

Transcriptional Profiling of Nitrogen Fixation in *Azotobacter vinelandii*^{∇†}

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Most biological nitrogen (N₂) fixation results from the activity of a molybdenum-dependent nitrogenase, a complex iron-sulfur enzyme found associated with a diversity of bacteria and some methanogenic archaea. *Azotobacter vinelandii*, an obligate aerobe, fixes nitrogen via the oxygen-sensitive Mo nitrogenase but is also able to fix nitrogen through the activities of genetically distinct alternative forms of nitrogenase designated the Vnf and Anf systems when Mo is limiting. The Vnf system appears to replace Mo with V, and the Anf system is thought to contain Fe as the only transition metal within the respective active site metallocofactors. Prior genetic analyses suggest that a number of *nif*-encoded components are involved in the Vnf and Anf systems. Genome-wide transcription profiling of *A. vinelandii* cultured under nitrogen-fixing conditions under various metal amendments (e.g., Mo or V) revealed the discrete complement of genes associated with each nitrogenase system and the extent of cross talk between the systems. In addition, changes in transcript levels of genes not directly involved in N₂ fixation provided insight into the integration of central metabolic processes and the oxygen-sensitive process of N₂ fixation in this obligate aerobe. The results underscored significant differences between Mo-dependent and Mo-independent diazotrophic growth that highlight the significant advantages of diazotrophic growth in the presence of Mo.

Biological nitrogen fixation, the reduction of dinitrogen (N₂) to ammonia, is an essential reaction in the global nitrogen (N) cycle. Biological N₂ fixation accounts for roughly two-thirds of the fixed N produced on Earth and is catalyzed by the nitrogenase complex (54). Homologs of nitrogenase have been identified in a diversity of bacteria and in several lineages of methanogenic archaea (8, 53). Most present-day biological N₂ fixation is catalyzed by molybdenum (Mo) nitrogenase (encoded by *nifHDK*), an oxygen-sensitive metalloenzyme complex composed of the Fe protein (product of *nifH*) and the MoFe protein (product of *nifDK*) (9). The Fe protein is a homodimer bridged by an intersubunit [4Fe-4S] cluster that serves as the obligate electron donor to the MoFe protein (23). The MoFe protein is an $\alpha_2\beta_2$ heterotetramer that houses the P clusters, [8Fe-7S] clusters that shuttle electrons to the FeMo cofactors (FeMo-co). The FeMo-co are [Mo-7Fe-9S-homocitrate] clusters where substrate reduction occurs (44). Two genetically distinct “alternative” forms of nitrogenase have been identified in a subset of diazotrophs that also encode *nif* (8, 53, 61). The nitrogenase encoded by the *vnfHDK* genes contains vanadium in place of molybdenum in the active-site cofactor,

whereas the nitrogenase encoded by the *anfHDK* genes appears to contain Fe as the only metal constituent of its active-site cofactor (11, 24). In some diazotrophs, the expression and activity of the alternative forms are regulated by the availability of Mo or V when fixed N is also limiting (34, 35); in contrast, at least one diazotroph synthesizes the alternatives in the absence of fixed N when the Mo-dependent nitrogenase is not functional, regardless of trace metal availability (48). Biochemical analysis indicates that the N₂ reduction rates for the Mo nitrogenase are much higher than the rates observed for the alternative forms (19). In addition, a larger proportion of the electron flux during nitrogen reduction is directed toward proton reduction by the alternative forms of nitrogenase than toward proton reduction by Mo nitrogenase (20). Although the alternative nitrogenases are genetically distinct, they are mechanistically similar to Mo nitrogenase. Because neither the Vnf nor the Anf system contains a complete suite of genes necessary for the maturation of the corresponding catalytic partners, it is likely that significant cross talk occurs either between or among the different systems during the assembly and regulation of these different enzyme complexes (36, 41).

Azotobacter vinelandii is an obligately aerobic member of the class *Gammaproteobacteria* that is common in terrestrial soils sampled from across the world (42) and is known to produce both alternative forms of nitrogenase in addition to Nif (32, 34, 35). Owing to its genetic tractability, *A. vinelandii* has emerged as the principal model organism for exploring the nitrogenase mechanism, the assembly of complex metalloclusters, and the

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regulatory features that coordinate the differential expression of the large number of genes associated with N₂ fixation (43). The recently completed *A. vinelandii* genome sequence (59), in combination with the results of extensive biochemical and genetic characterization (49, 54, 58), has implicated at least 82 gene products in the formation and regulation of the three forms of nitrogenase (17). In addition to the energetic demands associated with maintaining these 82 genes in *A. vinelandii*, the nitrogenase reaction is energetically demanding, requiring 16 molecules of ATP and 8 electrons for the reduction of a single molecule of N₂. These high metabolic costs, in conjunction with the demands for fixed N in growing cells and the requirement to protect the nitrogenase system from oxidative inactivation, presumably imposed a strong selective pressure to efficiently integrate N₂ fixation with other cellular metabolic processes.

In the present study, we examined patterns of global gene expression in *A. vinelandii* when it is cultured under N-replete conditions (fixed-N source is readily available) or when cells were grown under N₂-fixing conditions (fixed-N source is limiting) in several experimental iterations that individually favored the expression of the *nif*, *vnf*, or *anf* system. Mo-dependent diazotrophy was maintained by growing cells in medium lacking fixed N and amended with excess molybdate (Mo-dependent diazotrophy). Diazotrophic growth using the V-dependent nitrogenase was achieved by growth in medium lacking fixed N and molybdate and amended with excess vanadate (V-dependent diazotrophy). Diazotrophic growth using the Fe-only nitrogenase was achieved by growth in medium lacking fixed N, molybdate, and vanadate (heterometal-independent diazotrophy). Because alternative nitrogenase-dependent diazotrophic growth of *A. vinelandii* occurs only in the absence of even trace levels of Mo, reverse transcriptase PCR (RT-PCR) was used to confirm the presence of transcripts of the appropriate alternative nitrogenase structural genes under the given cultivation conditions prior to transcriptome analysis. High-throughput cDNA sequencing revealed distinct patterns of transcript levels in cells cultivated under the various growth conditions examined. These results have broad implications for the physiology of the diazotrophic growth of *A. vinelandii* and the evolution of nitrogenase.

MATERIALS AND METHODS

Strains and growth conditions. *A. vinelandii* DJ was cultured at 30°C with rotary shaking (200 rpm) in modified liquid Burk medium (6). Medium designed to favor growth under N-replete conditions was amended with 25 mM filter-sterilized ammonium acetate (NH₄OAc). For alternative nitrogenase-dependent diazotrophic growth, Mo-deficient medium was prepared as previously described and NH₄OAc was not added (6). For V-dependent diazotrophy, V₂O₅ was added to N- and Mo-deficient medium to a final concentration of 1 μM. Cells were grown in triplicate under each condition, and nitrogenase activity was monitored by acetylene reduction (25). Expression of genes encoding each type of nitrogenase was confirmed by RT-PCR using the AccessQuick RT-PCR system (Promega Corp., Madison, WI) according to the manufacturer's protocol. The targets and primers used for RT-PCR are listed in Table S10 in the supplemental material.

Preparation of total RNA, cDNA library constructions, and SOLiD sequencing. After subculturing for at least three transfers under the specified growth conditions, cells from 30-ml cultures at an optical density at 600 nm of 0.7 were harvested by centrifugation at 10,000 × g at 4°C for 10 min. Cell pellets were flash-frozen in liquid N₂ and stored at -80°C until further processed. Total RNA was prepared from cell pellets from each of the four conditions as previously described (45). Total RNA (0.5 μg) from each of the four cell growth conditions

was submitted to the Genomics Core Facility at The Pennsylvania State University (University Park, PA) for cDNA library construction and SOLiD sequencing (45).

Data processing and analysis. Raw sequencing reads were mapped against the *A. vinelandii* genome as previously described (45). Reads that mapped to more than one region of the genome (2 to 5% of the total) could not be unambiguously mapped and were excluded from subsequent analyses (see Table S7 in the supplemental material). Summary information for sequences obtained by SOLiD is provided in Table S7 in the supplemental material. Statistical analyses were performed using *DESeq* (2) (version 1.2.0) from the Bioconductor R statistical packages (<http://www.r-project.org/contributors.html>) (22). Transcript level differences with adjusted *P* values of <0.01 (generally, genes with more than 20 transcripts detected) and also a fold change ratio of 2.0 or greater (see reference 45) were considered to be significant (see Fig. 3). Previous comparisons of biological replicates have shown that 2-fold changes in transcript levels are significant and that much smaller changes are probably significant for highly expressed genes (45). The 15 genes most highly transcribed under each condition are summarized in Table S8 in the supplemental material.

Quantitative RT-PCR (qRT-PCR). To confirm the results of SOLiD sequencing, 14 genes were chosen for qRT-PCR analyses that spanned the range of coverage depth from the four transcriptomes (see Table S9 in the supplemental material). qRT-PCR was performed using the *Power SYBR Green RNA-to-CT 1-Step* Kit from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol, and reaction products were assayed on a RotorGene-Q real-time PCR detection system from Qiagen (Valencia, CA). Reactions were performed in triplicate with 25 ng of total RNA quantified as described above, with 200 nM forward and reverse primers (see Table S10 in the supplemental material) in a final reaction volume of 20 μl. Control reaction mixtures contained either no RT or no template RNA. Results of the qRT-PCR assays are summarized in Fig. S1 in the supplemental material.

RESULTS AND DISCUSSION

Transcript levels for nearly 30% of the *A. vinelandii* genes changed more than 2-fold during diazotrophic growth relative to those in the fixed-N replete control. This included 884 genes for which the changes in expression were shared for all of the diazotrophic growth conditions tested (Mo-dependent, V-dependent, or heterometal-independent diazotrophy) compared to expression in fixed-N replete control cultures (Fig. 1A). Mo-dependent diazotrophic growth resulted in the differential expression of 398 genes (at least a 2-fold change compared to expression in fixed-N replete control cultures) that did not undergo a similar change in abundance under conditions of V-dependent or heterometal-independent diazotrophy (Fig. 1A). In addition to the 884 genes that changed under all nitrogen-fixing conditions, V-dependent or heterometal-independent diazotrophic growth resulted in an additional set of 661 common differentially expressed genes relative to the fixed-N replete control that were not observed to be differentially expressed (at least a 2-fold change relative to the control) under Mo nitrogenase-dependent diazotrophic growth. However, the number of genes uniquely affected by either V-dependent or heterometal-independent diazotrophy was much smaller than for Mo-dependent growth: 161 or 175, respectively (Fig. 1A).

The largest changes in transcript abundance were observed during growth requiring expression of the alternative nitrogenases compared to Mo-dependent diazotrophy, when transcript abundance increased for over 1,000 genes and more than 700 decreased (by at least 2-fold) compared to the fixed-N replete control (Fig. 1B). Mo-dependent diazotrophic growth resulted in a smaller change in the transcriptome: transcripts for 788 genes increased and 685 decreased more than 2-fold (Fig. 1B). Interestingly, only 160 genes were differentially ex-

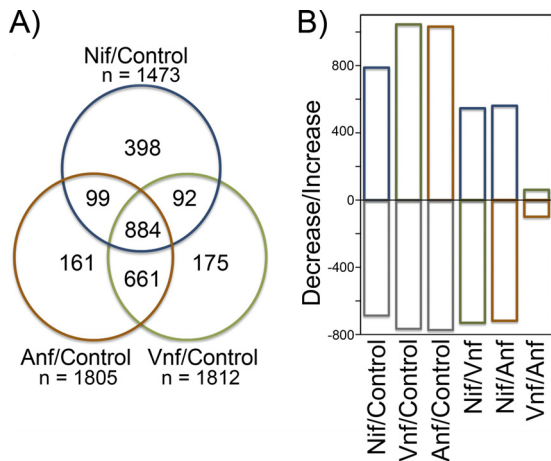


FIG. 1. Global response of the *A. vinelandii* transcriptome during diazotrophic growth. (A) Venn diagram comparing the total number of mRNA species undergoing at least a 2.0-fold change in transcript level under Mo-dependent diazotrophy (Nif, blue circle), under V-dependent diazotrophy (Vnf, green circle), and under heterometal-independent diazotrophy (Anf, rust circle) compared to a fixed-N replete (control) culture. Values in overlapping circles indicate the numbers of mRNA species that undergo a change in transcript level under both or all three conditions. n is the total number of mRNA species undergoing a change in transcript level in each culture. (B) Bar graph of the total number of mRNA species that undergo a larger-than-2-fold increase or decrease in transcript abundance as a result of metal availability and nitrogen limitation. Nif, Mo-dependent diazotrophy; Vnf, V-dependent diazotrophy; Anf, heterometal-independent diazotrophy; Control, N-replete culture.

pressed as a result of diazotrophic growth dependent on the alternative nitrogenases when V-dependent diazotrophic growth was compared to heterometal-independent diazotrophic growth (Fig. 1B). Collectively, the results indicated that the patterns of gene expression under conditions of V-dependent or heterometal-independent diazotrophic growth are very similar to one another but distinctly different from the patterns of gene expression under Mo-dependent diazotrophic growth.

Expression of *nif* genes under Mo-dependent nitrogen-fixing conditions compared to nondiazotrophic growth. The genetic complexity of nitrogenase assembly and the regulation of *nif* gene expression of nitrogenase have been extensively characterized using the model diazotroph *A. vinelandii* (43). Previous specific gene deletion (28, 29, 33, 36) and Northern blot hybridization analyses (30, 33) provided key insights into the genes involved in biological N_2 fixation by *A. vinelandii*. The genes encoding Mo nitrogenase and the machinery to assemble the enzyme are colocalized in a major *nif* cluster and a secondary cluster that contains a few key biosynthetic and regulatory genes (28, 33) (Fig. 2A). Transcription of *nif* genes is activated by a σ^{54} -dependent activator, NifA, and a number of *nif*-specific promoter sequences have been identified within the two *nif* clusters (15, 28).

Under N-fixing conditions, nitrogenase components make up nearly 10% of the total cellular proteins, the majority of which are structural proteins (14). The catalytic components necessary for FeMo-co biosynthesis are presumably necessary in much smaller amounts. A large increase in the transcript

levels of the structural genes *nifH* (Avin01380), *nifD* (Avin01390), and *nifK* (Avin01400) was observed under Mo-dependent diazotrophy compared to those in the fixed-N replete (control) culture (Fig. 2A; also see Table S1 in the supplemental material). The transcript levels of *nifH* increased much more (143-fold) than those of *nifD* and *nifK* (~54-fold) (Fig. 2A). This observation is consistent with previous analyses and supports the observation that nitrogenase activity is optimal under conditions of high Fe protein to MoFe protein ratios (13, 14). Interestingly, the abundances of transcripts of nonessential *nif* genes (28) downstream of *nifK* increased by only 2- to 14-fold (Fig. 2A). The different abundances of these apparently cotranscribed genes suggested that these gene-specific regions of the transcripts have different processing and segmental stabilities or that their synthesis may be regulated by transcriptional attenuation or processing.

The operon adjacent to the *nifHDK* structural genes contains *nifE* (Avin01450), *nifN* (Avin01470), and *nifX* (Avin01480), encoding the scaffold protein necessary for FeMo-co biosynthesis (NifEN) and a carrier protein (NifX) (54) (Fig. 2A; also see Table S1 in the supplemental material). The Shethna protein (FeSII, encoded by *fesII* [Avin01520]), which is involved in protecting the Fe protein from O_2 damage (46), is also encoded within this operon. Transcripts mapping to these genes increased 2- to 6-fold under Mo-dependent nitrogen fixation conditions (Fig. 2A).

The majority of the remaining genes within the major *nif* cluster encode proteins important for FeMo-co and P cluster biosynthesis and maturation of nitrogenase (54). Transcripts mapping to the genes *iscA^{nif}* (Avin01610) and *nifUSV* (Avin01620 to Avin01640) increased (~6- to 24-fold) under N_2 -fixing conditions (Fig. 2A; also see Table S1 in the supplemental material). NifU, a scaffold protein, and NifS, a cysteine desulfurase, are similar to proteins of the Isc and Suf house-keeping systems for [Fe-S] cluster biosynthesis and facilitate the assembly of Nif-specific metal clusters (38). NifU and NifS are required for the maturation of nitrogenase in *A. vinelandii* and, despite the similarity to IscU and IscS and evidence that some cross talk may exist (18), are unable to supplant the function of the ISC (iron-sulfur cluster) system (39). NifV is a homocitrate synthase and is required for synthesis of homocitrate for FeMo-co in *A. vinelandii* (66). Previous biochemical and genetic analyses indicate that these gene products are necessary for optimal diazotrophic growth in *A. vinelandii* (29). *orf8* (Avin01660) and *nifZW* (Avin01670 and Avin01680) transcript levels, also important for Mo-dependent diazotrophic growth (29), increased ~5- to 9-fold (Fig. 2A). Increases of ~6- to 8-fold were observed for transcripts mapping to *nifM* (Avin01690), the product of which is necessary for Fe protein maturation (26), and *clpX^{nif}* (Avin01700), which is cotranscribed (Fig. 2A), as well as the adjacent, independently transcribed gene *nifF* (Avin01710), which encodes a flavodoxin (4) (Fig. 2A). Transcripts mapping to genes encoding several hypothetical proteins located within the major *nif* operon also increased 3- to 11-fold (Fig. 2A; also see Table S1 in the supplemental material). However, previous gene deletion studies indicated that these products are not required for diazotrophic growth (28, 29).

The minor *nif* cluster contains two operons that include the genes encoding the *nif*-regulatory proteins NifL (Avin50990)

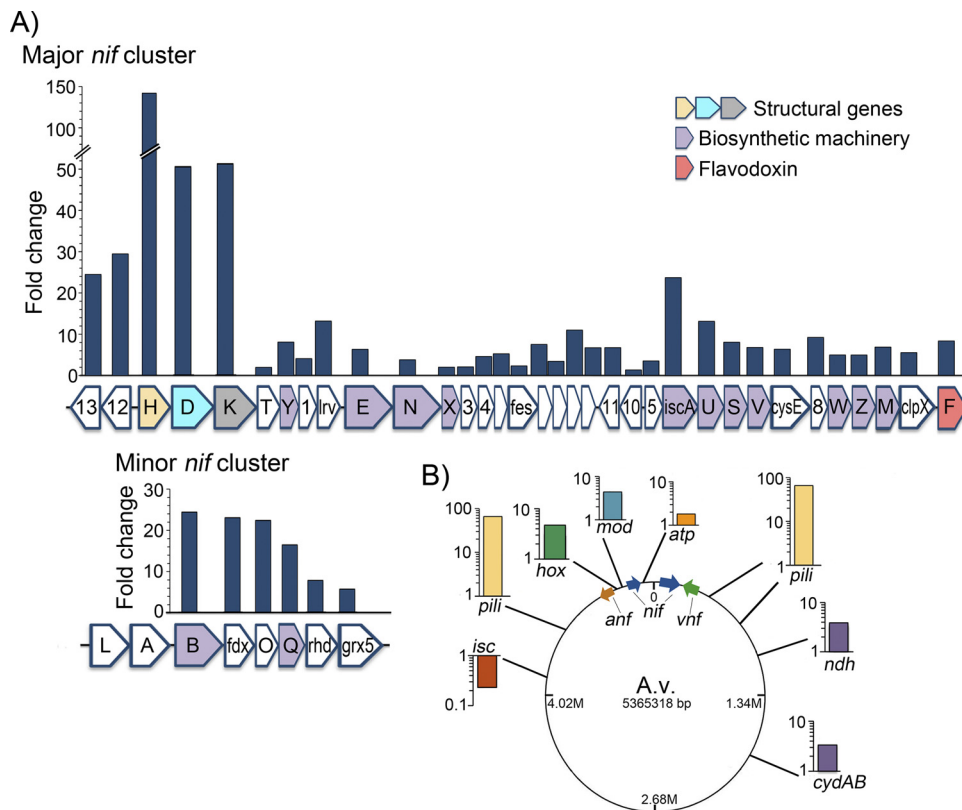


FIG. 2. Differential expression of proteins necessary for Mo-dependent diazotrophy. (A) The ratio of transcript levels (fold change) of genes encoding structural proteins, assembly machinery, and regulatory proteins for Mo-dependent diazotrophy (Nif, blue) are compared to transcript levels in fixed-N replete (control) cultures. Transcripts mapping to the regulatory genes *nifA* and *nifL* were detected at very low levels in both Mo-dependent diazotrophic and fixed-N replete cultures and, as a result, are not included here (see Table S1 in the supplemental material). (B) The ratio of transcript levels of genes or groups of genes encoding proteins important for the global response of *A. vinelandii* (A.v.) during Mo-dependent diazotrophic growth compared to the N-replete (control) culture, presented on a log scale. The genomic locations of these genes are indicated, as well as the genomic locations of the gene clusters encoding the Mo-dependent nitrogenase (*nif*, blue arrows) and the alternative nitrogenase (*vnf* [green arrow] and *anf* [rust arrow]). *isc*, ISC; *pili*, pilus machinery; *hox*, membrane-bound [NiFe] hydrogenase; *mod*, molybdate transport; *atp*, ATP synthase; *ndh*, NADH-dependent oxidoreductase; *cydAB*, cytochrome *bd* oxidase I. Each gene name is indicated by a letter(s). Full gene names, descriptions, and locus tags are given in Tables S1 and S2 in the supplemental material.

and NifA (Avin5100) and maturation proteins encoded by *nifB* (Avin51010) and *nifQ* (Avin51040), which are involved in FeMo-co biosynthesis. The genes *nifB*, *fdx*, *nifO*, *nifQ*, *rhd*, and *grx5* (Avin51010 to Avin51060) are cotranscribed. A large increase (~12- to 18-fold) in the number of transcripts mapping to *nifB*, *fdx*, *nifO*, and *nifQ* was detected, and a smaller increase (4- to 6-fold) in those for *rhd* and *grx5* was noted (Fig. 2A). NifB is a radical *S*-adenosylmethionine (SAM) protein and a key enzyme required for FeMo-co biosynthesis. NifQ (Avin51040) has been implicated in Mo acquisition and FeMo-co biosynthesis (54). The *nifL* and *nifA* regulatory genes are located upstream of *nifB* and are under the control of a separate promoter. As noted above, the σ^{54} -dependent activator NifA is required for transcriptional activation of Mo nitrogenase genes; NifA activity is controlled by its regulatory partner protein NifL in response to excess oxygen and fixed N, ensuring that Mo-dependent nitrogen fixation occurs only under appropriate physiological conditions (16). Low *nifL* and *nifA* transcript levels were detected under both conditions (see Table S1 in the supplemental material).

Differences in global patterns of gene expression implicated during Mo-dependent diazotrophic growth compared to non-diazotrophic growth. Fixed N is essential for the biosynthesis of cellular components and growth; however, when fixed N is limiting, biological N_2 fixation also imposes a high demand for ATP and reducing power on cells. These overlapping and competing requirements were expected to cause significant differences in the patterns of gene expression in the presence and absence of fixed N. Other than the major *nif* gene cluster, the largest increases (60- to >200-fold) in transcript abundance under Mo-dependent diazotrophic growth occurred for genes that encode type IV pili, namely, *pilG*, *pilE*, *pilA*, *pilN*, and *pilM* (Avin03180, Avin11830, Avin12104, Avin45290, and Avin45300, respectively) (Fig. 2B; also see Table S2 in the supplemental material). Type IV pili are involved in a number of processes, including cell motility, DNA uptake, sensing, attachment, and aggregation (55); the latter could be a previously unrecognized O_2 protection mechanism in *A. vinelandii*. Several strategies of O_2 protection for nitrogenase have been noted, including respiratory protection by active con-

sumption of oxygen (50), spatial decoupling, for which N₂ fixation occurs in differentiated anoxic heterocysts (21), and temporal separation by free-living cyanobacteria that fix nitrogen at night, when oxygenic photosynthesis cannot occur (62). When carbon is not limiting, *A. vinelandii* employs a respiratory protection mechanism to limit levels of cytoplasmic O₂, effectively protecting oxygen-sensitive enzymes such as nitrogenase from inactivation (50). The genome of *A. vinelandii* encodes four NADH:ubiquinone oxidoreductases and five terminal oxidases. Transcripts mapping to the genes coding for cytochrome *bd* oxidase I (CydAB I; Avin19880 and Avin19890) and the type II NADH-dependent oxidoreductase Ndh (Avin12000) increased 3- to 4-fold in nitrogen-fixing cells compared to those in the control (Fig. 2B; also see Table S2 in the supplemental material). These results are consistent with previous studies indicating a role for both CydAB I and Ndh in the diazotrophic growth of *A. vinelandii*, especially at high O₂ concentrations (5, 50). Small increases in transcript abundance for two other terminal oxidases, cytochrome *cbb*₃ oxidase (Cco; Avin19940 to Avin20010) and cytochrome *c* oxidase (Cox; Avin11170 and Avin11180), were also observed during diazotrophic growth (see Table S2 in the supplemental material).

The increased levels of transcripts for terminal oxidases and respiratory enzymes could be coordinately regulated to meet the energy demands for nitrogen fixation. Transcripts mapping to both ATP synthases (Avin19670 to Avin19750 and Avin52150 to Avin52230) also increased (~1- to 5-fold) under N₂-fixing conditions (Fig. 2B; also see Table S2 in the supplemental material). Transcript levels also increased ~5-fold for the well-characterized, membrane-bound [NiFe] hydrogenase (*hoxVTRQOLMZGK* [Avin50500 to Avin50590]) (Fig. 2B) (57), which presumably recycles the hydrogen produced as a by-product of nitrogenase-catalyzed N₂ reduction, thereby capturing reducing equivalents that would otherwise be lost.

In addition to *nif*-specific genes, whose products are necessary for the initial steps in targeting Fe and S for assembly of metalloclusters associated with the maturation of nitrogenase (*nifU* and *nifS*), the genome of *A. vinelandii* also includes a suite of genes that encode proteins required for the maturation of [Fe-S] cluster-containing proteins related to other metabolic functions. The more general components of the machinery responsible for maturation of "housekeeping" [Fe-S] proteins have been designated ISC components and include *iscR*, *iscU*, *iscS*, *iscA*, *hscB*, *hscA*, *fdx*, and *iscX* (Avin40340 to Avin40410) (38). Among the ISC proteins, *IscS* is paralogous to *NifS* (38% identity) and *IscU* is similar in sequence (49% identity) to the N-terminal domain of *NifU*. Biochemical studies have established that *NifS* and *IscS* have cysteine desulfurase activity associated with the mobilization of S for [Fe-S] cluster assembly and that both *IscU* and the N-terminal domain of *NifU* can serve as *in vitro* and *in vivo* scaffolds for [Fe-S] cluster formation (18, 60). Genetic and physiological studies have also established that there is some functional cross talk between the ISC and *Nif* systems for [Fe-S] cluster formation (18). This indicates that the *Nif* counterparts function primarily to satisfy the increased demand for the mobilization and targeting of Fe and S for [Fe-S] cluster formation associated with N₂ fixation. However, *NifU* and *NifS* cannot entirely replace the housekeeping [Fe-S] biogenesis supported by ISC in *A. vinelandii* (18, 39). The present work provides further evidence for func-

tional cross talk between the ISC and *Nif* systems because stimulation of *NifU* and *NifS* expression under N₂-fixing conditions is accompanied by a decrease in the expression of the ISC system (Fig. 2B; also see Table S2 in the supplemental material). This observation also extends to the expression of other housekeeping components associated with [Fe-S] protein maturation, including *ErpA* (Avin46030) and *NfuA* (Avin28760). Interestingly, *ErpA* has primary structural similarity to the *IscA*-like protein contained within the major *nif* cluster and *NfuA* has primary structural similarity to the C-terminal domain of *NifU*. Although the specific function of these proteins in *A. vinelandii* has not yet been clearly established, current evidence indicates that they could function as [Fe-S] cluster assembly scaffolds or as intermediate carriers of [Fe-S] clusters, a role that has been demonstrated for *NfuA* in *Synechococcus* sp. strain PCC 7002 (31). The observed ability of the *Nif*-specific [Fe-S] maturation components to replace the analogous housekeeping functions partially (18) may reflect the lower level of expression of the ISC components when cells are cultured under N₂-fixing conditions. However, expression of housekeeping [Fe-S] cluster biosynthetic proteins is subject to feedback regulation through the action of *IscR*, an [Fe-S] cluster-containing transcription regulator. It is also possible that the respiratory protection mechanism of nitrogen-fixing cells also protects other [Fe-S] proteins from oxygen damage, thus lowering the demand for [Fe-S] protein maturation.

A. vinelandii utilizes an efficient molybdate transport system, encoded by the *mod* genes (Avin50650 to Avin50690), to provide the Mo necessary for Mo-containing enzymes, including nitrogenase (47). The *A. vinelandii* genome contains genes encoding a high-affinity transport system (Avin50650 to Avin50690) located adjacent to the minor *nif* cluster and preceded by a putative σ^{54} -dependent promoter. Transcripts mapping to this molybdate transport system increased ~4-fold during N limitation (Fig. 2B; also see Table S2 in the supplemental material). In addition, smaller increases in transcript abundance (~1- to 5-fold) were also observed for the two other Mo transport systems (Avin01280 to Avin01300 and Avin50700 to 50730) (see Table S2 in the supplemental material).

Transcription profiles in cells expressing alternative nitrogenases. The alternative forms of nitrogenase were first discovered through the observation that strains of *A. vinelandii* in which *nif* structural genes had been deleted were still capable of diazotrophic growth in Mo-deficient, fixed-N-free medium (7). Subsequent genetic and DNA sequence analysis has revealed that distinct alternative (V and Fe-only) nitrogenases are encoded by *nif* paralogs within the *vnf* and *anf* clusters which are not colocalized with either of the *nif* gene clusters (Fig. 2A and 3) (59). The expression of the alternative systems in *A. vinelandii* is regulated by the availability of Mo (30). Transcripts of the *vnf* and *anf* structural genes have been observed by Northern blot hybridization in N-limited *A. vinelandii* cultures in the absence of Mo with added V (27) or in the absence of both Mo and V (51).

The alternative forms of nitrogenase are present only in the genomes of organisms that also harbor the *nif* system (8, 53, 61), and although the structural genes are distinct, the gene operons encoding the alternative enzymes do not include paralogs of all of the genes for the biosynthetic machinery neces-

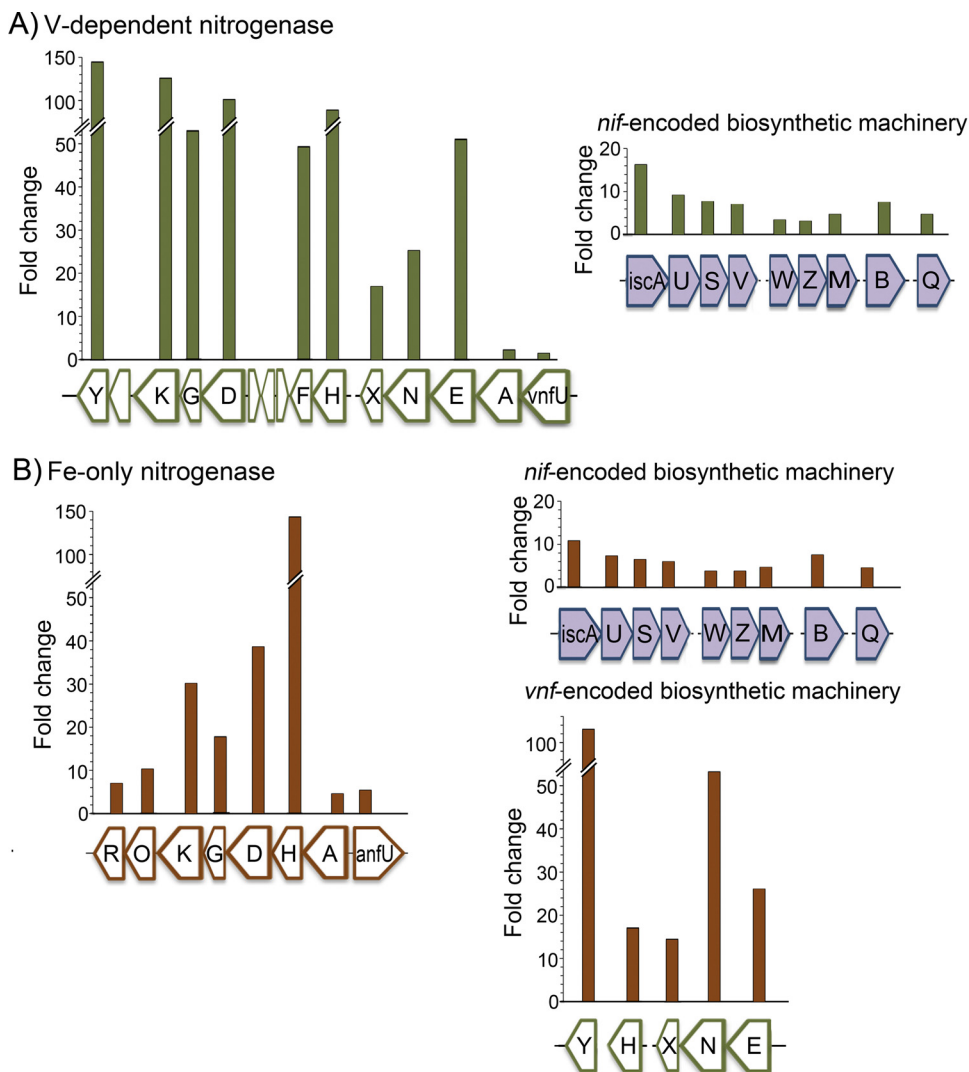


FIG. 3. Differential expression of proteins necessary for Mo-independent nitrogen fixation. (A) Ratios of transcript levels (fold changes) of genes encoding structural proteins, assembly machinery, and regulatory proteins during V-dependent diazotrophy (Vnf, green) to transcript levels in fixed-N replete (control) cultures. Transcript levels of *nif* genes observed to undergo large changes for V-dependent diazotrophy (Vnf, green) compared to transcript levels of the fixed-N replete (control) cultures are also indicated. (B) Ratios of transcript levels (fold changes) of genes encoding structural proteins, assembly machinery, and regulatory proteins for heterometal-independent diazotrophy (Anf, rust) to transcript levels in fixed-N replete (control) cultures. Transcript levels of *nif* and *vnf* genes observed to undergo large changes during heterometal-independent diazotrophy (Anf, rust) compared to transcript levels in fixed-N replete (control) cultures are also shown. Each gene name is indicated by a letter(s). Full gene names, descriptions, and locus tags are given in Table S3 in the supplemental material.

sary for the assembly and maturation of Mo-dependent nitrogenase. In *A. vinelandii*, limited biochemical evidence has suggested that VnfH can play a role analogous to that of NifH in FeMo-co maturation (10); however, diazotrophic growth is not supported in mutants containing deletions of each of the respective structural genes. Moreover, mutational analyses have indicated that some components of the *nif*-encoded biosynthetic machinery, such as *nifU*, *nifS*, *nifV*, *nifM*, and *nifB*, are required for nitrogen fixation by cells utilizing either of the alternative enzymes in *A. vinelandii* (36, 41) and a role for the NifEN scaffold protein has also been observed (65).

The transcriptome of cultures relying on V-dependent nitrogenase for diazotrophic growth revealed a large increase (68- to 137-fold) in the *vnfHF* and *vnfDGK* genes, compared to

the levels in fixed-N replete (control) cultures (Fig. 3A). The large difference in transcript abundance observed for *nifH* versus *nifDK*, which are cotranscribed from a single promoter, was not observed for *vnfH*, *vnfD*, and *vnfK*, which are located in separate operons (3). A small increase (~2 fold) was observed for *nifH* transcripts, but given the high *vnfH* transcript abundance, it is unclear what the potential role of *nifH*-encoded Fe protein might be during V-dependent diazotrophy. These genes encode an Fe protein specific to the V-dependent system (encoded by *vnfH*) and the VFe protein (encoded by *vnfDGK*) analogous to the MoFe protein which houses the active-site metalloclusters, the FeV cofactors (FeV-co). The *vnf* gene cluster in *A. vinelandii* also encodes a putative FeV-co assembly scaffold, VnfEN (Avin02770 and Avin02750), paralogous to

the NifEN scaffold protein necessary for FeMo-co biosynthesis. Mutant strains of *A. vinelandii* with knockouts in *vnfEN* can still grow diazotrophically under Mo-limiting but V-replete diazotrophic growth conditions (65). However, transcriptome analysis revealed a large increase (26- to 56-fold) in the expression of *vnfE* and *vnfN* transcripts (Fig. 3) and a very small increase in the expression of *nifEN* transcripts (~1- to 2.5-fold; see Table S3 in the supplemental material) during V-dependent diazotrophy. The large increase in *vnfEN* transcript levels supports the limited biochemical evidence that suggested that VnfEN, and not NifEN, is the preferred scaffold for FeV-co maturation (65). The *vnf* operon also encodes VnfX (Avin02740) and VnfY (Avin02570) (Fig. 2A), paralogs of NifX and NifY. VnfY is required for optimal V-dependent diazotrophy (55), and large increases in *vnfX* and *vnfY* transcript levels (15- and 117-fold) were observed during V-dependent diazotrophy (Fig. 3A). Interestingly, this is markedly different from the *nifX* (Avin01480) and *nifY* transcript levels observed under Mo-dependent diazotrophy, in which much smaller increases (<8-fold) were observed (see Tables S1 and S3 in the supplemental material). This implies that perhaps VnfX and VnfY have a role in FeV-co biosynthesis more significant than that of NifX and NifY in FeMo-co biosynthesis.

Transcripts mapping to the *nif*-encoded cluster biosynthesis machinery implicated in playing a role in the assembly of alternative cofactors such as *nifUSV*, *nifM*, and *nifB* also increased (5- to 10-fold), consistent with what was observed from previous deletion mutant analysis (36), indicating that these biosynthetic genes are also involved in V-dependent diazotrophy. Interestingly, an increase in transcript abundance of *nifQ*, which is necessary for Mo acquisition for FeMo-co, was also observed, and this may reflect the pressure to acquire Mo to utilize the Mo-dependent nitrogenase preferentially or may be simply the result of the transcriptional coupling of *nifQ* with *nifB*. Our analysis thus implicated the involvement of *nif*-encoded maturation proteins in V-dependent diazotrophy in *A. vinelandii*.

The Fe-only nitrogenase (AnfHDGK; Avin49000 to Avin48970) responsible for heterometal-independent diazotrophy is encoded by the *anf* gene cluster. This cluster includes the *anfHDGK* structural genes encoding an additional Fe protein, AnfH, specific to the Fe-only system and the genes encoding the Fe-only version of the substrate reduction component, the FeFe protein (AnfDGK), where the FeFe cofactors reside. In addition, this cluster includes *anfA* (Avin49020), a regulatory gene, and *anfO* (Avin48960) and *anfR* (Avin48950) (Fig. 3B). Transcripts mapping to the *anfHDGK* structural genes increased 18- to 144-fold in cultures dependent on the Fe-only nitrogenase for diazotrophic growth compared to the levels in the fixed-N replete cultures (Fig. 3B). The differences in transcript abundance of *anfH* versus *anfD* and *anfK*, which are presumably cotranscribed, mirrors what is observed for the *nif* structural genes, where the increase in *nifH* transcript abundance was much larger than the observed increases in *nifD* and *nifK* transcript abundance. Large increases (14- to 117-fold) in the *vnf*-encoded *H*, *E*, *N*, *X*, and *Y* transcripts were observed during heterometal-independent diazotrophy (Fig. 3B). This observation suggested that these *vnf*-encoded products are preferred over their *nif*-encoded counterparts for maturation of

the Fe-only nitrogenase. Therefore, both the V-dependent and Fe-only nitrogenases apparently utilize the VnfEN scaffold protein for cofactor assembly but the *nif*-encoded accessory proteins NifU, NifS, NifM, NifV, and NifB for maturation (41). Importantly, *vnfEN* in *A. vinelandii* is derived from *nifEN*, suggesting that the preferential utilization of the VnfEN scaffold over the NifEN scaffold in alternative systems is a recent evolutionary feature (8). In addition, VnfH has been demonstrated to be necessary for transcription of *anfHDGK*; however, in *vnfH* mutants, the presence of NifH is detected but lower growth rates of heterometal-independent diazotrophically grown cells are observed (37). An increase in *vnfH* transcripts (~17-fold) and *nifH* transcripts (~2.5-fold) was observed during heterometal-independent diazotrophy (see Table S3 in the supplemental material). Together, these results may suggest that Vnf preceded and is evolutionarily ancestral to Anf, which is consistent with evidence reported previously (8, 53).

Electron transport for Mo-, V-, and Fe-only nitrogenase-dependent diazotrophic growth. The energy and electron transport requirements differ for the three forms of nitrogenase, and both the *nif* and *vnf* systems encode specific flavodoxins, i.e., those encoded by the *nifF* (Avin01710) and *vnfF* (Avin02650) genes. Although *nifF* is not required for Mo-dependent diazotrophy (4), it is not known if *vnfF* is required for V-dependent or heterometal-independent diazotrophy. Both *vnfF* and *nifF* transcripts increased under Mo-independent diazotrophic conditions, and the increases in *vnfF* (47- to 102-fold) were much larger than the increase in *nifF* (~4-fold) (Fig. 3). During Mo-dependent diazotrophy, transcripts mapping to *nifF* increased 8-fold.

Three clusters of genes, *mf1* (Rnf1, Avin50930 to Avin50980), *mf2* (Rnf2, Avin19220 to Avin19270), and *fix* (Avin10510 to Avin10550), encode electron transport systems that are thought to provide reducing equivalents to nitrogenase in *A. vinelandii* (40, 56). The *mf* genes were originally identified and characterized in *Rhodobacter capsulatus*, and they encode a membrane-bound complex involved in electron transport to nitrogenase (56). The expression of the genes encoding the Rnf1 complex is NifA dependent, while the genes encoding Rnf2 are not regulated as a result of N availability (see Table S4 in the supplemental material) (12). In *A. vinelandii*, the *mf* genes are necessary for full Mo-dependent nitrogenase activity and have been suggested to be regulated by NifA, potentially by modulation of the redox state of NifL (12). In addition, Rnf1 proteins are required for accumulation of the [4Fe-4S] cluster in the *nifH*-encoded Fe protein (12). The *fix* operon, which is preceded by a putative σ^{54} -dependent promoter (R.D., unpublished data), encodes five proteins, FixP, -A, -B, -C, and -X, that form an electron transfer complex that seems to be required to support nitrogen fixation in some diazotrophs (40). Both *mf1* and *fixPABCX* transcript levels increased markedly during diazotrophic growth; however, during V-dependent or heterometal-independent diazotrophy, *fixPABCX* operon transcript levels were higher than *mf1* operon transcript levels (~231-fold versus ~18-fold) (see Table S4 in the supplemental material). In contrast, the increases in transcripts from the *mf1* operon and the *fixPABCX* operon were similar in magnitude during Mo-dependent diazotrophy (~40-fold). As

expected, levels of transcripts from the *nif2* operon, which is not NifA regulated, were relatively unchanged.

Regulation of gene expression during diazotrophic growth. The σ^{54} -dependent activator proteins VnfA and AnfA are required for expression of V-dependent nitrogenase and the Fe-only nitrogenase, respectively (52), but unlike the activity of NifA, their activity is not subject to regulation by a NifL-like protein. As observed for *nifA*, the *vnfA* and *anfA* transcripts were detectable at low levels under all of the growth conditions tested here; however, their transcript levels increased under V-dependent and heterometal-independent diazotrophic growth (Fig. 3) and decreased in the presence of Mo (see Table S5 in the supplemental material). This pattern of regulation of *vnfA* and *anfA* expression has been observed previously, and the Mo-responsive transcriptional regulator ModE has been shown to be at least partially involved in Mo-dependent repression of *anfA* (52). Transcriptional regulation of *vnfA* and *anfA* is likely to be responsible for the absence of the VFe- and Fe-only nitrogenases when molybdenum is available, because transcription of the genes encoding these alternative enzymes cannot be activated in the absence of VnfA or AnfA, respectively (64). The *A. vinelandii* genome contains an additional gene homologous to *nifA*, *nifA2* (Avin26490), and two additional *vnfA* homologs, *vnfA2* (Avin33440) and *vnfA3* (Avin47100) (59). Interestingly, the pattern of regulation of *vnfA2* and *vnfA3* expression was similar to that of *vnfA* and *anfA* expression, as transcripts for both increased under Mo-depleted diazotrophic conditions compared with those in cultures grown with molybdate (see Table S5 in the supplemental material). Although the precise role of these activator paralogs is unclear, the sequence similarity of their DNA binding determinants to those of the cognate NifA and VnfA activators (59) suggests that they may be able to provide additional regulatory input.

Differences in global patterns of gene expression implicated during Mo-independent nitrogen-fixing growth. The complexity of the nitrogenase enzyme and the absolute requirement for fixed N to meet cellular growth might be expected to result in a similar global response to fixed-N limitation, regardless of metal availability, but as noted above, the patterns of gene expression for Mo-dependent diazotrophy are significantly different from those in cells grown under Mo-independent diazotrophic conditions. The alternative forms of nitrogenase are markedly less efficient than the Mo nitrogenase (19), and coupled with the demand for fixed N levels necessary to maintain cell growth, this inefficiency would presumably result in significant differences in the transcriptional response of V-dependent or heterometal-independent diazotrophy from that in N-replete control cultures. Key differences are observed in the *A. vinelandii* transcriptome in response to N-limiting conditions and the availability of Mo. Most notably, transcripts for *hutU* (Avin26180), the gene encoding urocanate hydratase, increased dramatically (30- to 40-fold) under diazotrophic conditions in the absence of Mo (see Table S5 in the supplemental material). Urocanate hydratase catalyzes a key step in the degradation of histidine (63). The increased *hutU* transcript levels presumably reflect the low catalytic efficiency of the alternative forms and imply that cells seek to supplement the fixed N provided by the alternative forms by liberating fixed N during protein turnover by His degradation to maintain cell

viability until Mo or fixed N becomes available. This increase in enzymes capable of accessing fixed N was also noted in the transcriptome analysis of *Rhodospseudomonas palustris* under diazotrophic conditions requiring expression of the alternative nitrogenases (48). Transcript levels of other *hut* genes in the same operon, *hutG* (Avin26150), *hutI* (Avin26160), and the gene for a hypothetical protein (Avin26170), and just downstream, *hutH* (Avin26290) and *hutF* (Avin26300), were also observed to increase (~5- to 40-fold) under these conditions (see Table S5 in the supplemental material). Interestingly, the large increase in transcripts for the formation of pili was not observed during Mo-independent, diazotrophic growth. Also notable was an increase in the levels of transcripts for the genes (Avin04360 to Avin04410) encoding an uncharacterized soluble [NiFe] hydrogenase, which increased ~10-fold during V-dependent diazotrophy and ~20-fold during heterometal-independent diazotrophy (see Table S5 in the supplemental material). The increase in transcripts may reflect the need to recycle the larger amount of hydrogen produced by the activity of the alternative enzymes to counteract the catalytic inefficiency. The alternative nitrogenases have been shown to produce more hydrogen during dinitrogen reduction, which presumably provides additional pressure to avoid the loss of needed reducing equivalents (19). Finally, transcripts mapping to a number of genes annotated as hypothetical proteins were also observed to undergo large changes as a result of Mo-independent diazotrophy. For example, Avin02580, Avin16830, Avin47120, Avin46150, and Avin10510 transcript levels increased and Avin22850, Avin09340, Avin49410, and Avin47230 transcript levels decreased under these conditions (see Table S6 in the supplemental material).

Although our analysis indicates similar patterns of gene expression when cultures are grown either under conditions of V-dependent diazotrophy and heterometal-independent diazotrophy, several key differences were noted. Importantly, transcripts mapping to several genes near the *vnf* operon increased dramatically (53- to 100-fold change) during V-dependent diazotrophy (see Table S6 in the supplemental material). These genes, Avin02540, Avin02550, and Avin02560, encode proteins that are homologous to the inner membrane transport protein and substrate binding protein of an ABC transporter, which is annotated as a phosphonate transport system. Due to the proximity of these genes to the *vnf* gene cluster and due to the large increase in transcripts mapping to these genes during V-dependent diazotrophy, we hypothesize that these gene products are responsible for vanadate uptake.

Like Mo-dependent diazotrophy, both V-dependent and heterometal-independent diazotrophic growth resulted in an increased abundance of transcripts for CydAB I (~3- to 5-fold), Ndh (~3-fold), and both ATP synthases (see Table S5 in the supplemental material) compared to that in N-replete control cultures. Likewise, similar increases in levels of transcripts mapping to the *hox*-encoded, membrane-bound [NiFe] hydrogenase and the Mod transport systems were also observed in cultures expressing the alternative nitrogenases. Interestingly, a decrease in the ISC machinery was also noted, further supporting the hypothesis that *isc* gene expression levels observed under normal growth conditions are sufficient to provide [Fe-S] clusters for general cell metabolism (see Table S5 in the supplemental material). A significant decrease in

transcript abundance was noted for some genes regardless of metal availability during diazotrophic growth (see Table S6 in the supplemental material). The differential expression of these proteins presumably reflects changes in the transcriptome of *A. vinelandii* in response to fixed-N limitation; however, most of these transcripts mapped to proteins without defined functions (for example, those of Avin09340, Avin22850, Avin47230, and Avin49410).

Evolutionary implications. The results presented herein have fundamental implications for our understanding of *nif* evolution. Several phylogenetic studies have suggested that the alternative nitrogenases are evolutionary ancestors of Mo nitrogenase (8, 53, 61). This is considered to be consistent with early Earth history, because the bioavailability of Mo and perhaps V would have been very low prior to the advent of oxygenic photosynthesis and subsequent oxygenation of Earth's atmosphere (1). Interestingly, however, the V-dependent and Fe-only nitrogenases in extant organisms have not yet been observed in a background devoid of the Mo nitrogenase (8, 53). In this study, we clearly show that FeMo-co biosynthetic genes clustered with and required for the *nif* system are expressed as presumably essential components of alternative systems, extending mutant and biochemical analyses that have suggested this functional cross talk (36, 41) and in line with previous genetic experiments which indicated that the alternative nitrogenases are not functional in the absence of these *nif*-encoded gene products. The available information regarding alternative nitrogenase genomic occurrence, the results of previous genetic studies, and the transcription profiling results described here collectively make it difficult to consider the alternative nitrogenases as ancestral forms of the Mo nitrogenase. Instead, a simpler hypothesis is that the alternative nitrogenases evolved from Mo nitrogenase to expand the fundamental niche of diazotrophic populations under conditions where Mo may, in fact, be limiting.

Conclusion. The carefully controlled transcriptional analysis presented here has clearly defined the suite of genes associated with each of the three nitrogenase systems in *A. vinelandii*. In addition, these results have uncovered several interesting surprises regarding the evolutionary relationships between Mo nitrogenase and the alternative nitrogenases, which can be examined further through combined genetic, biochemical, and evolutionary analyses. The results of the global expression under N₂-fixing conditions suggest that Mo limitation, whether in the presence or in the absence of V, results in marked differences in gene expression in *A. vinelandii*. These results also support the hypothesis that the lower catalytic efficiencies of the V-dependent and Fe-only nitrogenases impose significant physiological constraints upon *A. vinelandii* when it is grown under N₂-fixing conditions in the absence of Mo. These studies will help to refine our understanding of the complex metabolic and regulatory interactions that control N₂ fixation in this important model organism.

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