

Expression of V-nitrogenase and Fe-nitrogenase in *Methanosarcina acetivorans* is controlled by molybdenum, fixed nitrogen, and the expression of Mo-nitrogenase

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ABSTRACT All nitrogen-fixing bacteria and archaea (diazotrophs) use molybdenum (Mo) nitrogenase to reduce dinitrogen (N₂) to ammonia, with some also containing vanadium (V) and iron-only (Fe) nitrogenases that lack Mo. Among diazotrophs, the regulation and usage of the alternative V-nitrogenase and Fe-nitrogenase in methanogens are largely unknown. *Methanosarcina acetivorans* contains *nif*, *vnf*, and *anf* gene clusters encoding putative Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase, respectively. This study investigated nitrogenase expression and growth by *M. acetivorans* in response to fixed nitrogen, Mo/V availability, and CRISPRi repression of the *nif*, *vnf*, and/or *anf* gene clusters. The availability of Mo and V significantly affected growth of *M. acetivorans* with N₂ but not with NH₄Cl. *M. acetivorans* exhibited the fastest growth rate and highest cell yield during growth with N₂ in medium containing Mo, and the slowest growth in medium lacking Mo and V. qPCR analysis revealed the transcription of the *nif* operon is only moderately affected by depletion of fixed nitrogen and Mo, whereas *vnf* and *anf* transcription increased significantly when fixed nitrogen and Mo were depleted, with removal of Mo being key. Immunoblot analysis revealed Mo-nitrogenase is detected when fixed nitrogen is depleted regardless of Mo availability, while V-nitrogenase and Fe-nitrogenase are detected only in the absence of fixed nitrogen and Mo. CRISPRi repression studies revealed that V-nitrogenase and/or Fe-nitrogenase are required for Mo-independent diazotrophy, and unexpectedly that the expression of Mo-nitrogenase is also required. These results reveal that alternative nitrogenase production in *M. acetivorans* is tightly controlled and dependent on Mo-nitrogenase expression.

IMPORTANCE Methanogens and closely related methanotrophs are the only archaea known or predicted to possess nitrogenase. Methanogens play critical roles in both the global biological nitrogen and carbon cycles. Moreover, methanogens are an ancient microbial lineage and nitrogenase likely originated in methanogens. An understanding of the usage and properties of nitrogenases in methanogens can provide new insight into the evolution of nitrogen fixation and aid in the development nitrogenase-based biotechnology. This study provides the first evidence that a methanogen can produce all three forms of nitrogenases, including simultaneously. The results reveal components of Mo-nitrogenase regulate or are needed to produce V-nitrogenase and Fe-nitrogenase in methanogens, a result not seen in bacteria. Overall, this study provides a foundation to understand the assembly, regulation, and activity of the alternative nitrogenases in methanogens.

KEYWORDS methanogens, nitrogen fixation, methanogenesis, metalloregulation, nitrogenase, *Methanosarcina*, archaea

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Microbes are the primary drivers of the global biological nitrogen (N) cycle (1, 2). For example, only select bacteria and archaea are capable of biological nitrogen fixation, whereby dinitrogen gas (N_2) is reduced to ammonia (NH_3), the preferred “fixed” form of N used directly by most organisms. The biological reduction of the triple bond of N_2 is difficult and is catalyzed by nitrogenase, a unique metalloenzyme (3, 4). To date, all known and predicted N_2 -fixing prokaryotes (diazotrophs) possess molybdenum (Mo) nitrogenase that contains a Mo atom within the unique iron (Fe) Mo-cofactor or M-cluster of the active site (5, 6). Mo-nitrogenase consists of two components; the Fe protein that contains a single iron-sulfur (Fe-S) cluster, and the MoFe protein that contains the active site FeMo-cofactor and the [8Fe-7S] P-cluster. The Fe protein, encoded by *nifH*, is the dinitrogenase reductase that donates electrons to the MoFe protein, the dinitrogenase composed of a heterotetramer of subunits encoded by *nifD* and *nifK*. Together NifH and NifDK catalyzes the energy intensive reduction of N_2 as shown: $N_2 + 16 \text{ ATP} + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16 \text{ ADP} + 16 p_i$ (7). As such, Mo-nitrogenase production and activity are highly regulated in diazotrophs and are only synthesized when a fixed N source is unavailable. When needed, Mo-nitrogenase is produced in high quantities and can comprise as much as 10% of the total protein of the cell (8).

In addition to having Mo-nitrogenase, some diazotrophs possess alternative nitrogenases that lack Mo (9, 10). The vanadium (V) nitrogenase and the Fe-only (Fe) nitrogenase contain an active site FeV-cofactor and FeFe-cofactor, respectively, instead of FeMo-cofactor (11, 12). The understanding of the genetic, biochemical, and catalytic properties of the alternative nitrogenases has primarily come from a few model bacteria (e.g., *Azotobacter vinelandii*). V-nitrogenase and Fe-nitrogenase have a similar subunit composition as Mo-nitrogenase, comprised of VnfH/VnfDK and AnfH/AnfDK subunits, respectively. However, a distinguishing feature of V-nitrogenase and Fe-nitrogenase is the presence of an additional subunit (G) that associates with the dinitrogenase component (i.e., VnfDGK and AnfdGK) (9, 11). The precise role of the G subunit is unknown, but it is required for diazotrophy in the absence of Mo (13). V-nitrogenase and Fe-nitrogenase are less efficient at reducing N_2 than Mo-nitrogenase. More electron flux is directed to H_2 production during reduction of N_2 by the alternative nitrogenases leading to substantially more ATP consumption. The V-nitrogenase and Fe-nitrogenase are estimated to produce 3 and 7 H_2 molecules and consume 24 ATPs and 40 ATPs, respectively, during the reduction of a single N_2 to 2 NH_3 (14, 15). As such, alternative nitrogenases in bacteria are only produced when insufficient levels of Mo are present to support usage of Mo-nitrogenase. In studied bacteria that possess all three nitrogenases, the expression and activity of each nitrogenase are highly regulated in response to metal and fixed N availability (9, 16). For example, metal-dependent expression of V-nitrogenase and Fe-nitrogenase in *A. vinelandii* involves the specific transcription regulators VnfA and AnfA that activate expression of V-nitrogenase and Fe-nitrogenase, respectively, in response to the absence of Mo (17, 18).

In addition to N_2 , nitrogenases from bacteria can reduce other double and triple-bonded substrates (e.g., CO, CO_2 , and acetylene). Moreover, in the absence of another substrate, nitrogenase reduces protons to H_2 , a feature that has been exploited to use nitrogenase to produce H_2 as a biofuel (19, 20). The substrate, product, and activity profiles are also different between the three nitrogenases. The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) is commonly used to measure nitrogenase activity (21). Mo-nitrogenase reduces acetylene at a higher rate than both V-nitrogenase and Fe-nitrogenase, both of which also further reduce ethylene to produce ethane (C_2H_6) as a minor product (22). Mo-nitrogenase does not produce ethane. Compared to Mo-nitrogenase, bacterial V-nitrogenase is also more adept at reducing CO to alkanes, and Fe-nitrogenase is better at reducing CO_2 to CH_4 (11, 23–25).

In contrast to bacterial diazotrophs, the regulation, assembly, and activity of nitrogenase, especially the alternative nitrogenases, are largely unknown in archaeal diazotrophs. Among archaea, only methanogens and the closely related anaerobic methanotrophs are known or predicted to fix N_2 (5, 26, 27). N_2 fixation has been studied

in a few species of methanogens. The primary models are the obligate CO₂-reducing methanogen *Methanococcus maripaludis*, and the more versatile species *Methanosarcina mazei* and *Methanosarcina barkeri* (28, 29). *Methanosarcina* species can grow using methylated compounds (e.g., methanol) and acetate, in addition to reducing CO₂ with H₂ (30). *M. maripaludis* and *M. mazei* only contain Mo-nitrogenase, whereas strains of *M. barkeri* are predicted to contain all three nitrogenases (31, 32). Mo-dependent and V-dependent N₂ fixation has been demonstrated in *M. barkeri* (33–35). To our knowledge, diazotrophy under Fe-only conditions using the Fe-nitrogenase has not been documented for any methanogen. Previous research has primarily focused on elucidating the mechanisms that regulate the production and activity of Mo-nitrogenase in methanogens, revealing that the regulatory proteins used to control transcription and activity of Mo-nitrogenase are distinct from those used by most bacteria (36, 37). Recently, small RNAs (sRNAs) have also been demonstrated to play roles in N₂ fixation and assimilation in methanogens (38, 39).

Methanosarcina acetivorans serves as an ideal model methanogen to understand the regulation and usage of the alternative nitrogenases in methanogens, since its genome encodes all three nitrogenases and it has a robust genetic system (40–43). Recently, it was shown that *M. acetivorans* can fix N₂ using Mo-nitrogenase. Like *M. maripaludis*, *M. mazei*, and *M. barkeri*, Mo-nitrogenase is only produced in *M. acetivorans* when cells are grown in the absence of a fixed N source (e.g., NH₄Cl). Silencing of the *nif* operon in *M. acetivorans* using the recently developed CRISPRi-dCas9 system confirmed that Mo-nitrogenase is required for diazotrophy when cells are supplied Mo (43). However, to our knowledge, the ability of *M. acetivorans* to fix N₂ when Mo is not available has not been documented nor have the activities of *M. acetivorans* V-nitrogenase or Fe-nitrogenase been reported. Presumably, *M. acetivorans* produces V-nitrogenase and/or Fe-nitrogenase when both fixed N and Mo are limiting. In this study, we show that *M. acetivorans* can grow by fixing N₂ in the absence of Mo with growth dependent on the production of V-nitrogenase and/or Fe-nitrogenase. These results provide a foundation to understand the regulation and properties of the three nitrogenases in methanogens.

RESULTS

Organization of nitrogenase genes in *M. acetivorans* and prevalence of alternative nitrogenases in methanogens

The annotated genome of *M. acetivorans* contains three separate nitrogenase gene clusters (Fig. 1), designated *nif*, *vnf*, and *anf*, encoding putative Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase, respectively (40). The gene arrangement of the *nif* cluster is similar to the characterized *nif* operons from *M. maripaludis*, *M. barkeri*, and *M. mazei* (32, 44, 45). In addition to encoding the nitrogenase structural components (NifH and NifDK), the operon also encodes the regulatory proteins Nif1 and Nif2 and the FeMo-cofactor scaffold proteins NifEN (12, 46). The *M. acetivorans vnf* cluster contains the same gene arrangement as *nif*, including its own regulatory and scaffold genes, but also includes *vnfG* and a homolog of *nifX*, designated *vnfX*. Additional copies of *nifX* are found in the genome of *M. acetivorans*. NifX is involved in FeMo-cofactor assembly in bacteria (12). The gene arrangement of the *M. acetivorans anf* cluster is like the *vnf* cluster, except *anfH* encoding the putative Fe-protein is divergent and downstream of *anfK*. The *anf* and *vnf* gene clusters are opposite each other in the chromosome of *M. acetivorans* (Fig. 1). Interestingly, the amino acid sequences of VnfH and AnfH are identical, indicating the same Fe-protein functions with both V-nitrogenase and Fe-nitrogenase. Also unique to the *anf* cluster is the presence of homologs of Anf3 and AnfO found in *anf* operons of bacteria. Anf3 is essential for diazotrophy with the Fe-nitrogenase in *Rhodobacter capsulatus* (47). An Anf3 homolog characterized in *A. vinelandii* is a heme-binding and FAD-binding oxidase that may protect the Fe-nitrogenase from oxygen (48). Recently, AnfO was shown to control misincorporation of the FeV-cofactor in AnfDGK (49).

The *nif*, *vnf*, and *anf* gene clusters are widely distributed within genera of bacteria. However, nitrogenase genes are found only in a subset of archaea, restricted to

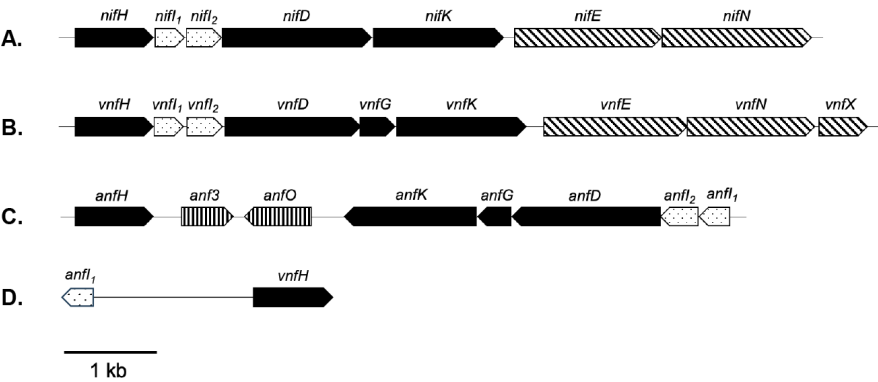


FIG 1 Arrangement of nitrogenase gene clusters in the genome of *M. acetivorans*. (A) *nif*; Mo-nitrogenase, (B) *vnf*; V-nitrogenase, (C) *anf*; Fe-nitrogenase. Locus tags: *nifH*₁₁₂*DKEN* (*ma3895-3901*), *vnfH*₁₁₂*DGKENX* (*ma1213-1221*), *anfI1*₂*DKEN* (*ma1212-1205*). Black arrows: nitrogenase subunits, diagonal striped arrows: cofactor assembly proteins, dotted arrows: regulatory proteins and vertical striped arrows: unknown function. (D) The *vnf* and *anf* gene clusters are opposite each other in the chromosome as shown.

methanogens and closely related anaerobic methanotrophs. The *nif* operon is distributed across six of the seven orders of methanogens, whereas the *vnf* and *anf* genes are restricted to the Methanosarcinales, with few exceptions, namely *Methanobacterium lacus*, which contains an *anf* gene cluster (5, 26, 27). Like bacteria, all methanogens that contain putative *vnf* and *anf* clusters also contain the *nif* operon. Out of the 41 complete Methanosarcinales genome sequences analyzed from the NCBI database, ~66% contain the *nif* genes. Of those containing *nif*, ~44% contain the *vnf* and/or *anf* genes (Table S1). The arrangement of the *vnf* and *anf* gene clusters are similar across the Methanosarcinales (Fig. S1). Of note is a hypothetical protein encoded by a gene between *vnfD*GK and *vnfE*N in several *Methanosarcina* species.

Molybdenum and vanadium availability affect diazotrophic growth of *M. acetivorans*

To ascertain the effect of molybdenum and vanadium availability on nitrogenase utilization by *M. acetivorans*, the wild-type strain WWM73 (Table 1) that is commonly used for genetic analysis (42) was passed in high-salt (HS) standard medium lacking Mo for >100 generations to deplete molybdate to <1 ppb as determined by ICP-MS. Molybdate is the biological available form of Mo. Vanadium is not present in standard HS medium. Mo-depleted cells were used to inoculate Mo-depleted HS medium devoid of NH₄Cl (fixed N source). Methanol was used as the carbon and energy source in all experiments. Molybdate, vanadate, and NH₄Cl were added from sterile anaerobic stocks to separate cultures to compare the effect of Mo, V, and fixed N on growth and nitrogenase expression. Neither the depletion of Mo nor the addition of V affects the generation time, or cell yield when NH₄Cl is supplied as the fixed N source (Fig. 2; Table 2). However, the depletion of Mo and the addition of V significantly affect the generation time and cell yield in cultures without NH₄Cl (diazotrophic). When *M. acetivorans* is provided Mo

TABLE 1 *M. acetivorans* strains used in this study

Strain	Description	gRNA	References
WWM73	Wild-type strain used for genetic analysis	None	(42)
DJL72	WWM73 with integrated CRISPRi plasmid	None	(43)
DJL74	WWM73 with integrated CRISPRi plasmid	gRNA- <i>nifH</i>	(43)
DJL107	WWM73 with integrated CRISPRi plasmid	gRNA- <i>vnfH</i>	This study
DJL120	WWM73 with integrated CRISPRi plasmid	gRNA- <i>anfI1</i>	This study
DJL119	WWM73 with integrated CRISPRi plasmid	gRNA- <i>vnfH</i> + gRNA- <i>anfI1</i>	This study

TABLE 2 Effect of metal and NH₄Cl availability on growth of *M. acetivorans* with methanol

Relevant metals	Nitrogen source	Lag time (h) ^a	Generation time (h) ^b	Cell yield (cells/mL) ^b
Mo + Fe	NH ₄ Cl	30	8.2 ± 0.5	3.02 ± 0.2 × 10 ⁸
	N ₂	48	28.5 ± 4	1.92 ± 0.3 × 10 ⁸
V + Fe	NH ₄ Cl	30	8.5 ± 0.1	3.08 ± 0.2 × 10 ⁸
	N ₂	90	44.5 ± 4.1	1.53 ± 0.6 × 10 ⁸
Fe-only	NH ₄ Cl	30	8.7 ± 0.1	3.34 ± 0.2 × 10 ⁸
	N ₂	96	82 ± 4.1	9.88 ± 0.4 × 10 ⁷

^aApproximate time when culture entered exponential phase.

^bGeneration time and cell yield represent the mean ± 1 SD from at least three biological replicates. Cell yield was determined by cell counts (initial cells/mL = 6.0 × 10⁶).

in the absence of NH₄Cl, the generation time increases approximately threefold, and the cell yield decreases by approximately 37% compared to non-diazotrophic cultures (Table 2). Diazotrophic cultures lacking Mo but provided V have an even longer generation time and further reduction in cell yield (~50% that of non-diazotrophic cultures). Diazotrophic growth is further impacted by the absence of both Mo and V, with an ~10-fold increase in generation time and an ~70% reduction in cell yield compared to non-diazotrophic cultures (Fig. 2; Table 2). Diazotrophic cultures lacking Mo also have an extended lag phase compared to diazotrophic cultures containing Mo (Fig. 2; Table 2). These data reveal that *M. acetivorans* is capable of diazotrophy in the absence of Mo, and that V availability impacts N₂ fixation. These results are consistent with *M. acetivorans* utilizing Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase to fix N₂ according to Mo and V availability.

Methylotrophic methanogenesis is not altered by diazotrophy or the availability of molybdenum or vanadium

Growth of *M. acetivorans* with methanol utilizes the methylotrophic pathway of methanogenesis, where one methyl group of methanol is oxidized to CO₂, and the resulting three electron pairs are used to reduce three additional methyl groups to CH₄ (50). To determine if diazotrophy and metal availability affect the flow of carbon during methylotrophic methanogenesis, contributing to the slower growth rate and lower cell yields in the absence of Mo, total CH₄ was determined after the cessation of growth of nondiazotrophic and diazotrophic cultures. Similar amounts of CH₄ were observed across all growth conditions (Table 3), revealing N₂ fixation and differences in Mo and V availability does not significantly alter the flow of carbon during methylotrophic methanogenesis. Therefore, the observed hierarchical decrease in cell yields during

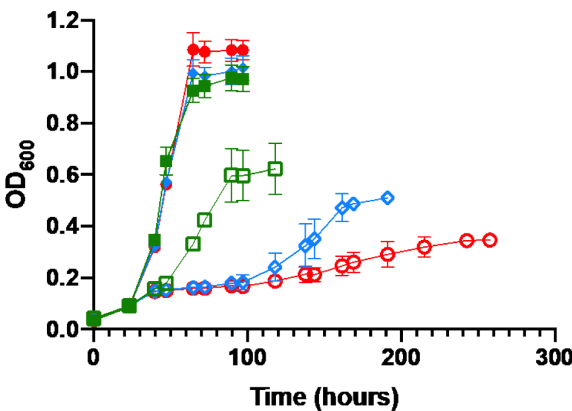


FIG 2 Comparison of the growth of *M. acetivorans* in the presence (closed) or absence (open) of NH₄Cl in HS medium with Mo + Fe (green squares), V + Fe (blue diamonds), or Fe alone (red circles). Inoculum: stationary phase cells grown in Mo-depleted HS medium with NH₄Cl. Error bars represent mean ± 1 SD from at least three biological replicates.

TABLE 3 Effect of metal and NH₄Cl availability on total CH₄ production by *M. acetivorans* with methanol

Relevant metals	Nitrogen source	CH ₄ produced (μmol) ^a
Mo + Fe	NH ₄ Cl	1,004 ± 109
	N ₂	1,092 ± 58
V + Fe	NH ₄ Cl	926 ± 193
	N ₂	823 ± 24
Fe-only	NH ₄ Cl	1,031 ± 48
	N ₂	1,079 ± 41

^aData represent the mean ± 1 SD from at least three biological replicates (10 mL cultures).

diazotrophic growth under Mo + Fe, V + Fe, or Fe-only conditions (Table 3) is not due to altered methanogenesis but is likely due to the increased ATP consumption needed to support N₂ reduction by Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase, as seen in bacteria (9).

Molybdenum availability affects the expression of V-nitrogenase and Fe-nitrogenase but not Mo-nitrogenase in *M. acetivorans*

Previous results demonstrated that Mo-nitrogenase is not produced in *M. acetivorans* cells grown in the presence of NH₄Cl. Removal of NH₄Cl results in a modest increase in *nif* transcription and production of Mo-nitrogenase, allowing growth with N₂. Moreover, CRISPRi repression of the *nif* operon abolished the ability to grow with N₂ in medium containing Mo (43). To determine the effect of fixed N and Mo depletion on Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase expression, qPCR was performed using primers specific for *nifD*, *vnfD*, and *anfD* to analyze transcript abundance in cells grown in medium with or without NH₄Cl and containing Mo + Fe, V + Fe, or Fe-only (Fig. 3). An increase in transcript abundance for *nifD* and *vnfD* was observed in cells grown in Mo + Fe medium without NH₄Cl, relative to the transcript abundance in cells grown with NH₄Cl (Fig. 3A). However, only the fold change for *vnfD* was significant. Comparison of *nifD*, *vnfD*, and *anfD* transcript abundance from cells grown with V + Fe showed a significant fold change for *vnfD* and *anfD* (Fig. 3B). The transcript abundance of *vnfD* is ~180-fold higher in cells grown in V + Fe medium without NH₄Cl compared to cells grown with NH₄Cl. Transcript abundance for *anfD* is ~60-fold higher in cells grown in V + Fe medium without NH₄Cl compared to cells grown with NH₄Cl. In contrast, only a slight increase (~threefold) was observed for *nifD* transcript abundance. Like the transcript abundance of *vnfD* and *anfD* in cells grown with V + Fe, cells grown in Fe-only medium lacking NH₄Cl had a significant increase in *vnfD* and *anfD* transcript abundance compared to cells grown with NH₄Cl (Fig. 3C). No change in the expression of *nifD* was detected in cells grown in Fe-only medium lacking NH₄Cl relative to that with NH₄Cl (Fig. 3C).

To further determine the effect of Mo depletion on transcription of each nitrogenase gene cluster, the fold change in *nifD*, *vnfD*, and *anfD* transcript abundance was also calculated by comparing the relative abundance in cells grown in V + Fe or Fe-only medium to the transcript abundance in cells grown in Mo + Fe medium (Fig. 4). The expression of *nifD* did not significantly change in cells grown in medium with or without Mo, regardless of the presence or absence of NH₄Cl (Fig. 4A). However, the removal of Mo significantly affected the transcription of both *vnfD* and *anfD* in cells grown with or without NH₄Cl (Fig. 4B and C). The transcript abundance of *vnfD* is highest in cells grown in Fe-only medium, with the fold change higher than when V is present. A similar pattern was observed for the expression of *anfD*. However, the fold change in expression of *anfD* in cells grown with Fe-only compared to Mo + Fe was much higher (~300- to 600-fold). These results indicate there is significant regulatory control of transcription of the *vnf* and *anf* gene clusters, whereas there is only modest transcriptional control of the *nif* operon. The results also show that the depletion of Mo is the key signal that increases transcription of the *vnf* and *anf* gene clusters. Removal of a fixed N source (NH₄Cl) when

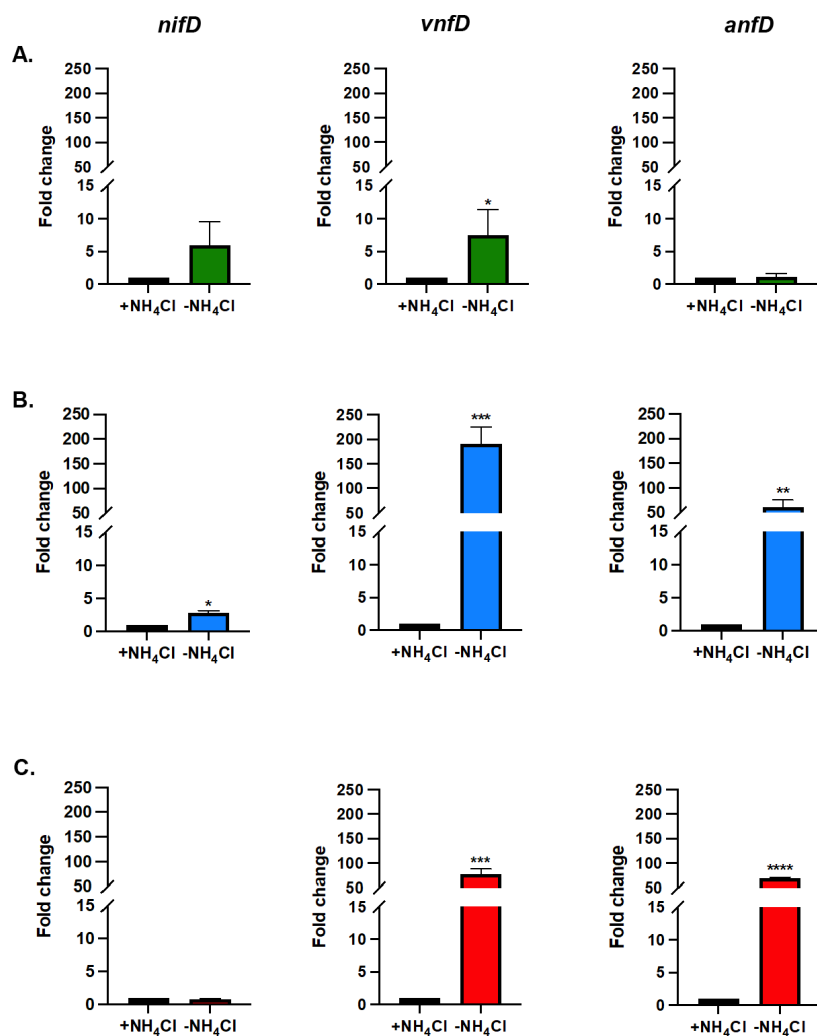


FIG 3 Effect of fixed N availability on the transcription of the *nif*, *vnf*, and *anf* gene clusters in *M. acetivorans* as determined by qPCR. The relative abundance of *nifD*, *vnfD*, and *anfD* transcripts in *M. acetivorans* cells grown with NH₄Cl (normalized to one) were compared to cells grown without NH₄Cl and 16S rRNA was used as the control. *M. acetivorans* was grown with methanol in HS medium containing (A) Mo + Fe (B) V + Fe, or (C) Fe-only. Error bars represent mean $\pm$ 1SD for two technical replicates and three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Mo is available has only a slight effect on the transcription of the *vnf* and *anf* gene clusters (Fig. 3A).

The production of Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase in *M. acetivorans* grown under the same conditions for qPCR analysis was determined by Western blot using antibodies specific to NifD, VnfD, and AnfD (Fig. 5), and a loading control SDS-Page gel is shown in Fig. S2. Consistent with previous results (43), NifD was only detected in lysate from *M. acetivorans* cells grown in Mo + Fe medium lacking NH₄Cl. Neither VnfD nor AnfD was detected in lysate from cells grown in Mo + Fe medium regardless of the presence or absence of NH₄Cl. However, both VnfD and AnfD were detected in lysate from cells grown in Mo-depleted medium lacking NH₄Cl. Interestingly, NifD was also detected in lysate from cells grown in Mo-depleted medium. The availability of V does not appear to affect the production of VnfD or AnfD. These results indicate that both the depletion of fixed N and Mo are required for production of V-nitrogenase and Fe-nitrogenase in *M. acetivorans*.

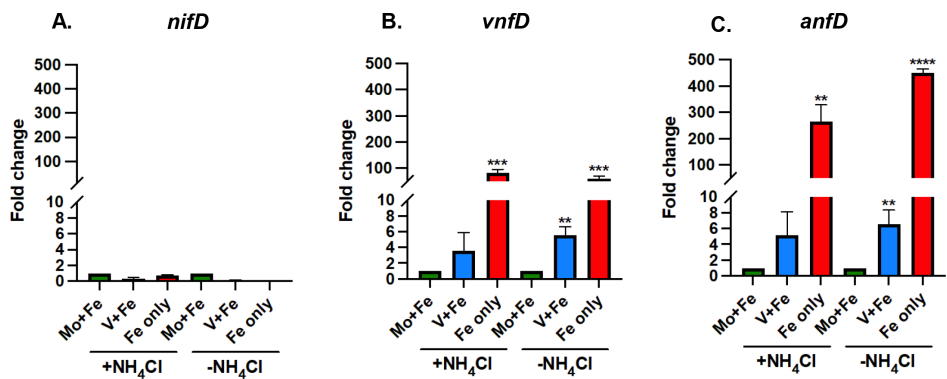


FIG 4 Effect of molybdenum availability on the transcription of the *nif*, *vnf*, and *anf* gene clusters in *M. acetivorans* as determined by qPCR. The relative abundance of (A) *nifD*, (B) *vnfD*, and (C) *anfD* transcripts in cells grown with Mo (normalized to 1) were compared to cells grown without Mo, and 16S rRNA was used as the control. Error bars represent mean $\pm$ 1SD for two technical replicates and three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Mo-nitrogenase is required for V-nitrogenase and Fe-nitrogenase expression

To determine the role of each nitrogenase during diazotrophy in Mo-repleted and Mo-depleted medium, growth studies were performed with the previously described *nif* operon CRISPRi repression strain DJL74 and new *vnf* and/or *anf* operon CRISPRi repression strains (Table 1). As described previously, strain DJL74 is unable to grow in the absence of fixed nitrogen and presence of Mo (Fig. 6A). Surprisingly, strain DJL74 is also unable to grow in the absence of Mo when V-nitrogenase and/or Fe-nitrogenase should support N₂ fixation (Fig. 6A). This result reveals that Mo-nitrogenase expression is required for diazotrophy under all conditions tested, consistent with NifD detection when fixed nitrogen is depleted regardless of the presence/absence of Mo (Fig. 5). VnfD is not detected by Western blot (loading control gel in Fig. S4) in lysates from strain DJL107 (Fig. 7), consistent with complete repression of the *vnf* operon. However, strain DJL107 grows comparable to the control strain DJL72 under all conditions (Fig. 6B). Similarly, AnfD is not detected by Western blot (loading control gel in Fig. S4) in lysates from strain DJL120 (Fig. 7). Strain DJL120 also grows similar to strain DJL72, except under Fe-only conditions, where it is unable to grow, indicating that expression of the Fe-nitrogenase is required for diazotrophy in the absence of Mo and V. Taken together, these data indicate the V-nitrogenase likely only supports diazotrophy in presence of V, while the Fe-nitrogenase supports diazotrophy in the presence and absence of V. Importantly, neither VnfD nor AnfD is detected by Western blot (loading control gel in Fig. S4) in cell lysates of the dual *vnf/anf* repression strain DJL119, with these cells only producing NifD (Fig. 7). Strain DJL119 grows similar to the control strain in the presence of Mo, but is unable to grow in medium lacking Mo, regardless of the presence/absence of V (Fig. 6D), revealing that the alternative nitrogenases are required for diazotrophy

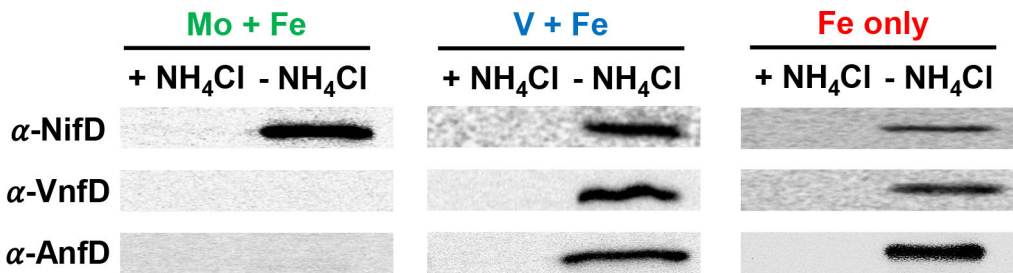


FIG 5 Western blot analysis using NifD-specific, VnfD-specific, and AnfD-specific antibodies on lysate from *M. acetivorans* cells grown with or without NH₄Cl and the indicated metals.

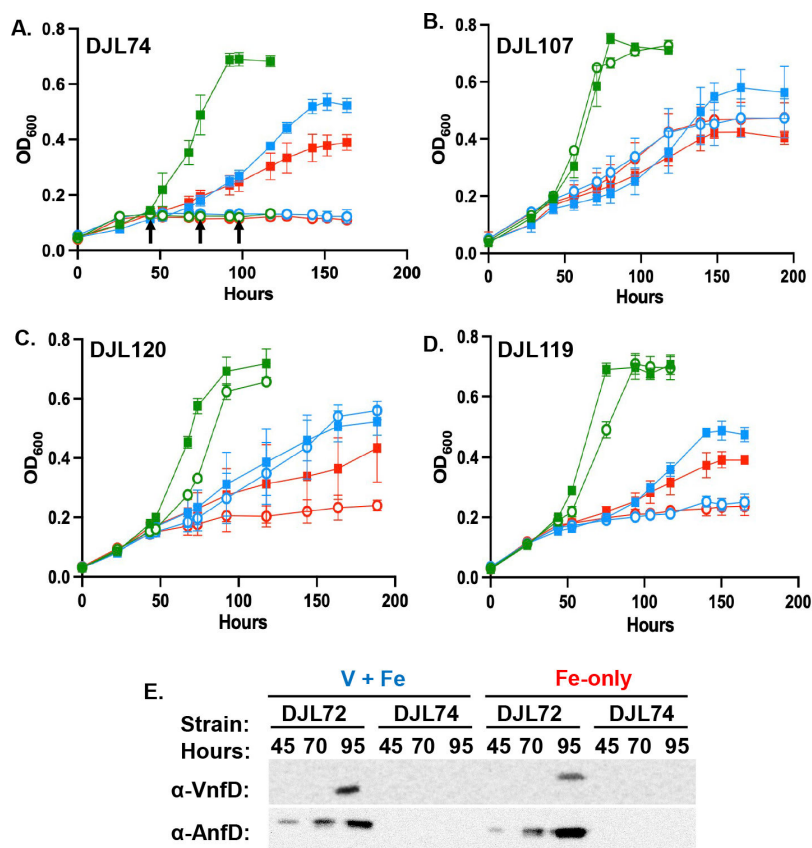


FIG 6 Comparison of the growth of *M. acetivorans* CRISPRi repression strains. (A) DJL74 (*gRNA-nifH*), (B) DJL107 (*gRNA-vnfH*), (C) DJL120 (*gRNA-anf1*), and (D) DJL119 (*gRNA-vnfH/anf1*) to the control strain DJL72 in the absence of NH_4Cl in HS medium with Mo + Fe (green), V + Fe (blue), or Fe alone (red). In all panels, strain DJL72 is represented by squares and the CRISPRi repression strain by circles. Error bars represent mean $\pm$ 1SD from at least three biological replicates. (E) Western blot of lysates from cells of strains DJL72 and DJL74 taken at the timepoints indicated by the arrows in panel (A).

in the absence of Mo. This result also demonstrates that Mo-nitrogenase alone in strain DJL119 is unable to support diazotrophy in the absence of Mo.

To test if *nif* operon expression is required for alternative nitrogenase production, V-nitrogenase and Fe-nitrogenase expression over time in strains DJL72 and DJL74 grown in Mo-depleted medium was probed by Western blot (loading control gel in Fig. S5) using antibodies specific to VnfD and AnfD (Fig. 6E). Whereas the detection of both VnfD and AnfD occurs in cells of strain DJL72 grown in V + Fe and Fe-only medium, both VnfD and AnfD are not detected in strain DJL74. This result clearly demonstrates that the expression of the *nif* operon is required for production of both V-nitrogenase and Fe-nitrogenase and explains why strain DJL74 is unable to grow fix N_2 in the absence of Mo (Fig. 6A).

DISCUSSION

The regulation, assembly, and activity of the three forms of nitrogenase are well understood in diazotrophic bacteria, especially in the principal model *A. vinelandii* that contains all three nitrogenases. *A. vinelandii* is an obligate aerobe; thus, in addition to nitrogenase structural proteins, *A. vinelandii* requires accessory proteins to prevent oxidative damage to nitrogenase and to integrate nitrogen fixation into central metabolism. At least 82 genes are predicted to be involved in the formation and regulation of Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase in *A. vinelandii* (16).

Moreover, there is complex regulatory control over hierarchal nitrogenase expression, with only one nitrogenase produced at a time. When fixed N is absent and Mo is available, Mo-nitrogenase is preferentially produced over V-nitrogenase and Fe-nitrogenase, followed by V-nitrogenase if Mo is absent and V is present. If neither Mo nor V is available, then Fe-nitrogenase is produced (26). Among anaerobic methanogens, the alternative nitrogenases are restricted primarily to the Methanosarcinales, the most metabolically diverse methanogens with the largest genomes. Nonetheless, the genomes of sequenced Methanosarcinales contain simpler nitrogenase gene clusters and lack many of the accessory and regulatory proteins found in *A. vinelandii* and other diazotrophic bacteria (27). The formation and regulation of the alternative nitrogenases are likely simpler in methanogens compared to aerobic diazotrophic bacteria. The results presented here demonstrate that *M. acetivorans* produces all three nitrogenases and is capable of diazotrophy in the absence of available Mo and V (Fe-only condition). Results from the CRISPRi repression strains reveal that diazotrophy in the absence of Mo requires V-nitrogenase and/or Fe-nitrogenase. To our knowledge, this is the first direct evidence of a methanogen using a Fe-nitrogenase.

Like other diazotrophs, *M. acetivorans* only produces nitrogenase in the absence of fixed N. The diazotrophic growth profiles of *M. acetivorans* correlate with reported ATP requirements by Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase from bacteria (14). *M. acetivorans* has the fastest growth rate and highest cell yield during diazotrophic growth when utilizing only Mo-nitrogenase. Only a modest increase in transcription of the *nif* operon was observed in response to fixed N depletion, even though NifD is only detected in cells not provided fixed N. The high basal level of transcription of the *nif* operon likely allows *M. acetivorans* to be poised for rapid Mo-nitrogenase production. The relatively short lag time before the onset of diazotrophic growth in Mo + Fe medium (Table 2; Fig. 2) supports the rapid production of Mo-nitrogenase.

The results indicating minimal transcriptional control of the *nif* operon further support that post-transcriptional regulation is a key factor controlling Mo-nitrogenase production. Previous studies investigated the role of NrpR in regulating the expression of

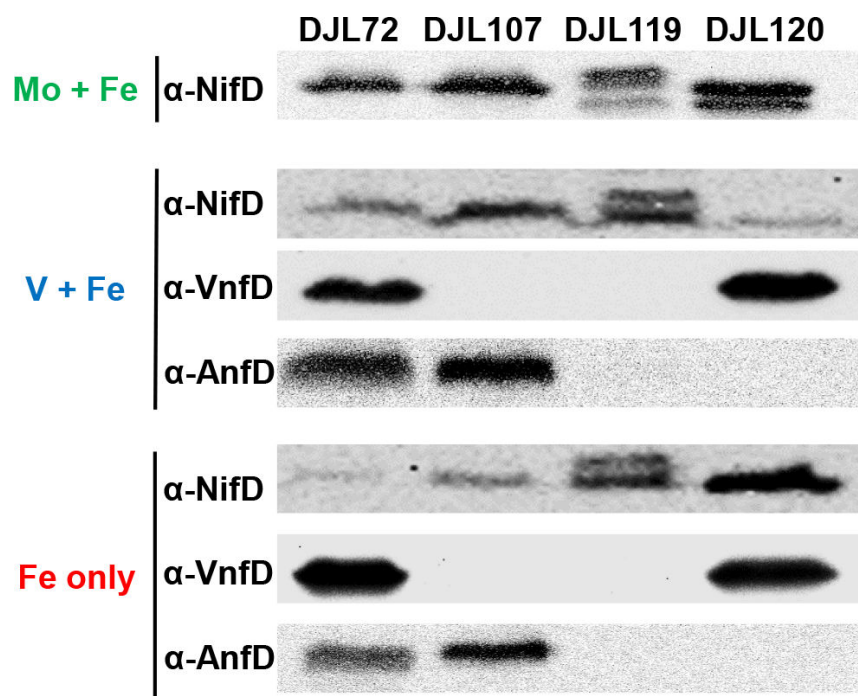


FIG 7 Western blot analysis using NifD-specific, VnfD-specific, and AnfD-specific antibodies on lysate from cells of *M. acetivorans* CRISPRi repression strains DJL107, DJL120, DJL119, and control strain DJL72 grown without NH_4Cl and the indicated metals.

Mo-nitrogenase in *M. acetivorans*. NrpR is the repressor of the *nif* operon in methanogens and indirectly senses fixed N availability by directly sensing intracellular 2-oxoglutarate levels (51). A mutant strain of *M. acetivorans* where *nrpR* transcription was silenced using the CRISPRi-dCas9 system revealed that the depletion of NrpR results in an increase in the transcription of the *nif* operon, but the mutant still fails to produce detectable nitrogenase when grown with fixed N (43). In *M. mazei*, a small RNA (sRNA₁₅₄) is exclusively expressed when fixed N is limiting and functions to stabilize the polycistronic mRNA produced from the *nif* operon (38). The genome of *M. acetivorans* encodes a sRNA₁₅₄ homolog, indicating similar post-transcriptional regulation of the *nif* operon. Interestingly, removal of Mo did not significantly alter transcription of the *nif* operon or the production of nitrogenase (Fig. 4A and 5). Therefore, the critical and likely only signal for Mo-nitrogenase production in *M. acetivorans* is fixed N limitation. This is distinct from diazotrophic bacteria that contain V-nitrogenase and Fe-nitrogenase. For example, *A. vinelandii* and the purple nonsulfur phototroph *Rhodospseudomonas palustris* both stop producing Mo-nitrogenase when Mo is depleted (26, 52).

While Mo-depletion had little effect on Mo-nitrogenase expression, it is critical for the expression of V-nitrogenase and Fe-nitrogenase in *M. acetivorans*. Both fixed N and Mo depletion are required for production of V-nitrogenase and Fe-nitrogenase (Fig. 5). Importantly, Mo depletion resulted in a significant increase in the relative transcript abundance of *vnfD* and *anfD* (Fig. 3 and 4). Thus, unlike production of Mo-nitrogenase, transcriptional regulation is a key mechanism to control production of V-nitrogenase and Fe-nitrogenase in *M. acetivorans*. The overall transcript abundance profiles for *vnfD* and *anfD* are similar across all growth conditions. Mo depletion appears to be a key effector as cells grown with NH₄Cl exhibited a significant increase in transcript abundance of *vnfD* and *anfD* (Fig. 4). Nonetheless, neither VnfD nor AnfD was detected in cells grown with NH₄Cl in Mo-depleted medium (Fig. 5), indicating post-transcriptional regulation of *vnf* and *anf* genes is also likely involved. Unexpectedly, in the absence of Mo, the presence of V does not increase the transcript abundance of *vnfD* and *anfD* as much as the increase during Fe-only conditions (Fig. 4). The role V plays in nitrogenase regulation is unknown in most diazotrophs. Nevertheless, when comparing the effect of fixed N depletion, a large relative fold change in transcript abundance for *vnfD* and *anfD* was observed in cells grown in V + Fe medium (Fig. 3B). Expression of the *vnf* and *anf* operons in *A. vinelandii* in the absence of Mo results in the production of either V-nitrogenase or Fe-nitrogenase depending on V availability, but not both. In contrast, V availability had no effect on V-nitrogenase or Fe-nitrogenase production in *M. acetivorans*, as each was produced in cells grown in Mo-depleted medium (Fig. 5). Notably, VnfH and AnfH are identical in amino acid sequence, indicating a single dinitrogenase reductase (VnfH/AnfH) can support the *in vivo* activities of separate dinitrogenases (VnfDGK and AnfDGK).

Production of both V-nitrogenase and Fe-nitrogenase in *M. acetivorans* clearly requires fixed N depletion since neither VnfD nor AnfD was detected by immunoblot in lysate from cells grown with NH₄Cl regardless of Mo availability. Regulation of V-nitrogenase and Fe-nitrogenase expression in response to fixed N availability does not likely involve direct control of *vnf* and *anf* transcription since fixed N depletion in the presence of Mo did not alter *anfD* transcript abundance and only had a modest effect on *vnfD* transcript abundance (Fig. 3A). These results are consistent with the promoter regions of both the *vnf* and *anf* gene clusters lacking the identified NrpR operator sequence (53). The promoter regions also lack identified binding sites for NrpA, an activator of the *nif* operon in *M. mazei*, for which *M. acetivorans* encodes two homologs (MA0545 and MA0546) (54). Thus, post-transcriptional regulation is likely the primary mechanism of control of V-nitrogenase and Fe-nitrogenase production in response to fixed N availability. It is possible sRNA₁₅₄, or another sRNA, is responsive to fixed N depletion and functions to stabilize *vnf* and *anf* mRNAs, which allows for V-nitrogenase and Fe-nitrogenase production only when fixed N is depleted.

Mo availability is the key factor controlling transcription of both the *vnf* and *anf* gene clusters in *M. acetivorans*. In non-diazotrophic (e.g., *Escherichia coli*) and

diazotrophic bacteria, the molybdate-responsive transcriptional regulator ModE controls the expression of the high-affinity molybdate transporter ModABC as well as Mo-dependent enzymes (55). In *A. vinelandii*, ModE indirectly represses the expression of both V-nitrogenase and Fe-nitrogenase by directly repressing the transcription of the genes encoding the regulators VnfA and AnfA. VnfA activates transcription of the *vnf* operon and AnfA activates transcription of the *anf* operon in *A. vinelandii* (55). The genome of *M. acetivorans* encodes several homologs of ModABC (MA0325-27, MA1235-37, and MA2280-82), including additional homologs of ModBC (MA3902-03) downstream of the *nif* operon. *M. acetivorans* contains a ModE homolog (MA0283) but lacks homologs to VnfA and AnfA. Potential ModE-binding sites are located upstream of *vnfH* and *anfI*₁, the first genes in the *vnf* and *anf* gene clusters (56). Therefore, it is highly plausible that ModE is responsible for repressing transcription of *vnf* and *anf* when sufficient Mo is available to support Mo-nitrogenase activity. Depletion of Mo (corepressor) likely results in removal of DNA-bound ModE and de-repression of transcription of the *vnf* and *anf* gene clusters, leading to the simultaneous production of V-nitrogenase and Fe-nitrogenase in *M. acetivorans*. The results are consistent with this regulatory mechanism. Interestingly, the starter inoculum used in all expression studies was maintained in Mo-depleted medium, which should result in an increase in *vnf* and *anf* transcription even during growth with NH₄Cl (Fig. 4). As such, the starter inoculum should be primed to use the alternative nitrogenases once fixed N is depleted, yet there was a much longer period before the onset of growth in Mo-depleted medium compared to the onset of growth in Mo-depleted medium with added Mo (Table 2; Fig. 2). This result indicates that there are likely other unknown regulatory factors involved in controlling the production of V-nitrogenase and Fe-nitrogenase in response to fixed N and Mo depletion.

To our knowledge, *M. acetivorans* is the first diazotroph shown to simultaneously produce all three nitrogenases when grown in Mo-depleted conditions, indicating that the function of the alternative nitrogenases is intimately connected to Mo-nitrogenase. Although *M. acetivorans* may continue to produce Mo-nitrogenase when fixed N is limiting regardless of Mo availability to be poised to use the most efficient nitrogenase, since energy conservation (i.e., ATP generation) during methanogenesis by *M. acetivorans* is significantly lower compared to studied diazotrophic bacteria (57), the results with strain DJL74 clearly demonstrate that *nif* operon expression is required for expression of functional V-nitrogenase and Fe-nitrogenase. Of the proteins encoded in the *nif* operon (Fig. 1), NifH could be the critical factor, since in addition to providing electrons to NifDK during N₂ reduction, it serves multiple roles in nitrogenase maturation in bacteria. For example, NifH is involved in the synthesis of the complex metalloclusters within NifDK (e.g., P-cluster) (3, 12, 58). Therefore, NifH could be required for metallocluster synthesis in VnfDGK and AnfDGK. Although VnfEN scaffold proteins are encoded in the *vnf* gene cluster, it is also possible NifEN is needed for metallocluster synthesis in VnfDGK and/or AnfDGK. Alternatively, inactive NifDK may serve a regulatory role in controlling the production of active V-nitrogenase and Fe-nitrogenase. We are currently investigating which *nif* operon proteins are required for alternative nitrogenase production.

Overall, the results from this study reveal that the expression and usage of the alternative nitrogenases in *M. acetivorans* is distinct from studied bacteria, and that Mo-nitrogenase expression is directly linked to the production of functional alternative nitrogenases. Finally, the results highlight the utility of *M. acetivorans* as a model to understand the regulation, maturation, and activity of the three forms of nitrogenase in methanogens.

MATERIALS AND METHODS

M. acetivorans strains and growth

M. acetivorans strain WWM73, a pseudo-wild type strain used for genetic manipulation (42), was used for all experiments. Anoxic HS medium was prepared as previously described with some modifications (59). To prepare Mo-depleted HS medium, all glassware was washed twice with 1 M HCl, once with 1 M H₂SO₄, and then rinsed with ultrapure water to remove any residual molybdate prior to use. NH₄Cl and molybdate were omitted and the HS medium was reduced with 1.5 mM DTT. The concentration of molybdate in Mo-depleted HS medium was determined <1 ppb by ICP-MS (University of Arkansas Stable Isotope Facility). Methanol, NH₄Cl, sodium sulfide (Na₂S), sodium molybdate (Na₂MoO₄), and sodium vanadate (Na₃VO₄) were added from anoxic sterile stocks using sterile syringes prior to inoculation. *M. acetivorans* strain WWM73 was grown in Balch tubes containing 10 mL of HS medium with 125 mM methanol and 0.025% Na₂S (wt/vol). Molybdate (1 μM), vanadate (1 μM), and NH₄Cl (18 mM) were added to cultures as indicated. *M. acetivorans* strain WWM73 was grown for more than 100 generations in Mo-depleted HS medium containing methanol and NH₄Cl prior to the growth experiments. The inoculum used in growth experiments was stationary phase cells grown in Mo-depleted HS medium with NH₄Cl. Growth was measured by monitoring optical density at 600 nm (OD₆₀₀) using a spectrophotometer. Cell density was determined from OD₆₀₀ using a standard curve generated by direct cell counts with a hemocytometer.

Quantitative PCR analysis of gene expression

M. acetivorans cells were harvested during mid-log phase (0.3–0.4 OD₆₀₀) by anaerobic centrifugation of 4–8 mL of culture. Cell pellets were resuspended in 1 mL TRIzol and frozen at –80°C. RNA was extracted using the Zymo Direct-zol Miniprep kit (#R2052) and further purified using the Invitrogen DNA-free DNA Removal Kit (#AM1906). cDNA was generated using the Bio-Rad iScript Select cDNA Synthesis Kit (#1708896). qPCR primers were designed using Geneious Prime to analyze *nifD* (Forward: 5'-CGC-CCGCTGTGAAGGATATA-3', Reverse: 5'-TTATGTCAAAGGGAGTGGGGTC-3'), *vnfD* (Forward: 5'-GTCGGAAAGAGTTGCAGCTA-3', Reverse: 5'-GCTTCGTGTGCCAGGTATCA-3'), *anfD* (Forward: 5'-GTCTCCCTGATGGCCGAATT-3', Reverse: 5'-AGATCTGTCTCTGGCCTGGT-3'), and 16srRNA (Forward: 5'-GGTACGGTTGTGAGAGCAA-3', Reverse: 5'-CTCGGTGTCCCC-TTATCACG-3').

qPCR of three biological replicates and two technical replicates was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #1725271). Relative quantification was determined using the 2^{–ΔΔC_q} method with 16srRNA as a standard as described (43).

Western blot analysis

Separate custom polyclonal antibodies specific for *M. acetivorans* NifD, VnfD, or AnfD were generated using the PolyExpress Silver package (two epitopes) from Genscript. Specificity of the antibodies was confirmed using recombinant NifD, VnfD, and AnfD expressed in *E. coli* (data not shown). *M. acetivorans* cells were harvested during mid-log phase (0.3–0.4 OD₆₀₀) by aerobic centrifugation (8500 × *g* for 10 min at 4°C) of 6 mL of culture. The cell pellet was resuspended in 50 mM Tris, 150 mM NaCl pH 7.2 with 1 mM PMSF and 1 mM benzamidine, normalized based on OD₆₀₀, and frozen at –80°C. Whole-cell lysate was generated by five freeze/thaw cycles and a 1-h DNase (5 μg) treatment at 37°C. Protein concentration was determined using the Bradford assay. After blocking for 1 h in TBST (20 mM Tris, 150 mM NaCl, and 0.1% Tween pH 7.6) with 5% milk, membranes were incubated for 18 h with the primary antibodies specific for NifD, VnfD, or AnfD, then washed three times with TBST. Membranes were then incubated with an HRP-conjugated secondary antibody (Promega) for 1 h, followed by three washes with TBST. Finally, membranes were visualized using an enhanced chemiluminescent reagent (Thermo Scientific) and an Alpha Innotech imaging system.

Methane determination by gas chromatography

After the cessation of growth, the total volume of gas produced by each culture was measured using a glass syringe, which also normalized the pressure to 1 atm. The amount of CH₄ produced was determined by injection of 50 µL of headspace gas into a Shimadzu Nexis GC-2030 gas chromatograph fitted with an Rt-Q-BOND fused silica PLOT column with a 0.32-mm internal diameter, a 30-m length, and a 10.00-µm film thickness (Restek, VWR #89166-308) and BID detector. The sample split ratio was 42.6, and the carrier gas was helium at 4.44 mL/min. The injection port temperature was 100°C, column temperature was 27°C, and BID temperature was 220°C. Peak integration was performed using Shimadzu LabSolutions software and moles of CH₄ were determined using methane standards.

Construction of CRISPRi gene repression strains

CRISPRi repression of the *vnf* and *anf* operons in *M. acetivorans* was designed and strains constructed as previously described for CRISPRi repression of the *nif* operon in strain DJL74 (43). Briefly, 20 bp guide RNAs (gRNA) targeting dCas9 to the *vnf* (*gRNA-vnfH*-5'-CCCGTAAAAAGCAATTTTC-3') or *anf* (*gRNA-anfI*-5'-ATAACCTTCGTACTACCTTT-3') gene cluster were designed using Geneious Prime (Fig. S3). Synthetic DNA oligos (gBlocks) (IDT) were designed for assembly with CRISPRi-dCas9 plasmid pDL734. gBlocks and pDL734 were assembled using the Gibson assembly Ultra Kit (Synthetic Genomics). The assembly mix was used to transform *E. coli* strain WM4489, transformants were screened for assembled plasmids using standard methods, and final plasmids were sequenced. Each plasmid was used to transform *M. acetivorans* strain WWM73 as described (60). Integration of each plasmid into the chromosome of strain WWM73 was screened using PCR. Repression of the *vnf* and/or *anf* operons in the resultant CRISPRi repression strains DJL107, DJL120, and DJL119 (Table 1) was confirmed by Western blot as described above.

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Melissa Chanderban, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing | Christopher A. Hill, Investigation | Ahmed E. Dhamad, Formal analysis, Investigation | Daniel J. Lessner, Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – review and editing

DATA AVAILABILITY

The raw data from growth studies and qPCR will be available upon request.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Table S1, Figures S1 to S5 (AEM01033-23-s0001.pdf). Supplemental tables and figures.

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