presence of Mo. Overexpression of the V-nitrogenase in the double *vnfR1 vnfR2* mutant resulted in decreased activity of the Mo-nitrogenase. VnfR1 bound specifically, *in vitro*, to a region upstream of the *ava4025* promoter.**

Introduction

Filamentous heterocyst-forming cyanobacteria are capable of aerobic nitrogen fixation because the oxygen-sensitive nitrogenase is expressed only in specialized micro-oxic cells called heterocysts that differentiate in filaments in response to nitrogen deprivation (reviewed: Thiel, 2004; Flores and Herrero, 2010; Maldener and Muro-Pastor, 2010). The strain *Anabaena variabilis* ATCC 29413 is unusual among the well-studied cyanobacteria in that it has an alternative V-nitrogenase that functions in heterocysts under conditions of both nitrogen and molybdate limitation, when the Mo-nitrogenase is non-functional (Thiel, 1993). The V-nitrogenase has been best characterized in *Azotobacter vinelandii* (Bishop and Premakumar, 1992; Ruttimann-Johnson *et al.*, 2003; Lee *et al.*, 2009). It is similar in structure to the Mo-nitrogenase; however, it has a FeV cofactor in place of the FeMo cofactor found in the Mo-nitrogenase (Ruttimann-Johnson *et al.*, 2003). *A. variabilis* has a third nitrogenase, a Mo-nitrogenase, encoded by the *nif2* gene cluster that functions in vegetative cells under anoxic conditions (Thiel *et al.*, 1995; 1997).

V-nitrogenase genes *vnfDG* (a single gene), *vnfK*, *vnfE*, *vnfN* and *vnfH* have been identified in *A. variabilis* (Thiel, 1996, Thiel, 1993, Pratte *et al.*, 2006) as well as the *vupABC* genes encoding a high-affinity vanadate transport system (Pratte and Thiel, 2006) (Fig. 1A). These are part of a large cluster of Mo-regulated genes located within a region of about 32 kb in the genome. These genes are all repressed by fixed nitrogen and by Mo; however, the Mo- and V-nitrogenase systems are not totally independent; there is no evidence in *A. variabilis* for the existence of *vnfB*, *vnfS* or *vnfU* genes, which in the *nif* system encode accessory proteins for nitrogenase maturation and assembly (Rubio and Ludden, 2008), and these functions are provided instead by *nifB1*, *nifS1* and *nifU1* (Lyons and Thiel, 1995). Further, NifH1 and VnfH can both function as dinitrogenase reductase for activity of the Mo- or V-nitrogenase (Pratte *et al.*, 2006).

In *A. vinelandii*, the *vnf* genes are regulated by a transcriptional activator VnfA (Walmsley *et al.*, 1994; Nakajima *et al.*, 2010; Yoshimitsu *et al.*, 2011). Little is known about the regulation of nitrogenase gene expression in cyanobacteria except that the genes are expressed late in heterocyst differentiation and that there are no genes similar to the NifA/VnfA activators that are present in the Proteobacteria. The *vnf* genes and the *nif1* genes probably share the same regulation, as their expression is restricted to mature heterocysts whose development is dependent on the co-ordinated actions of at least three regulatory proteins, NtcA, HetR and NrrA (Muro-Pastor *et al.*, 2002; Herrero *et al.*, 2004; Ehira and Ohmori, 2006; Kim *et al.*, 2011; Camargo *et al.*, 2012). However, the *vnf* genes are also regulated by Mo. Since the V-nitrogenase has only been found in this cyanobacterial strain, it appears likely that Mo-responsive regulatory factors would be unique to

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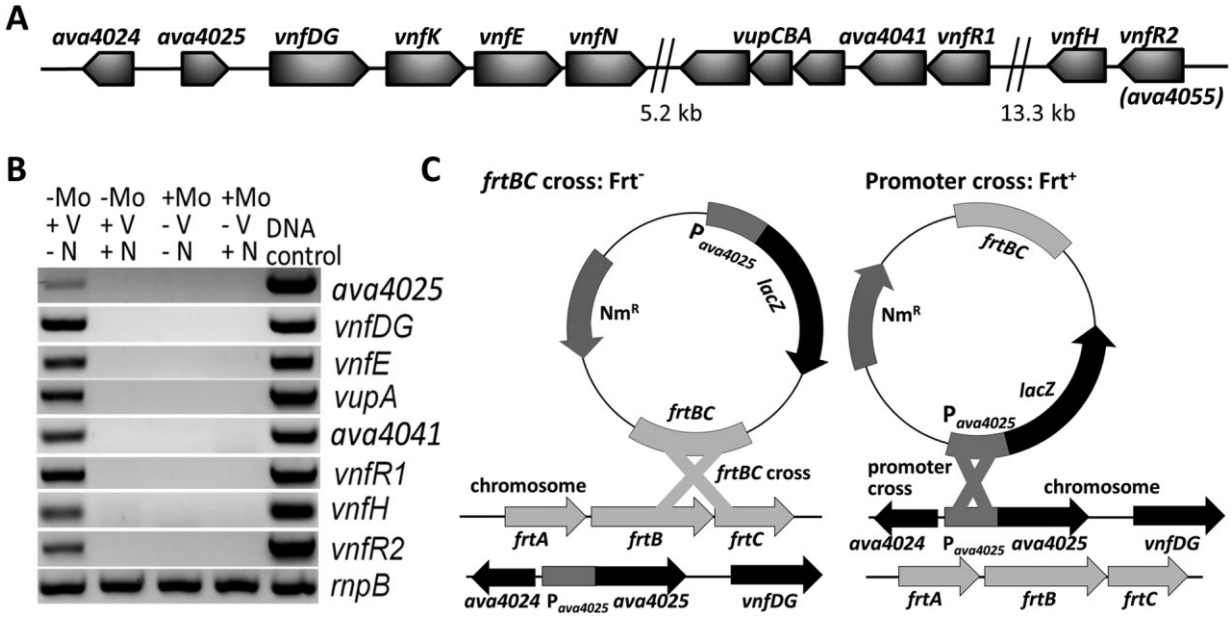

Fig. 1. Map and expression of V-nitrogenase-related genes.
A. Relative locations of V-nitrogenase-related genes described in this study. Distances between gene clusters are shown.
B. RT-PCR analysis of indicated genes with RNA isolated from cells grown with or without ammonium (N) in the presence of either Mo or V.
C. Construction of upstream promoter region of the gene fused to *lacZ* with integration in the fructose transport genes in the chromosome, leading to inability of the cells to use fructose (left panel), or plasmid integration in the upstream promoter region of the gene in the chromosome with cells retaining their ability to transport and use fructose (right panel). A simplified version of the plasmid, pJU411, used for construction of all the fusions is shown in (C) with the upstream promoter region of *ava4025* shown fused to *lacZ*.

this strain and should be located near the known *vnf* genes. We provide evidence here that *vnfDGKEN* form a single operon with the upstream gene, *ava4025*, and that all these genes are under the dual control of two newly described Mo-dependent repressors, VnfR1 and VnfR2, encoded by nearby genes.

Results

Transcription of vnfDGKEN is controlled by the ava4025 promoter

The *vnfDGKEN* genes are found in the genome of *A. variabilis* at one end of several clusters of genes that appear to be regulated by Mo. These Mo-regulated clusters include *vnfDGKEN*, separated by 5.2 kb from the vanadate transport genes, which are in turn separated by 13.3 kb from *vnfH* (Fig. 1A) (Thiel, 1993; Thiel, 1996; Pratte and Thiel, 2006; Pratte *et al*., 2006). Within this 32 kb region there are many genes of unknown function. Upstream of two of the structural genes for the V-nitrogenase, *vnfDG* and *vnfK* is *ava4025*. I-Tasser (Zhang, 2008; Roy *et al*., 2010; Xu *et al*., 2011) results suggested several periplasmic molybdate-binding proteins (ModA) with good matches to the predicted protein Ava4025 (see *Experimental procedures* for details): ModA from *Archaeoglobus fulgidus* (Hollenstein *et al*., 2007), from *Methanosarcina acetivorans* (Chan *et al*., 2010) and from *A. vinelandii* (Lawson *et al*., 1998). However, it is unlikely that *ava4025* encodes ModA because the *modA* gene in *A. variabilis* has been described and its product has been shown to function in Mo transport (Zahalak *et al*., 2004) and because of the apparent lack of a signal sequence in Ava4025 (see *Experimental procedures* for evidence), which is characteristic of other ModA proteins (Lawson *et al*., 1998; Hollenstein *et al*., 2007; Chan *et al*., 2010). The predicted structure for Ava4025, as predicted by I-Tasser (Xu *et al*., 2011), shows two globular domains linked by a hinge region, forming a cleft that could bind molybdate, a structure that is typical of the characterized ModA structures of *A. fulgidus, M. acetivorans* and *A. vinelandii* (Lawson *et al*., 1998; Hollenstein *et al*., 2007; Chan *et al*., 2010).

RT-PCR data (Fig. 1B) indicated that *ava4025* expression was repressed either by Mo or by fixed nitrogen, suggesting that it might be involved in the V-nitrogenase system. In order to identify the region controlling Mo repression of *ava4025*, strains with upstream fragments of *ava4025* fused to *lacZ* were constructed as shown in Fig. 1C (*frtBC* cross) and assayed for β-galactosidase activity in the absence of fixed nitrogen, in the presence of Mo or V, or in the presence of ammonium, in the absence of Mo and V (Fig. 2). Fragments of 196 bp (CH16; not shown), 367 bp (CH14; not shown) and 575 bp (CH12; Fig. 2) showed only background β-galactosidase activity.

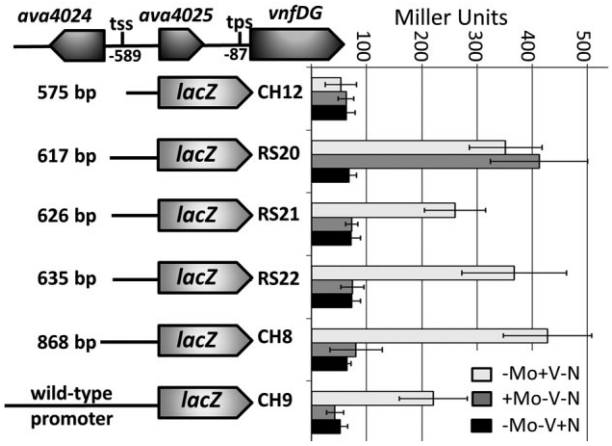

Fig. 2. β-Galactosidase assays of *ava4025* promoter fusions to *lacZ*. The indicated regions upstream of *ava4025* were fused to *lacZ* and inserted in the chromosome in the *frt* gene region, as shown in Fig. 1C (left panel). The wild-type promoter:*lacZ* strain (CH9) was the result of a promoter region cross (Fig. 1C, right panel) using the same plasmid that was used to make CH8. tss: transcriptional start site; tps: transcriptional processing site.

Transcription of *ava4025* required a region that began 617 bp upstream of the translation start site and repression by Mo required a slightly larger region of 626 bp. Fragments larger than 626 bp did not significantly affect expression of *lacZ*, suggesting that the region up to –626 bp was necessary and sufficient for Mo-regulated expression of *ava4025* (Fig. 2).

Based on these experiments, primers were designed to (i) identify the transcription start site of *ava4025* and (ii) reassess the previously described transcription start site of *vnfDG* located 87 bp upstream of the gene (Thiel, 1993). Transcript ends were determined by 5′ RACE (Bensing *et al*., 1996), which can distinguish between primary and processed transcripts because primary transcripts have a 5′ triphosphate that prevents efficient ligation of an RNA adapter to the 5′ end of the transcript. Therefore, ligation requires treatment of the RNA with tobacco acid pyrophosphatase (TAP), which hydrolyses the triphosphate to a monophosphate. Processed transcripts with a 5′ monophosphate do not require TAP for ligation to the adapter. If the ligation reaction works equally well with or without TAP, then the transcript is likely to be processed. We performed RNA ligase-mediated RT-PCR with primers specific for the regions upstream of *ava4025* and *vnfDG* and then recovered and sequenced the PCR-amplified cDNA bands. As shown in Fig. 3, the transcript for *ava4025*, beginning 589 bp upstream of the gene, required TAP and thus represents a primary transcript. However, the *vnfDG* product did not require treatment of the RNA with TAP (Fig. 3), indicating that the transcript was processed, probably from the transcript made from the promoter located upstream of *ava4025*. The processing site for *vnfDG*, located 87 bp upstream of the gene, corresponds to the site that was previously identified as the transcription start site, but is identified here as a transcript processing site (tps).

To determine the importance of *ava4025* in growth using the V-nitrogenase, a mutant with a deletion of 382 bp within *ava4025*, CHL4, was constructed (Fig. 4A). This ensured that while the gene itself was absent, the promoter for *ava4025*, driving expression of *vnfDG*, was present. The *ava4025* mutant grew as well as the parent strain, FD, using either the Mo-nitrogenase or the V-nitrogenase (Fig. 4B). However, a strain in which only the promoter region of *ava4025* was deleted (–803 to –242), RS40, (Fig. 4A) showed greatly impaired diazotrophic growth using the V-nitrogenase but normal growth using the Mo-nitrogenase (Fig. 4B). These data supported the hypothesis that the promoter of *ava4025* is required for good expression of the V-nitrogenase genes, but that Ava4025 itself is not required. In order to determine whether *ava4025* is required for growth using the V-nitrogenase, additional strains were constructed by fusing truncated upstream regions to *ava4025* and *vnfDG*. As shown in Fig. 1C, the same plasmids that were constructed to make upstream:*lacZ* fusions could also be

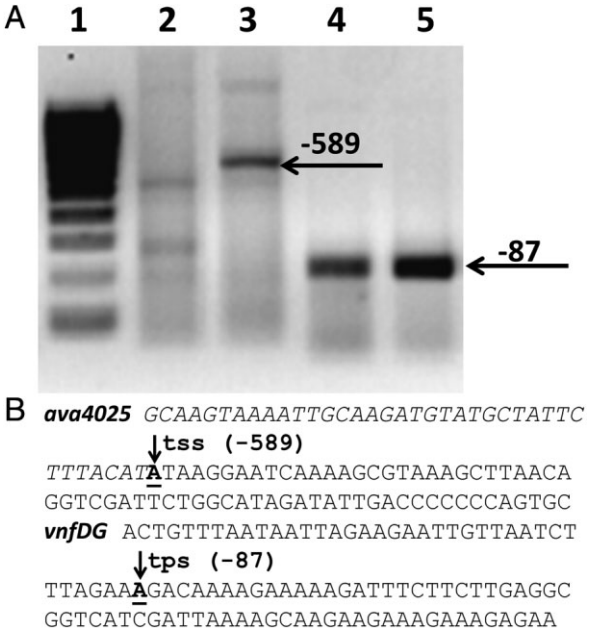

Fig. 3. Mapping transcriptional start sites or transcriptional processing sites.
A. 5′ RACE was used to amplify the 5′ ends of the RNA, pre-treated with TAP or not, for *ava4025* and *vnfDG*. 1. MW markers, 2. *ava4025* (–TAP), 3. *ava4025* (+ TAP), 4. *vnfDG* (-TAP), 5. *vnfDG* (+ TAP).
B. Sequences around the mapped sites as determined by sequencing the PCR products shown in (A). tss: transcriptional start site; tps: transcriptional processing site. The conserved motif upstream of the tss of *ava4025* (described in the Discussion section) is italicized.

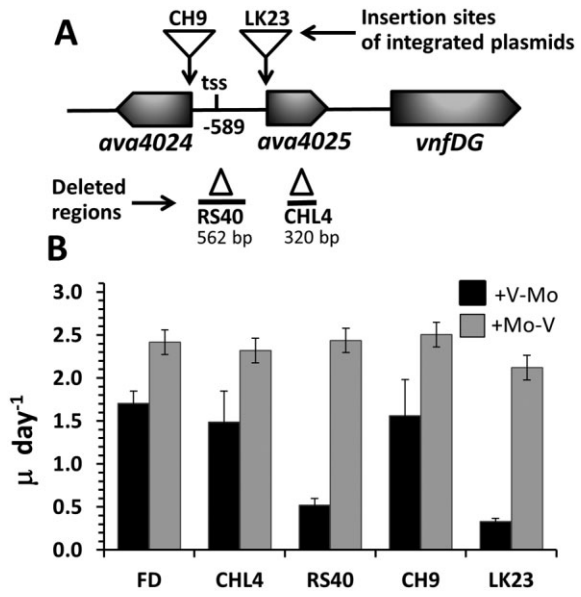

Fig. 4. Growth of *ava4025*/*vnfDG* promoter mutants.
A. Mutations in strains shown in graph. Lines below the map indicate the regions deleted (denoted by delta symbol) in CHL4 and RS40 by double-cross-over recombination. Insertion points above the map indicate where the vector portion of the integrating plasmid (pCH3 for strain CH9, pLK21 for strain LK23) integrated as a result of a promoter cross (see Fig. 1C).
B. Growth of mutants in media + Mo-V or + V-Mo, in the absence of fixed nitrogen. The rate constant μ was calculated as described in *Experimental procedures*.

used to construct strains in which these upstream regions drove expression of the native downstream genes in the chromosome. Exconjugant colonies were screened for the two alternative single-recombination events by their ability to grow using fructose in the dark (Frt^+). Strains that resulted from recombination in the upstream region (promoter cross) are Frt^+ and have the upstream fragment fused to the full *ava4025* gene in the chromosome. Strain CH9, in which a region beginning 868 bp upstream of *ava4025* drove expression of *ava4025* and *vnfDG* (Fig. 4A), showed normal growth with either the Mo-nitrogenase or the V-nitrogenase (Fig. 4B). However a strain, LK23, in which *vnfDG* was driven by a region beginning 1497 bp upstream of *vnfDG*, lacking the promoter of *ava4025* (Fig. 4A) grew very poorly using the V-nitrogenase, similarly to RS40 (see above) in which the *ava4025* promoter was deleted (Fig. 4B). Not only was growth very slow in strains RS40 and LK23, but the cultures became yellow, indicating nitrogen starvation. These data indicate that the promoter for *ava4025*, but not the *ava4025* gene product, is required for normal growth using the V-nitrogenase.

We confirmed that *vnfDG* was controlled by the promoter upstream of *ava4025* by qRT-PCR, measuring the amount of transcript for each gene in this apparent operon in the wild-type strain, FD, and in RS40, the strain lacking the *ava4025* promoter. RS40 had about 5% transcription of *vnfDG*, *vnfK*, *vnfE* and *vnfN* compared with FD, the parent strain (Fig. 5A). The fact that these five genes all require the functioning of the same promoter indicates that they are all part of the same operon, *ava4025-vnfDGKEN*. Comparison of the relative amounts of transcripts of all the genes in this operon revealed that *vnfDG* is present in much greater abundance than the other genes in the operon, particularly *ava4025* (Fig. 5B).

It is possible that Ava4025 is required for Mo-dependent repression of transcription of *vnfDG*, in which case the *ava4025* mutant would have no obvious phenotype. The amount of *vnfDG* mRNA was measured by qRT-PCR in the wild-type strain and the *ava4025* mutant (CHL4) grown in a medium with Mo. There was virtually no expression of *vnfDG* in either strain (data not shown) indicating that Ava4025 is not required for the repression of *vnfDG* by Mo.

Repression of the V-nitrogenase by VnfR1 and VnfR2

We have previously identified genes distant from *vnfDGKEN* that are important for the V-nitrogenase, including *vupABC*, encoding the vanadate transporter (Pratte and Thiel, 2006), and *vnfH*, which is one of the structural genes of the V-nitrogenase (Fig. 1A) (Pratte *et al*., 2006).

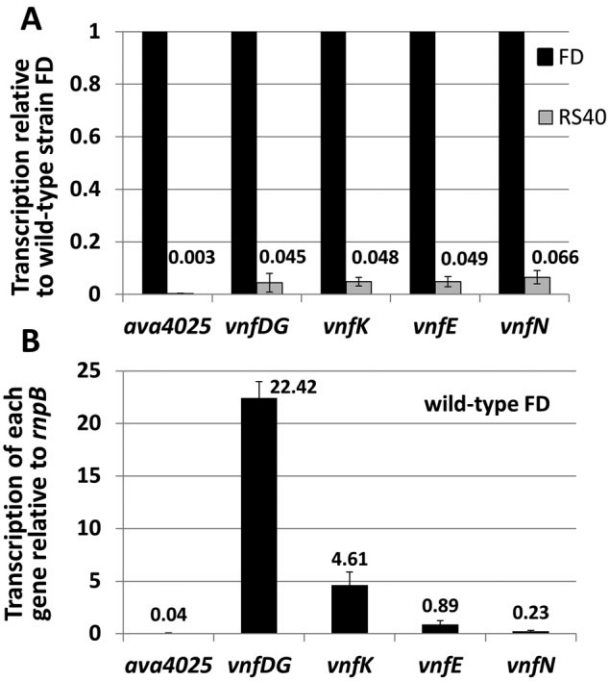

Fig. 5. qRT-PCR analysis of *vnf* gene expression.
A. The relative amount of transcript for each gene in RS40, a mutant lacking the promoter region of *ava4025*, compared with the parent strain FD.
B. The amount of transcript for each gene in strain FD normalized in each case to the constitutive gene *rnpB*.

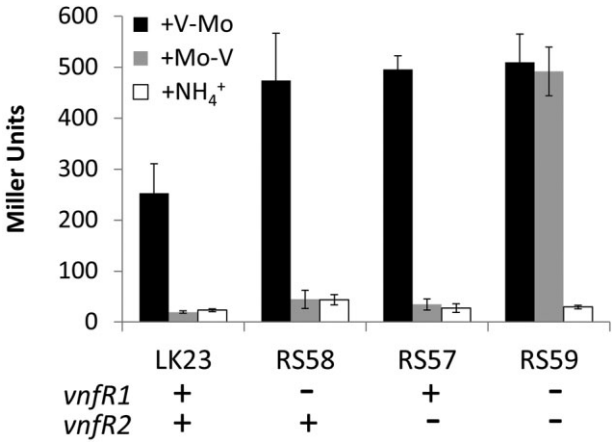

Fig. 6. Expression of *vnfDG* in *vnfR1* and *vnfR2* mutants. β-Galactosidase was measured in cells grown with V (no Mo), with Mo or with ammonium (no Mo). Using a promoter region cross (see Fig. 1C), the *vnfDG* gene was replaced in chromosome by *lacZ*, in a *vnfR1* mutant (RS58), in a *vnfR2* mutant (RS57), in a *vnfR1 vnfR2* double mutant (RS59) or in a wild-type background (LK23).

Upstream of *vupABC* is *ava4042*, which appears to be a regulatory gene, herein named *vnfR1* and upstream of *vnfH* there is a similar gene (73% amino acid identity), *ava4055*, herein named *vnfR2*. We have shown previously that the promoter for *vnfH* is located upstream of *vnfR2*, so these two genes are co-transcribed (Ungerer *et al*., 2010). Although these genes are not yet well characterized, both are repressed by Mo (Fig. 1B), and the protein products of the genes show a likely N-terminal helix–turn–helix (HTH) motif (see *Experimental procedures* for evidence) and, thus, are likely regulators of the *vnf* genes.

In order to determine whether either VnfR1 or VnfR2 represses transcription of the V-nitrogenase genes, we constructed *lacZ* fusions, replacing the *vnfDG* gene with *lacZ* in the chromosome by single-cross-over recombination between a plasmid containing a 1493 bp region upstream of *vnfDG* fused to *lacZ* (see Fig. 1C, promoter cross). These constructs were made in (i) a wild-type background producing strain LK23, (ii) a *vnfR1* partial deletion mutant background (BP396) producing strain RS58, (iii) a *vnfR2* insertion mutant (BP270) producing strain RS57 or (iv) a *vnfR1 vnfR2* double mutant background (BP484) producing strain RS59. In the presence of either VnfR1 or VnfR2 (LK23, RS57, RS58), expression of *lacZ* was inhibited by Mo or by fixed nitrogen; however, there was no inhibition by Mo in the double *vnfR1 vnfR2* mutant background (RS59) (Fig. 6). Repression by fixed nitrogen was not affected by the loss of either VnfR1 or VnfR2. Interestingly, expression of *lacZ* was lower in the absence of Mo in the strain that had both VnfR1 and VnfR2 (LK23), suggesting that these repressors may somewhat repress expression of *vnfDG* even in the absence of Mo. These *lacZ* fusion strains were also used for *in situ* localization of expression of β-galactosidase. The *vnfR1*$^{+}$ *vnfR2*$^{+}$ strain (LK23), the *vnfR1* (RS58) and the *vnfR2* (RS57) mutant strains all showed heterocyst-specific expression of *lacZ* that was repressed by Mo (Fig. 7). Only the *vnfR1 vnfR2* double mutant (RS59) showed expression of *lacZ* in the presence of Mo, and that expression was heterocyst specific, indicating that either VnfR1 or VnfR2 could repress expression from the *vnfDG* promoter in the presence of Mo.

The single and double mutants of *vnfR1* and *vnfR2* were used to determine their effect on diazotrophic growth. The single mutants of *vnfR1* (BP396) and *vnfR2* (BP270) showed no effect on growth using either the Mo-nitrogenase or the V-nitrogenase; however, the double mutant (BP484) was somewhat impaired in diazotrophic growth, especially when the strain was grown with Mo (Fig. 8B). VnfR1 and VnfR2 are not normally made in the presence of Mo so it is not clear why loss of both proteins would inhibit Mo-nitrogenase activity. Since overexpression of the V-nitrogenase in the double *vnfR1 vnfR2* mutant might inhibit Mo-nitrogenase activity, we compared growth of the double *vnfR1 vnfR2* mutant (BP484) with a *vnfR1 vnfR2 vnfDG* triple mutant (BP693). Loss of the V-nitrogenase in BP693 was evident from the inability of the strain to grow diazotrophically using V, but there was still inhibition of diazotrophic growth in the presence of Mo in BP693 (Fig. 8B). However, overexpression

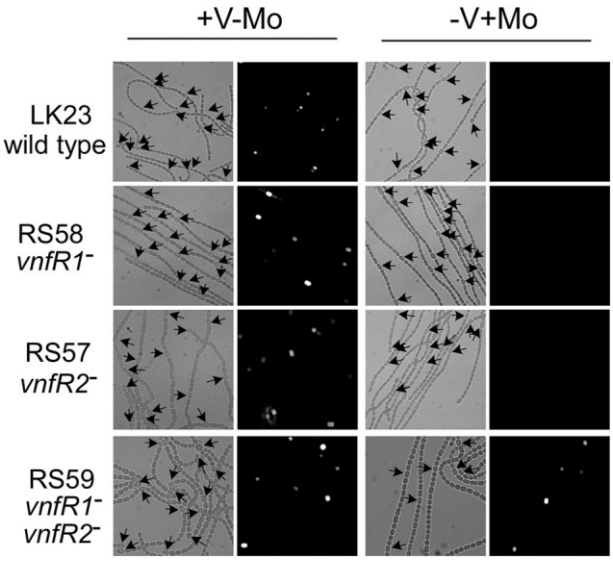

Fig. 7. *In situ* localization of expression of *vnfDG*. Using a promoter region cross (see Fig. 1C), the *vnfDG* gene was replaced in the chromosome by *lacZ* in a wild-type background (LK23), in a *vnfR1* mutant (RS58), in a *vnfR2* mutant (RS57) or in a *vnfR1 vnfR2* double mutant (RS59). β-Galactosidase was visualized with a fluorescent substrate in cells grown with V (no Mo) or with Mo. Left panels are bright-field images while the right panels are fluorescence images of the same field.

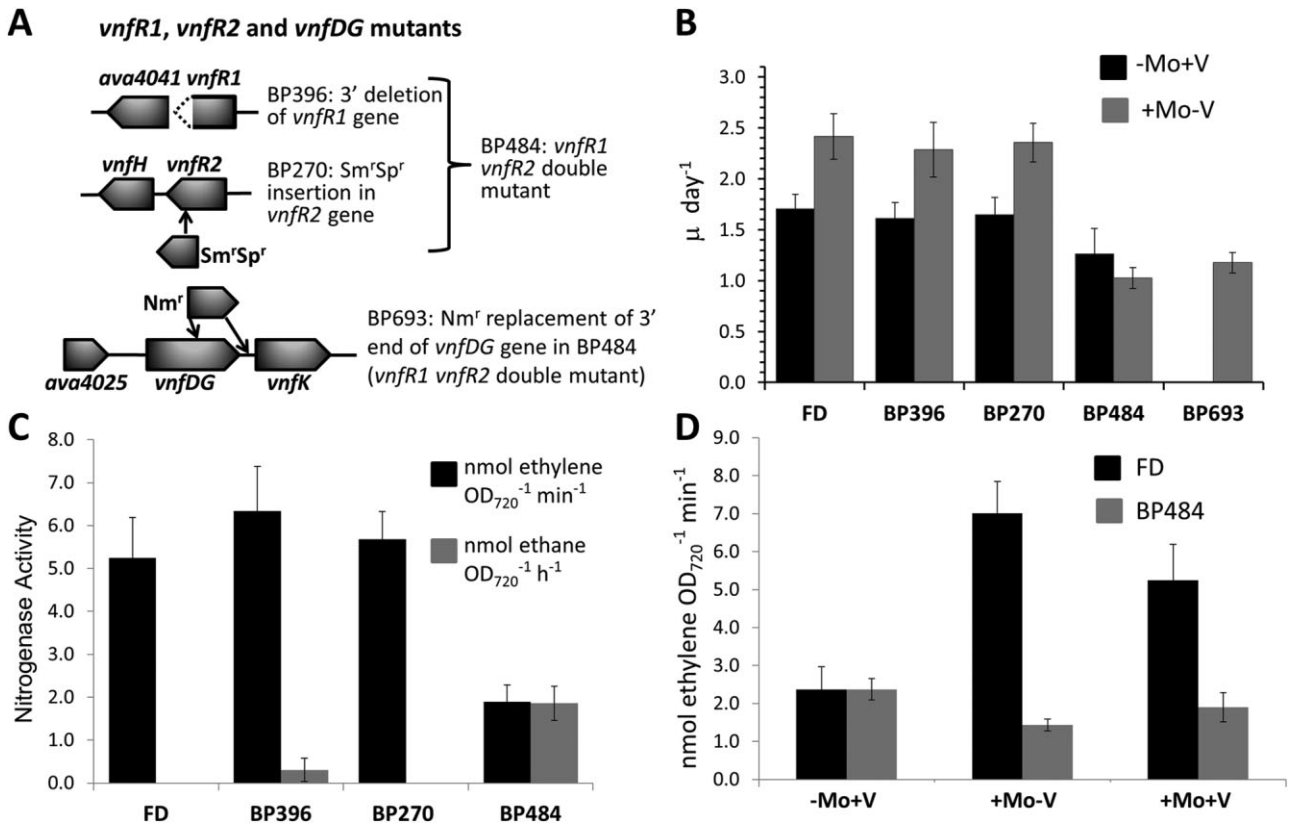

Fig. 8. Growth and nitrogenase activity of *vnfR1* and *vnfR2* mutants.
A. Map of the mutations.
B. Growth of the mutants. The rate constant μ was calculated (as described in *Experimental procedures*) for the wild-type FD, the *vnfR1* mutant (BP396), the *vnfR2* mutant (BP270), the *vnfR1 vnfR2* double mutant (BP484) and the *vnfR1 vnfR2* double mutant with a mutation in *vnfDG* (BP693).
C. Nitrogenase activity was measured by the reduction of acetylene to ethylene or ethane in the *vnfR1* mutant (BP396), the *vnfR2* mutant (BP270), the *vnfR1 vnfR2* double mutant (BP484) and the wild-type strain, FD in cells grown with V and Mo. (Note that ethane is measured per hour so that the numbers can be plotted on the same scale as ethylene, which is measured per min.)
D. Comparison of nitrogenase activity in the *vnfR1 vnfR2* double mutant (BP484) and the wild-type strain, FD, in cells grown with V, with Mo or with V and Mo.

of other *vnf*-associated genes that may also be controlled by VnfR1 or VnfR2 could affect diazotrophic growth in cells grown in the presence of Mo.

The effect of mutations in *vnfR1* and *vnfR2* was also measured by nitrogenase activity in the *vnfR1* (BP396), *vnfR2* (BP270), and the double mutant (BP484) grown with V or with Mo, which normally inhibits transcription of the V-nitrogenase genes (Fig. 1B). To distinguish between Mo-nitrogenase and V-nitrogenase activity, the reduction of acetylene to ethylene and to ethane was measured. While both enzymes reduce acetylene to ethylene, only the V-nitrogenase produces ethane (Dilworth *et al.*, 1988). Like the wild-type strain, the *vnfR2* mutant (BP270), produced no ethane in the presence of Mo and V, indicating no V-nitrogenase activity; however, the *vnfR1* mutant (BP396) produced small amounts of ethane and the double mutant (BP484) produced large amounts of ethane, indicating that the V-nitrogenase was functional (Fig. 8C). Consistent with the results of the growth experiments (Fig. 8B), the *vnfR1 vnfR2* double mutant (BP484) was impaired in nitrogen fixation and produced much less ethylene in the presence of Mo, with or without V, than the wild-type strain (Fig. 8D). Inhibition of nitrogenase activity was not observed in BP484 grown with V alone, consistent with the normal growth of this strain with V (Fig. 8B). Thus, the problem with growth in the *vnfR1 vnfR2* double mutant appears to be the result of impaired Mo-nitrogenase activity.

Since VnfR1 and VnfR2 appear to be regulatory proteins that repress expression of *vnfDG* from the promoter upstream of *ava4025*, we determined whether they bound to a 192 bp region upstream of the transcription start site of *ava4025* that included the region that was required for repression by Mo (–702 to –511; Fig. 2). VnfR1 bound specifically to this region; however, the binding was not affected by removing Mo from the reaction (Fig. 9A). In

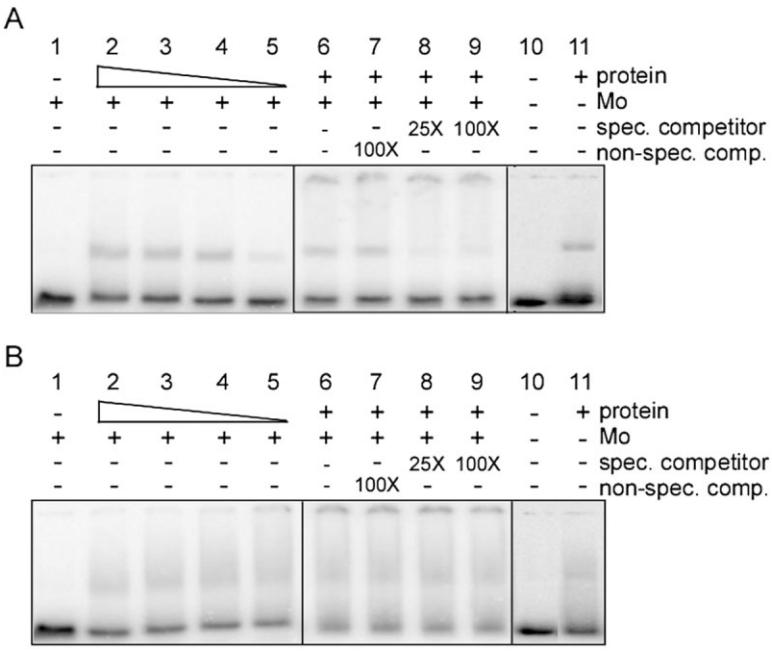

Fig. 9. Mobility shift assays with VnfR1 and VnfR2.
A. Purified VnfR1 protein was bound to a ^{32}P labelled, 192 bp fragment containing the promoter region of *ava4025*. Lanes: 1, control without protein; 2–5, decreasing protein (0.2, 0.15, 0.10, 0.05 nmoles); 6–9 and 11, with 0.2 nmoles VnfR1; 7, 100× non-specific competitor DNA; 8–9, increasing amounts of specific cold competitor DNA; 10–11, same as 1–2 except there was no Mo present.
B. Same as (A) except that purified VnfR2 protein was used. Lanes 2–5, decreasing VnfR2 protein (0.28, 0.21, 0.14, 0.10 nmoles); 6–9 and 11 with 0.28 nmoles VnfR2.

contrast binding of VnfR2 to this region was weak and did not appear to be specific (Fig. 9B). This could be due to the fact that both proteins were refolded from inclusion bodies and that only VnfR1 was refolded properly after the proteins were solubilized from inclusion bodies by urea (see *Experimental procedures*).

Discussion

A number of genes in a 32 kb region of the genome of *A. variabilis* are repressed by Mo and several are known to be involved in the ability of this strain to fix nitrogen in the absence of Mo using the V-nitrogenase, including the structural genes, *vnfDG* and *vnfK*, for dinitrogenase (Thiel, 1993) and *vnfH*, encoding dinitrogenase reductase (Pratte *et al*., 2006); *vnfE* and *vnfN*, likely encoding the scaffolding proteins, analogous to NifE and NifN (Thiel, 1996); and *vupABC*, encoding the transporter of vanadate (Pratte and Thiel, 2006). We have shown here that *ava4025*, immediately upstream of *vnfDG* is the first gene of this operon and that the operon includes *vnfEN*; however, *vnfDG* transcript is present in much greater abundance than is transcript for the other genes in the operon, including *ava4025*, *vnfE* and *vnfN*. Since *vnfE* and *vnfN* are made from the same promoter as *vnfDG* it is possible that the low levels of transcript for *vnfE* and *vnfN* reflect post-transcriptional control, such as another processing event upstream of *vnfEN* and this may explain the fact that a *vnfDG* probe failed to identify a transcript large enough to include all the genes in the operon by Northern hybridization (Thiel, 1993).

Although the function of *ava4025* is still not known, since the mutant had no observable phenotype, the fact that its promoter serves as the promoter for the operon suggests that it plays some role in diazotrophic growth with the V-nitrogenase. Its transcript is present in only very small amounts since it accumulates to only about 0.2% of the level of *vnfDG*. The similarity of the Ava4025 protein to the periplasmic Mo-binding protein of the Mo transport system suggests that it might play a role in sensing Mo in the cell, which could be important to the cell in making the decision of whether to switch from the Mo-nitrogenase to the V-nitrogenase; however, Ava4025 is not required for Mo repression of *vnfDGKEN*. The Mo repression of the *ava4025* promoter depends both on a region located about 35 bp upstream from the transcription start site and on the presence of at least one of the two regulatory proteins, VnfR1 or VnfR2, whose synthesis was also repressed by Mo. The region upstream of the transcription start site for *ava4025* includes a motif (GCAAGTAAAATTGCAAGAT GTATGCTATTCTTTACAT) (see italicized sequence in Fig. 3B) identified using the program MEME (Bailey *et al*., 2009) that is also conserved at the same position in the *vnfR2* promoter (*ava4055* gene in Ungerer *et al*., 2010) and was identified upstream of *vnfR1* and *ava4051*, a gene of unknown function, using the program FIMO (Grant *et al*., 2011). It is likely that this motif is involved in Mo repression, since only Mo and not V is involved in regulation of transcription of *vnfDGK* (Thiel, 1993) or *vnfH* (Pratte *et al*., 2006). For the strains in which *lacZ* was fused to the region upstream of *ava4025*, induction of expression in a medium lacking Mo and V gave the same levels of expression as

induction in a medium with V alone (data not shown), indicating that V is also not involved in regulation of the *ava4025* promoter. Inhibition of expression of *ava4025* by fixed nitrogen was not affected by loss of either the site or the proteins required for Mo repression. One possible explanation is that control by fixed nitrogen occurs only through a regulator or cellular conditions that are specific to heterocysts, and that nitrogenase genes in heterocysts are expressed by default, unless, like the *vnf* genes, they are repressed by Mo. The *in situ* localization pictures showed that the *vnf* genes were expressed only in heterocysts, even in the absence of both repressor proteins, supporting the idea that localization of *vnf* gene expression to heterocysts is controlled independently from Mo regulation.

VnfR1 and VnfR2 represent novel repressors. They are similar proteins with an N-terminal HTH motif and their expression is repressed by Mo. Since repression of the *ava4025* promoter depends on a specific DNA region, at least one of these proteins, and on Mo, it is possible that these proteins bind Mo *in vivo*. It is not clear why there are redundant proteins to control expression of *vnfDGKEN*, but the nitrogenase assays suggest that VnfR1 might be the stronger repressor of the *vnfDGKEN* genes. The *vnfR2* mutant, with only VnfR1, produced no ethane in the presence of Mo indicating that the V-nitrogenase was not made, while the *vnfR1* mutant with only VnfR2 present produced some ethane in the presence of Mo indicating that the V-nitrogenase was made in low amounts. Thus, the two proteins may bind with different affinities to the promoter upstream of *ava4025*.

We have shown previously that the 5′ ends of the transcripts carrying *vnfH* and *nifH1* are the result of transcriptional processing (Ungerer *et al*., 2010). In the case of *vnfH*, the true promoter is just upstream of *vnfR2* (Fig. 1). In the case of *nifH1*, the primary promoter appears to be several kb upstream, before the *nifB1* gene, with a secondary weak promoter in *nifU1*, the gene upstream of *nifH1* (Ungerer *et al*., 2010). We have shown here that there is a transcriptional processing site upstream of *vnfDG*, and that transcription is driven by the *ava4025* promoter. It is notable that *nifH* is known to be a very highly expressed gene compared with *nifB* (Ehira *et al*., 2003; Flaherty *et al*., 2011) and we have shown here by qRT-PCR that *vnfDG* transcript accumulates to high levels relative to *ava4025*. Since the amount of transcript should remain constant or possibly decline somewhat with distance from the promoter, it appears that the transcriptional processing site may increase the stability of the *vnfDG* transcript compared with *ava4025*. The data provided here indicate that *ava4025*, *vnfDG*, *vnfK*, *vnfE* and *vnfN* form an operon, all under the control of the *ava4025* Mo-repressible promoter. Regulation of this operon includes developmental control by heterocyst formation, transcriptional control by Mo-dependent repressors, and post-transcriptional control by RNA processing.

Experimental procedures

Growth

Anabaena variabilis strain FD, a derivative of *A. variabilis* ATCC 29413 that can grow at 40°C, was maintained on agar-solidified Allen and Arnon (AA) medium (Allen and Arnon, 1955) supplemented, when appropriate, with 5 mM NH_4Cl, 10 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), pH 7.2, 25–40 μg ml^{-1} neomycin sulphate (Nm), 5 μg ml^{-1} erythromycin (Em) or 3 μg ml^{-1} each of spectinomycin (Sp) and streptomycin (Sm). Strains were grown photoautotrophically in liquid cultures in an eightfold dilution of AA medium (AA/8) or in AA/8 supplemented with 5 mM NH_4Cl and 10 mM TES, pH 7.2, at 30°C, with illumination at 100–120 μE m^{-2} s^{-1}. Antibiotics, when used, included Nm (5 μg ml^{-1}) and Sp (0.3 μg ml^{-1}) and Em (5 μg ml^{-1}). For experiments performed in the absence of Mo, strains were starved for Mo for at least 10 generations in Mo-free medium that was first prepared as described previously (Thiel, 1993) and then conditioned (by growth with strain FD followed by filtration to remove the cyanobacteria) to remove residual Mo. This medium contained less than 5×10^{-10} M Mo as determined by ICP-AES spectroscopy. For growth with V, Mo-starved cells were supplemented with 1 μM Na_3VO_4.

For growth assays, OD_{720} was measured during exponential growth over three cell divisions, which required that growing cells be diluted to an OD_{720} of about 0.02 in appropriate medium (2.0 ml of diluted culture in each well of a 12-well plate) at the start of the experiment. Growth was observed to be exponential up to an OD_{720} of about 0.2 and then became linear, but growing eventually to OD_{720} of about 2.5, as the cells became light limited by self-shading. Growth was measured every 3 h and the exponential growth data were used to calculate $\mu = [\ln(N2) - \ln(N1)]/(t2 - t1)$ where $N = OD_{720}$ and t = time in days. Growth experiments were performed using triplicate cultures; the average and standard deviation are shown.

Construction of strains

Cyanobacterial mutants were created using the same general method described here. Specific information for the construction of each strain is included in Supplement Table S1. Generally the gene or region of interest was cloned into appropriate restriction sites in a vector, inactivated by deletion of a region or by the insertion of an antibiotic resistance gene, and then made mobilizable for conjugation by the insertion a 5 kb fragment of pRL2948a or the similar plasmid pRL1075, containing the oriT site for mobilization, *sacB* for sucrose selection of double recombinants, and an Em^r gene (Black *et al*., 1993). The corresponding cyanobacterial mutant strain was created by introducing the mobilizable plasmid into strain FD, or an existing mutant, by conjugation as previously described (Elhai and Wolk, 1988). Single recombinants were selected by Em^r, followed by *sacB* selection on 10% sucrose

plates for double recombinants. PCR was used to verify that there were no wild-type copies of the gene or region of interest.

The strains with promoter fusions to *lacZ* were created as follows: FD chromosomal DNA was used with specific primers (Supplement Table S2) to PCR-amplify 185 bp, 356 bp, 564 bp, 606 bp, 615 bp, 624 bp and 856 bp fragments from the *ava4025* upstream region and 1497 bp and 2364 bp fragments from the *vnfDG* upstream region. PCR products were ligated into the BglII and SmaI sites of pJU411 located just upstream of the promoterless *lacZ* (a simplified diagram of pJU411 is shown in Fig. 1C), sequenced and conjugated into FD, selecting for Nm^r exconjugants. Strains resulting from a single cross-over in the *frt* region were identified by screening for colonies that were unable to grow in the dark with fructose (Fig. 1C).

RNA isolation, RT-PCR and qRT-PCR

Prior to RNA isolation strains were first starved for Mo in the absence of fixed nitrogen, then switched to Mo-free AA/8 supplemented with 5 mM NH_4Cl and 10 mM TES, pH 7.2 for five generations. Cultures, OD_{720} of 0.2–0.3, were washed free of nitrogen and then grown for an additional 24 h with or without ammonium and containing either (i) 1 μM Na_3VO_4 or (ii) 1 μM Na_2MoO_4. RNA was isolated using Tri-Reagent (Sigma) as previously described (Ungerer *et al.*, 2010). DNA was removed using a Turbo DNA-free kit (Ambion, Austin, TX). Reverse transcription-PCR (RT-PCR) was performed on RNA as previously described (Ungerer *et al.*, 2008). Gene-specific primers (described in supplemental Table S2) were used: *ava4025*, Ava4025-L/Ava4025-R; *vnfDG*, vnfD1-L/vnfD1-R; *vnfE*, VnfERT3-L/VnfERT3-R; *vupA*, wabcA-L/wabcA-R; *ava4041*, Ava4041-L/Ava4041-R; *vnfR1*, Moelike1-L/Moelike1-R; *vnfH*, nifH3L/nifH123R(New); and *vnfR2*, Moelike2-L/Moelike2-R.

qRT-PCR was performed to quantify *ava4025*, *vnfDG*, *vnfK*, *vnfE* and *vnfN* transcripts. cDNA was made from 500 ng of DNase-treated RNA from FD and RS40 grown with either V or Mo using Bio-Rad iScript Reverse Transcription Super Mix (170–8841) in a 10 μl reaction. cDNA was diluted to 5 ng μl^{-1}. qPCR reactions were done using SsoAdvanced SYBR® Green SuperMix in a 10 μl reaction containing 20 ng of cDNA, 5 pmol of gene-specific primers, and 1x reaction supermix. The following gene-specific primers (described in supplemental Table S2) were used: *ava4025*, qava4025-R/qava4025-L; *vnfDG*, qvnfDG-R/qvnfDG-L; *vnfK*, qvnfK-R/qvnfK-L; *vnfE*, qvnfE-R/qvnfE-L; and *vnfN*, qvnfN-R/qvnfN-L. qPCR reactions were run with initial denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 10 s, 55°C for 30 s and 72°C for 30 s. Data analysis was performed using Bio-Rad CFX Manager 3.0. The average and standard deviation of triplicate biological samples are shown.

5′ RACE

5′ RACE, with and without tobacco acid pyrophosphatase (TAP), was performed as described previously (Bensing *et al.*, 1996; Ungerer *et al.*, 2010). RNA was reverse transcribed with Superscript III (Invitrogen) using the following gene-specific primers (described in supplemental Table S2): *ava4025*, Ava4025-R(-mut1); and *vnfDG*, vnfD-PE1R. The P1 left primer was used for PCR along with the following right primers: *ava4025*, pAva4025-R; and *vnfDG*, vnfD-PE2R. The PCR bands were excised and sequenced to determine the 5′ ends.

Bioinformatics analysis

A summary of protein characteristics, as determined by bioinformatics software, is provided for all deduced proteins in sequenced genomes at the JGI/DOE genome website IMG (Markowitz *et al.*, 2012). In addition, several programs were used to supplement or verify the data provided by IMG. Analysis of Ava4025 by I-Tasser (Xu *et al.*, 2011) indicated that several periplasmic molybdate-binding proteins are good matches to Ava4025. These include ModA from *A. fulgidus* (Hollenstein *et al.*, 2007) (PDB entry: 2onsA; Tm = 0.832), from *M. acetivorans* (Chan *et al.*, 2010) (PDB entry: 3k6xA; TM = 0.826) and from *A. vinelandii* (Lawson *et al.*, 1998) (PDB entry: 1ATG, TM-0.823). A TM score above 0.5 indicates that the model has the correct topology and a TM score less than 0.17 indicates only random similarity (Xu and Zhang, 2010). The I-Tasser alignments showed that these bacterial ModA proteins with strong similarity to Ava4025 matched well structurally along about 90% of the length, but there was no match between Ava4025 and ModA proteins for the first 20 aa that comprise the signal sequence of ModA. The absence of a signal sequence in Ava4025 was further supported by analysis of the protein using the program SignalP (Dyrløv Bendtsen *et al.*, 2004), which indicated that Ava4025 lacks a signal sequence (D = 0.165; cut-off = 0.57). In contrast, SignalP predicted an N-terminal signal peptide for the ModA protein *of A. variabilis* (D = 0.786; cut-off = 0.57). Ava4042 (VnfR1) and Ava4055 (VnfR2) show a likely N-terminal HTH motifs similar to the XRE family of proteins, according to the program SMART (Letunic *et al.*, 2012) ($E = 1.6 \times 10^{-14}$ for VnfR1 and $E = 9.1 \times 10^{-16}$ for VnfR2), and both belong to the PFAM (Finn *et al.*, 2010) periplasmic binding domain protein (PBP) superfamily ($E = 3.3 \times 10^{-61}$ for VnfR1 and $E = 4.2 \times 10^{-56}$ for VnfR2). The HTH motif spans aa 10–65 in both proteins while the PBP domains cover aa 160–349 (VnfR1) and 161–350 (VnfR2). A conserved DNA motif in the promoter region of *ava4025* (GCAAGTAAAATT-GCAAGATGTATGCTATTCTTTACAT) was identified using the program MEME (Bailey *et al.*, 2009). Other instances of very similar motifs (*P*-values of 10^{-17} to 10^{-22}) were identified upstream of *vnfR1*, vnfR2 and *ava4051* using the program FIMO (Grant *et al.*, 2011).

Acetylene reduction assays

Strains for acetylene reduction assays were starved for Mo by growing in Mo-free AA/8. Mo-starved cells were switched to Mo-free AA/8 supplemented with 5 mM NH_4Cl and 10 mM TES, pH 7.2, for approximately 5 generations to an OD_{720} of 0.2–0.3, washed and used to start cultures containing 1) 1 μM Na_3VO_4, 2) 1 μM Na_2MoO_4, or 3) 1 μM Na_3VO_4 and 1 μM Na_2MoO_4. Acetylene reduction assays were performed after 24 h by gas chromatography as previously described (Shah *et al.*, 1975; Pratte *et al.*, 2006). The average and standard deviation of triplicate biological samples are shown.

β-Galactosidase assays and in situ *localizations*

Strains for β-galactosidase assays were starved in Mo-free AA/8, and then switched to Mo-free AA/8 supplemented with 5 mM NH_4Cl and 10 mM TES, pH 7.2 for approximately 5 generations. Cultures, (OD_{720} of 0.2–0.3) were washed free of nitrogen. Cells were used to start cultures containing (i) 1 μM Na_3VO_4, (ii) 1 μM Na_2MoO_4 or (iii) 5 mM NH_4Cl and 10 mM TES. These cultures were grown for 24 h before being used for β-galactosidase assays. β-Galactosidase assays were performed as described previously (Miller, 1972; Thiel *et al*., 1995). The average and standard deviation of triplicate biological samples are shown.

For *in situ* localization, the cells were washed twice with water and fixed for 15 min at 25°C with 0.04% glutaraldehyde. Cells were washed twice with water to remove glutaraldehyde and incubated in the dark at 37°C for 30 min with 100 μM 5-dodecanoyl-aminofluorescein di-β-D-galactopyranoside in 25% dimethyl sulphoxide. Excess substrate was removed by washing twice with water, and cells were resuspended in Vectashield® (Vector Laboratories), an antibleaching agent. The cells were imaged on a Zeiss epifluorescence microscope using a fluorescein filter set (excitation, 450–490 nm; dichroic, 510 nm; barrier, 520 nm) with a 560 nm short-pass filter. Images were taken using a Retiga EXi (QImaging) cooled charge-coupled device camera with IP Labs 4.0 software (BD Biosciences).

VnfR1 and VnfR2 protein production

PCR products containing *vnfR1* and *vnfR2* open reading frames were cloned into pET22b and overexpressed in BL21 Tuner DE3 cells (EMD Biosciences). Each protein was overexpressed and purified from inclusion bodies essentially as previously described (Sambrook, 2001). Cells were harvested after IPTG induction at 37°C and resuspended in lysis buffer [25 mM Tris pH 8.0, 100 mM NaCl, 0.02% sodium azide, 1 mM phenylmethanesulphonyl fluoride (PMSF), 10 mM $MgCl_2$, 100 U ml^{-1} DNase I, 1 mM dithiothreitol (DTT), 1% Triton X-100, 30% glycerol, and 3.0 mg ml^{-1} lysozyme] and incubated at 4°C with one min vortexing every two min until the viscosity of the solution increased. The lysates were centrifuged at 4°C for 5 min at 5000 *g* to pellet the inclusion bodies, which were then washed twice in wash buffer (25 mM Tris pH 8.0, 4 M urea, 100 mM NaCl, 1 mM PMSF, 10 mM $MgCl_2$, 1 mM DTT, 1% triton X-100 and 30% glycerol) with a 5 min incubation between each centrifugation. The washed inclusion bodies were resuspended in inclusion body solubilization buffer (50 mM Tris-HCl pH 8.0, 6 M guanadine-HCl, 300 mM NaCl, 10% glycerol, 2 mM $Na_2MoO_4{\cdot}2H_2O$, 10 mM DTT, 1 mM PMSF). The dissolved protein was then dialysed against refolding buffer (50 mM Tris pH 8.0, 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 20% glycerol) with decreasing concentrations of urea (6 M for one h, 5 M for 1 h, 4 M for 1 h, 3 M for 1.5 h, 2 M for 1.5 h and 1 M for 3 h), after which the protein solutions were dialysed overnight against protein storage buffer (50 mM Tris pH 8.0, 200 mM KCL, 40% glycerol). The proteins were stored at –80°C. Protein concentrations were quantified using a Bradford assay (Sambrook, 2001).

Electrophoretic mobility shift assays

A 192 bp DNA fragment that included the promoter and the region upstream of the promoter of *ava4025* was amplified using the primers ava_4025MS-(L/R), phosphatased, and end-labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The reaction was desalted and 5 fmoles of γ-^{32}P-labelled DNA was incubated with varying amounts of VnfR1 or VnfR2 recombinant native protein (see legend to Fig. 9 for amounts used in experiments) for 30 min in EMSA binding buffer [12.5 mM Tris-HCl pH 7.2, 40 mM NaCl, 12.5% glycerol, 40 μg ml^{-1} poly(deoxyinosinic-deoxycytidylic) acid sodium salt, 5 mM $Na_2MoO_4{\cdot}2H_2O$, and 0.4 mg ml^{-1} BSA]. Competition experiments utilized 25-fold or 100-fold excess DNA. The non-specific competitor was a PCR product amplified from *rnpB* of *A. variabilis* using the primers rnpB(L/R), and the specific competitor was unlabelled DNA amplified from the promoter of *ava4025* using the primers ava_4025MS-(L/R) (described in supplemental Table S2). After incubation the reactions were analysed on 4% polyacrylamide gels in 1× TGB (25 mM Tris, 192 mM glycine, pH 8.3) supplemented with 1 mM $Na_2MoO_4{\cdot}2H_2O$ and run at 50 V at 4°C. For Mo-free conditions $Na_2MoO_4{\cdot}2H_2O$ was replaced with 1 mM Na EDTA in the EMSA binding buffer, the gel and the running buffer.

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