Reconstruction and minimal gene requirements for the alternative iron-only nitrogenase in *Escherichia coli*

Jianguo Yang[a], Xiaqing Xie[a], Xia Wang[a], Ray Dixon[b,1], and Yi-Ping Wang[a,1]

[a]State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China; and [b]Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, United Kingdom

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All diazotrophic organisms sequenced to date encode a molybdenum-dependent nitrogenase, but some also have alternative nitrogenases that are dependent on either vanadium (VFe) or iron only (FeFe) for activity. In *Azotobacter vinelandii*, expression of the three different types of nitrogenase is regulated in response to metal availability. The majority of genes required for nitrogen fixation in this organism are encoded in the nitrogen fixation (*nif*) gene clusters, whereas genes specific for vanadium- or iron-dependent diazotophy are encoded by the vanadium nitrogen fixation (*vnf*) and alternative nitrogen fixation (*anf*) genes, respectively. Due to the complexities of metal-dependent regulation and gene redundancy in *A. vinelandii*, it has been difficult to determine the precise genetic requirements for alternative nitrogen fixation. In this study, we have used *Escherichia coli* as a chassis to build an artificial iron-only (Anf) nitrogenase system composed of defined *anf* and *nif* genes. Using this system, we demonstrate that the pathway for biosynthesis of the iron-only cofactor (FeFe-co) is likely to be simpler than the pathway for biosynthesis of the molybdenum-dependent cofactor (FeMo-co) equivalent. A number of genes considered to be essential for nitrogen fixation by FeFe nitrogenase, including *nifM*, *vnfEN*, and *anfOR*, are not required for the artificial Anf system in *E. coli*. This finding has enabled us to engineer a minimal FeFe nitrogenase system comprising the structural *anfHDGK* genes and the *nifBUSV* genes required for metallocluster biosynthesis, with *nifF* and *nifJ* providing electron transport to the alternative nitrogenase. This minimal Anf system has potential implications for engineering diazotrophy in eukaryotes, particularly in compartments (e.g., organelles) where molybdenum may be limiting.

Nitrogen is a critical limiting element for plant growth and production (1). Biological nitrogen fixation, the process that converts gaseous nitrogen to ammonia, offers a natural means of providing fixed nitrogen to plants (2). Three genetically and biochemically distinct types of nitrogenase systems have been demonstrated to exist in bacteria and archaea (3). The most widespread and intensively characterized system is the classical molybdenum-dependent (MoFe) nitrogenase, but two types of alternative nitrogenase are also found in some diazotrophs. One is the vanadium-dependent (VFe) nitrogenase system, which contains V instead of Mo in the heterometal cofactor (4). The third nitrogenase contains neither Mo nor V, and the heterometal atom in the cofactor is replaced by Fe. This enzyme is designated as nitrogenase-3 or the iron-only (FeFe) nitrogenase (5).

The structural genes encoding FeFe nitrogenase are organized as a single operon *anfHDGKOR* in *Azotobacter vinelandii* (6, 7). The subunits of dinitrogenase reductase are encoded by *anfH*, whereas the α- and β-subunits of dinitrogenase are encoded by *anfD* and *anfK*, respectively. An additional δ-subunit encoded by *anfG*, which is specific for alternative nitrogenases, forms an $\alpha_2\beta_2\delta_2$ hexameric structure in FeFe nitrogenase (8). The predicted protein products of *anfO* and *anfR* genes do not show overall similarity to any known *nif* gene products, and the exact role of these two genes in the function of FeFe nitrogenase is unknown. However, the components encoded by the *anfHDGKOR* operon do not provide all of the requirements for the biosynthesis of FeFe nitrogenase, and several genes required for iron-sulfur cluster biosynthesis and metallocluster assembly are shared by all three nitrogenase systems in *A. vinelandii*. These genes include *nifB* (9), which is essential for biosynthesis of the active-site nitrogenase cofactor and encodes a radical S-adenosylmethionine (SAM)-dependent enzyme that inserts the central carbon atom into the eight-Fe core of NifB cofactor (NifB-co) (10, 11). Likewise, homocitrate synthase encoded by *nifV* provides the organic moiety in all three nitrogenase cofactors, and the *nifU* and *nifS* genes function to assemble Fe-S clusters and the iron-sulfur core of the cofactor in all three enzymes (12). The *anf* system is unusual in that there are no additional homologs in the *A. vinelandii* genome of the *nifENX* or *vnfENX* operons. These genes provide scaffolds and carriers for the final maturation of the cofactor, enabling insertion of the heterometal (Mo or V, respectively) and homocitrate in a reaction catalyzed by dinitrogenase reductase (Fe protein). Genetic analysis and transcriptome profiling of *A. vinelandii* indicate that the *anf* system is dependent on *vnfEN* for maturation of the FeFe cofactor in the iron-only nitrogenase (13, 14). Finally, *nifM*, which potentially encodes a peptidyl-prolyl *cis-trans* isomerase required for proper folding of the Fe protein (15), is thought to be required for all three types of nitrogenase (16).

Difficulties in determining the precise genetic requirements for the FeFe nitrogenase system arise from the complexities of metal-dependent gene regulation in *A. vinelandii* and the sharing of genes among all three nitrogenase systems in this organism.

Significance

To date, three different nitrogenase systems [molybdenum (MoFe), vanadium (VFe), and iron-only (FeFe)] have been found in nature. The MoFe nitrogenase has been studied extensively, but the alternative vanadium-dependent (Vnf) and iron-only (Anf) systems are less well characterized, particularly with respect to components required for their biosynthesis and activity. We have engineered an artificial FeFe nitrogenase system in *Escherichia coli* that combines *anf* structural genes with accessory nitrogen fixation genes (*nif*) to provide a minimal 10-gene cluster that supports the biosynthesis and activity of the FeFe nitrogenase. Our findings underscore the potential for the future engineering of nitrogen fixation in eukaryotes because the Anf system can bypass limitations in molybdenum availability in plant organelles.

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[1]To whom correspondence may be addressed. Email: ray.dixon@jic.ac.uk or wangyp@pku.edu.cn.

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We and others have established that *Escherichia coli* can provide a useful host for engineering diazotrophy (17–19), determining the minimum requirements for nitrogen fixation in evolutionary distant diazotrophs (20), and redesigning *nif* gene clusters for synthetic biology (21, 22). Recently, we developed a modular operon-based system for functional analysis of the *Klebsiella oxytoca nif* gene cluster in *E. coli* (21). In the current study, we have adapted this approach to develop an artificial FeFe nitrogenase system in which the *K. oxytoca* Mo nitrogenase structural genes in our reassembled *nif* cluster are replaced by the *anf* structural genes from *A. vinelandii*. The nitrogenase activity of this reconstructed Anf system, as measured by acetylene reduction and ^{15}N assimilation, is not dependent on the presence of *nifENX*, *nifM*, *nifQ*, or *anfOR*. Our results define a minimal requirement of eight genes for synthesis of the iron-only nitrogenase in *E. coli* in the absence of Mo, thus bypassing the need for components that appear specific to the assembly of FeMo-co. Two additional genes, *nifF* and *nifJ*, which provide a physiological electron transport pathway to nitrogenase, are required to support the artificial Anf system but can be partially substituted by homologs present in *E. coli.*

Results

Reconstruction of the FeFe Nitrogenase System in *E. coli*. In a previous study, we used BioBrick interfaces to reconstruct a gene cluster containing the seven *nif* operons from *K. oxytoca*, in which regulation is dependent on the native σ^{54}-dependent promoters and is regulated by the *nifL* and *nifA* genes (21) (Fig. 1*A* and Table S1). To replace the Mo structural genes with Anf components, the *anfHDGKOR* operon was amplified from the genome of *A. vinelandii* and then fused to the *nifH* promoter of *K. oxytoca* (details are provided in *Materials and Methods*). The resultant cassette [P_{nifH}-*anfHDGKOR*] was used to replace the *nifHDKTY* operon to derive the artificial FeFe nitrogenase system (Fig. 1*A* and Table S1). When transformed into *E. coli* JM109, the reconstructed Anf system (pKU7801) exhibited nitrogenase activity as measured by C_2H_2 reduction (~3 nmol of C_2H_4 per minute per milligram of protein) and ^{15}N assimilation assay [~840 ($\delta^{15}N/^{14}N$) ‰]. This output corresponds to ~10% and ~35% of the activity, respectively, from the MoFe system (pKU7017) (Fig. 1 *B* and *C*).

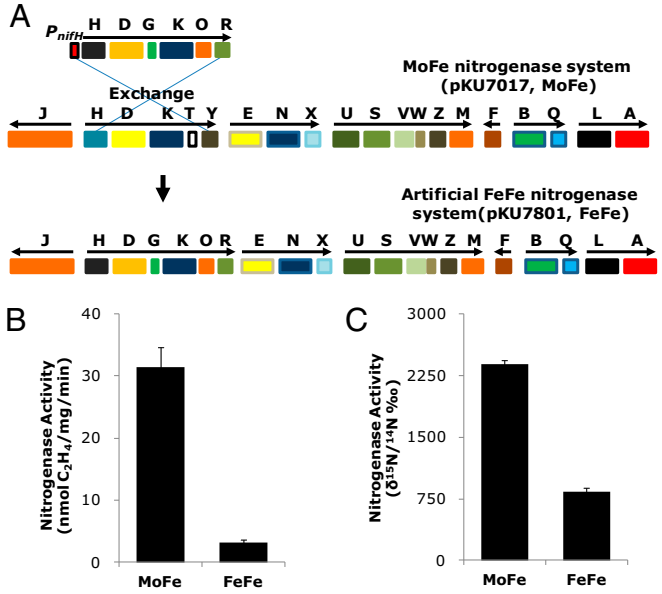

Fig. 1. Construction of the artificial FeFe nitrogenase system and measurement of nitrogenase activity. (*A*) Schematic map of the construction process of the artificial FeFe system. Nitrogenase activity of the artificial FeFe system was compared with nitrogenase activity of the MoFe system, as measured by the C_2H_2 reduction assay (*B*) or the ^{15}N assimilation assay (*C*), respectively. Bars marked as MoFe represent the MoFe nitrogenase system (pKU7017), and bars marked as FeFe represent the artificial FeFe nitrogenase system (pKU7801). Error bars indicate the SD observed from at least three independent experiments.

Minimal Gene Requirements for the Artificial Anf System in *E. coli*. The inventory of genes required for diazotrophy varies greatly among diverse genera (23–25). A minimal core of three catalytic (*nifHDK*) and three biosynthetic (*nifENB*) genes is conserved in most diazotrophs (23, 26), reflecting the core protein components required for FeMo-co biosynthesis in vitro (27). Other genes required for nitrogenase biosynthesis and activity are less well conserved or are substituted by other genes in the host. Furthermore, some *nif* genes (e.g., electron transfer components) are specifically adapted to support nitrogenase activity in accordance with the physiological requirements of the host organism. Notably, alternative nitrogenases are only found in species that also encode a molybdenum-dependent nitrogenase (14, 23).

To determine the minimal *nif* genes required for the FeFe nitrogenase system in *E. coli*, we constructed a series of deletions in the reconstituted Anf system (Fig. 2*A* and Table S1). Neither C_2H_2 reduction activity nor ^{15}N incorporation activity was detected in the *nifB* deletion mutant (Fig. 2 *B* and *C*, bars 3 and 8), as anticipated, because *nifB* performs a core function in assembling the eight-Fe core of the cofactor and insertion of the central carbon atom (28, 29). However, when the *K. oxytoca nifENX* operon was removed, no reduction in either C_2H_2 reduction or ^{15}N assimilation activity was observed compared with the complete system (Fig. 2 *B* and *C*, bars 1 and 5). This result is surprising, given previous observations that either *vnfEN* or *nifEN* is necessary for diazotrophic growth associated with the FeFe nitrogenase in *A. vinelandii* (13). Removal of the complete *nifUSVWZM* operon or deletion of *nifU*, *nifS*, or *nifV* in combination with Δ*nifENX* leads to a dramatic decrease in nitrogenase activity, as measured by C_2H_2 reduction and ^{15}N assimilation assays (Fig. 2 *B* and *C*, bars 4, 10, 15, 16, and 17), which is expected because *nifU and nifS* are required for biosynthesis of Fe-S clusters (30) and *nifV* enables synthesis of the homocitrate moiety of the cofactor (28, 31). In contrast, deletion of *nifWZM* had no apparent influence on nitrogenase activity (Fig. 2 *B* and *C*, bar 12), which contradicts previous results in *A. vinelandii*, where *nifM* is required for all three nitrogenase systems (16). As expected, the *nifQ* gene product, which specifically donates Mo for FeMo-co biosynthesis (11), was not required for the activity of the FeFe nitrogenase (Fig. 2 *B* and *C*, bar 9). The δ-subunit of FeFe nitrogenase, encoded by *anfG*, is a specific feature of alternative nitrogenases. Although deletion of this gene led to only a ~20% reduction in acetylene reduction (Fig. 2*B*, bar 13), activity decreased to ~5% of the activity observed with the complete system when ^{15}N assimilation was measured (Fig. 2*C*, bar 13). This observation concurs with previous results in *A. vinelandii*, which demonstrated that *anfG* is crucial for reduction of dinitrogen by FeFe nitrogenase but is not essential for acetylene reduction (8). In contrast, only minor effects on nitrogenase activity were observed with the *anfOR* deletion mutant (Fig. 2 *B* and *C*, bar 14). This finding is inconsistent with the phenotype of *anfO* and *anfR* mutants in *A. vinelandii*, which are essential for reduction of dinitrogen but not for acetylene (7). Finally, deletion mutations in *nifF* and *nifJ*, which provide electron transport to MoFe nitrogenase in *K. oxytoca*, reduced both the level of acetylene and nitrogen reduction by the reconstituted FeFe nitrogenase in *E. coli* (Fig. 2 *B* and *C*, bars 1, 6, and 7). Overall, these results suggest that some components essential for biosynthesis of MoFe nitrogenase are not required for the FeFe system or can be substituted

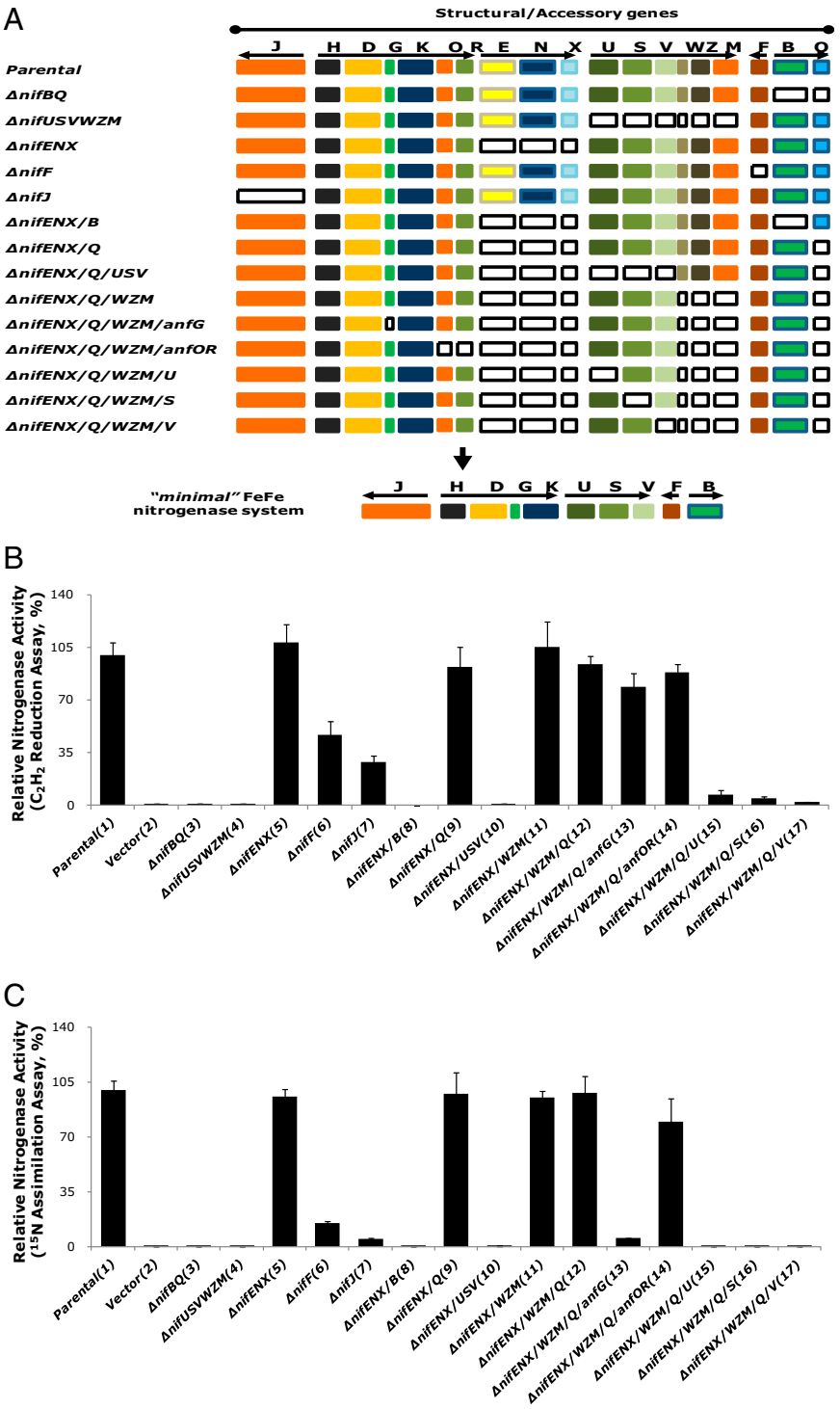

Fig. 2. Deletion analysis of the genes present in the artificial FeFe nitrogenase system. (*A*) Scheme showing the genetic organization of the artificial FeFe nitrogenase and its deletion derivatives. The black and white rectangles represent the deleted genes, and the colored rectangles represent the remaining genes. (*B* and *C*) Nitrogenase activity of the deletion mutants compared with the artificial FeFe nitrogenase system (pKU7801, bars marked as Parental) and the empty vector (pACYC184, bars marked as Vector) in *E. coli* JM109. Strains were grown anaerobically in nitrogen-deficient conditions and assayed for C_2H_2 reduction (*B*) or ^{15}N assimilation (*C*), respectively. Error bars indicate the SD observed from at least three independent experiments.

by homologs encoded in the genome of *E. coli*. We have therefore defined a minimal cluster of 10 structural/accessory genes (*anfHDGK*, *nifB*, *nifUSV*, *nifF*, and *nifJ*) required to support FeFe nitrogenase activity in *E. coli*. We will refer to this construct as the minimal FeFe system for the remainder of this paper (Fig. 2*A*, plasmid pKU7815, and Table S1).

***nifENX or vnfENX* Gene from *A. vinelandii* Does Not Increase the Activity of the Minimal FeFe System in *E. coli*.** The results presented above indicate that the presence of the *K. oxytoca nifENX* operon does not influence the nitrogenase activity of the reconstituted Anf system, which implies that biosynthesis of FeFe-co can take place in the absence of the scaffold and carrier proteins

normally required for FeMo-co assembly in conventional MoFe nitrogenase. However, we considered the possibility that heterogeneous components (i.e., *A. vinelandii anf* structural genes in combination with noncognate biosynthetic *nif* genes from *K. oxytoca*) might not function optimally in FeFe-co biosynthesis. To address this issue, the *A. vinelandii vnfENX* operon (controlled by the *nifE* promoter from *K. oxytoca*) and the *nifENX* genes (controlled by the original *A. vinelandii* promoter) were cloned and inserted into the minimal FeFe system (details are provided in *Materials and Methods* and Table S1). When introduced into *E. coli* and assayed by C_2H_2 reduction, no enhanced activity was observed in either the *A. vinelandii nifENX*$^+$ or the *A. vinelandii vnfENX*$^+$ strain compared with the minimal FeFe strain or the *KpnifENX*$^+$ strain (Fig. 3). These results confirm that scaffold proteins, which are essential for the biosynthesis of FeMo-co and VFe-co, are not required for the biosynthesis of FeFe-co in *E. coli.*

Next, we examined the effects of heterometals on the activity of the reconstituted Anf system in the presence and absence of the *nifENX* or *vnfENX* operon. It has been previously demonstrated that under certain conditions, FeMo-co can be incorporated into FeFe nitrogenase, resulting in the synthesis of a hybrid enzyme, which produces increased levels of ethane, in addition to ethylene, as products of acetylene reduction (32, 33). When V (30 μM) was added to the culture medium, we noticed slight inhibition of acetylene reduction activity (~10%) in the minimal FeFe system and in the reconstituted systems containing either *A. vinelandii nifENX* of *vnfENX* (Table 1). Whereas the addition of Mo (30 μM) had no influence on the activity of the minimal FeFe system, it resulted in a considerable decrease in acetylene reduction when either *K. oxytoca nifENX* or *A. vinelandii nifENX* was also present (~36% and ~16%, respectively, of the activity observed with iron alone). In addition, the production of ethane increased to 8% and 5%, respectively, of the ethylene formed by these strains in iron-only conditions (Table 1). In contrast, Mo had a far less inhibitory effect on acetylene reduction in the presence of *A. vinelandii vnfENX* (~72% of the activity with iron alone), and ethane formation was not detected in this case. These experiments reinforce the conclusion that synthesis of FeFe-co is not dependent on either *nifENX* or *vnfENX.* Furthermore the results suggest that FeMo-co can be incorporated into FeFe nitrogenase by NifENX, when Mo is available.

Table 1. Influence of V or Mo on the activity of the minimal FeFe nitrogenase system when *nifENX* or *vnfENX* is also present

Genotype	Condition[†]	Relative nitrogenase activity (C_2H_2 reduction assay)* C_2H_4, %	C_2H_6, %
"Minimal"	+Fe	100 ± 16	ND
	+Fe/+V	90 ± 6	ND
	+Fe/+Mo	101 ± 15	ND
KpnifENX$^{+‡}$	+Fe	100 ± 8	ND
	+Fe/+V	101 ± 6	ND
	+Fe/+Mo	36 ± 4	8 ± <0.5
AvnifENX$^{+§}$	+Fe	100 ± 12	ND
	+Fe/+V	87 ± 13	ND
	+Fe/+Mo	16 ± 3	5 ± <0.5
AvvnfENX$^{+¶}$	+Fe	100 ± 10	ND
	+Fe/+V	89 ± 6	ND
	+Fe/+Mo	72 ± 3	ND

ND, not detectable (ethane/ethylene ratio below 0.020).
*Amount of C_2H_4 detected under iron-only conditions (+Fe) represents 100% activity in each genetic background.
[†]Medium contained only iron (+Fe, 150 μM) or additional vanadium (30 μM) or molybdenum (30 μM) as indicated.
[‡]Minimal FeFe system with additional *K. oxytoca nifENX* (plasmid pKU7819).
[§]Minimal FeFe system with additional *A. vinelandii nifENX* (plasmid pKU7820).
[¶]Minimal FeFe system with additional *A. vinelandii vnfENX* (plasmid pKU7821).

NifM Is Not Required for the Accumulation and Maturation of AnfH. The gene product of *nifM* is predicted to be a peptidyl-prolyl *cis-trans* isomerase (15) belonging to the PpiC/parvulin rotamase family. The C-terminal PpiC domain of NifM is thought to be essential for activation of the NifH protein (34). NifM is required for the accumulation of active Fe protein in *K. oxytoca* (35) and *E. coli* (36). Although *nifM* is apparently required for all three types of nitrogenase in *A. vinelandii*, our data imply that *nifM* is not necessary to activate AnfH in the reconstructed FeFe system expressed in *E. coli* (Fig. 2). To investigate this issue further, we compared the requirements for *nifM* in our reconstructed MoFe and FeFe systems (plasmid pKU7017 with its Δ*nifM* derivative pKU7017-Δ*nifM* and pKU7815 with its *nifM*$^+$ derivative pKU7822, respectively) (Table S1). Whereas acetylene reduction by the MoFe system was clearly dependent on *nifM*, the activity of the FeFe system was not altered by the presence of this gene (Fig. 4*A*). It has been demonstrated previously that the level of NifH expressed in *E. coli* is reduced significantly when *nifM* is absent, suggesting that NifM is required to stabilize NifH. The presence of *nifM* is also required for Fe protein activity in *E. coli* (36). In accordance with these results, we observed that the proportion of soluble NifH expressed from the MoFe system decreased dramatically in the absence of NifM (Fig. 4*B*). In contrast, the level of soluble AnfH protein expressed from the artificial FeFe system was not affected by the presence of NifM (Fig. 4*B*).

Although our data clearly demonstrate that *nifM* is not required for the maturation of AnfH, it is feasible that housekeeping counterparts of this gene, encoded in the *E. coli* genome, are competent to perform a similar function. Three proteins, encoded by *ppiC*, *ppiD*, and *surA* in *E. coli*, share a isomerase domain similar to the isomerase domain of NifM. To exclude the possibility that these orthologs are responsible for AnfH maturation, we constructed single and multiple deletions of the respective genes. (We were unable to construct the triple mutant *ppiC*, *ppiD*, and *surA*; *Materials and Methods*). When the minimal FeFe system was introduced into these mutant strains and assayed for acetylene reduction, only minor effects on activity were observed compared with the WT counterpart (Fig. 4*C*). These minor effects on activity may result from the pleiotropic influence of these mutations on cellular physiology, because these mutant strains grow slower than the WT strain. Taken together, the results indicate that NifM is required for the accumulation and the maturation of NifH, but not for AnfH, in *E. coli.*

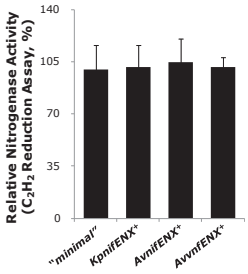

Fig. 3. Genetic analysis of the requirement for the *nifENX* or *vnfENX* gene. The C_2H_2 reduction activity of the minimal FeFe nitrogenase system and constructions in which either *nifENX* or *vnfENX* was also present were compared. The minimal FeFe nitrogenase system (pKU7815) is marked as "minimal." The minimal FeFe nitrogenase system with three different kinds of additional *ENX* genes is represented as *KpnifENX*$^+$ (pKU7819), *AvnifENX*$^+$ (pKU7820), and *AvvnfENX*$^+$ (pKU7821), respectively. The nitrogenase activity observed from the minimal system represents 100% activity. Error bars indicate the SD observed from at least three independent experiments.

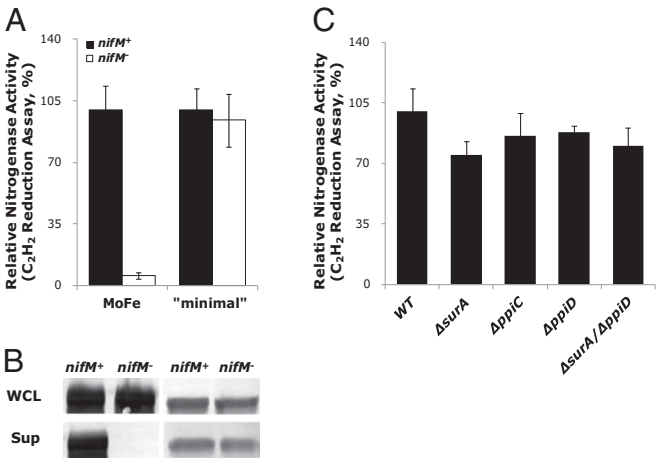

Fig. 4. Genetic analysis of the requirement for *nifM*. (*A*) Comparison of the C_2H_2 reduction activity of MoFe nitrogenase and the minimal FeFe system in *E. coli* strain JM109, in the presence or absence of *nifM* (solid and open bars, respectively). Activity on the *y* axis is represented as a percentage of either the $nifM^+$ MoFe system (pKU7017, bars indicated as MoFe) or the $nifM^+$ derivative of the minimal FeFe system (pKU7822, bars marked as "minimal"). The $nifM^-$ equivalents of these constructs are pKU7017-Δ*nifM* and pKU7815, respectively (Table S1). (*B*) Influence of *nifM* on the expression of NifH and AnfH as measured by Western blotting of the cultures indicated in *A*. Sup, supernatant; WCL, whole cell lysate. (*C*) Relative nitrogenase activity of the minimal FeFe system (expressed from plasmid pKU7815) in different *E. coli* mutants. WT indicates strain JM109; Δ*surA*, Δ*ppiC*, Δ*ppiD*, and Δ*surA*/Δ*ppiD* indicate deletion mutants of strain JM109. Error bars indicate the SD observed from at least three independent experiments.

Electron Transport Pathway to Nitrogenase Can Be Partially Substituted by Orthologs in *E. coli*. NifJ and NifF constitute a nonbranched electron transport pathway to nitrogenase in *K. oxytoca* and other diazotrophs, providing electron donation from pyruvate via a pyruvate:flavin oxidoreductase encoded by *nifJ* and a flavodoxin encoded by *nifF* (37–39). Both genes are dispensable under nondiazotrophic conditions, yet mutants display a clear Nif^- phenotype. However, the nitrogenase activity of the artificial FeFe system is not completely ablated by deletion of either *nifF* or *nifJ* (Fig. 2), suggesting other genes in *E. coli* may partially substitute for their function in electron transport. In accord with this hypothesis, we identified two *nifF* orthologs encoding flavodoxins (*fldA* and *fldB*) and one *nifJ* ortholog (*ydbK*) in *E. coli.* Amino acid sequence identity is 38.5% between FldA and NifF, 35.7% between FldB and NifF, and 47.7% between YdbK and NifJ (Table S2).

A deletion mutation in *fldB* had no effect on the activity of the minimal FeFe system, either in the presence or absence of *nifF* (Fig. 5*A*), and expression of this gene from a multicopy plasmid did not increase nitrogenase activity (Fig. 5*B*). As observed previously (40), we were unable to construct a deletion mutant of *fldA*, which is apparently an essential gene. However, expression of multicopy *fldA* partially rescued the decreased nitrogenase activity observed when *nifF* was absent from the minimal FeFe system (Fig. 5*B*). Taken together, these results suggest that *fldA*, but not *fldB*, can partially substitute for the function of *nifF* in *E. coli*. Deletion of the NifJ homolog encoded by *ydbK* had no effect on nitrogenase activity when *nifJ* was present, but activity decreased to 3% when both *ydbK* and *nifJ* were absent. Furthermore, activity could be rescued in the double *ydbK*, *nifJ* deletion by introducing a plasmid carrying *ydbK* (Fig. 5*C*). Thus, it would appear that YdbK and FldA can at least partially provide an electron donation pathway to FeFe nitrogenase in *E. coli*.

Discussion

The alternative FeFe nitrogenase was initially identified in *A. vinelandii* grown under combined Mo and V limitation about 25 y ago (41). As a second/third option for diazotrophs, it shares many genes with the conventional MoFe nitrogenase system, and expression of the FeFe nitrogenase is completely inhibited by traces of Mo or V (32, 41). Gene redundancy in *A. vinelandii* and the complexities of metal-dependent regulation in this obligate aerobic diazotroph make analysis of the FeFe system difficult. We have circumvented these limitations by engineering an artificial FeFe system in the defined genetic background of *E. coli*. Furthermore, we have demonstrated that this hybrid system, containing a combination of *A. vinelandii anf* structural genes, together with accessory *nif* genes derived from *K. oxytoca*, requires a minimum of 10 components (encoded by *anfHDGK*, *nifUSV*, *nifB*, *nifF*, and *nifJ*) to support FeFe nitrogenase activity in *E. coli*. Although *E. coli* has proven to be an extremely useful host for analyzing components necessary for FeFe nitrogenase biosynthesis and activity, we identified housekeeping equivalents of the *nifJ* and *nifF* genes, which can at least partially substitute for their function in electron donation to nitrogenase.

Several genes that are essential for the biosynthesis and activity of MoFe nitrogenase are not required for the minimal FeFe system in *E. coli*. One of the notable differences is the number of components necessary for cofactor biosynthesis. Our data demonstrate that in the physiological background of *E. coli*, the synthesis of FeFe-co does not require either *nifENX* or *vnfENX*, in contrast to the requirements for FeMo-co assembly. This observation suggests that following synthesis of NifB-co by the radical SAM-dependent enzyme NifB, further steps in the maturation of FeFe-co are carried out on the AnfDK structural subunits rather than on the NifEN scaffold. It is possible that this pathway represents an ancestral route for cofactor biosynthesis, because a number of diazotrophic organisms lacking *nifEN* (e.g., *Roseiflexus* spp.) have been identified (42). However, the NifDK subunits in strains that lack *nifEN* or *nifN* represent a separate phylogenetic group, which appears to be associated with a thermophilic lifestyle (23). In *Rhodobacter capsulatus*, diazotrophic growth dependent on the alternative FeFe nitrogenase is not influenced by mutations in either *nifE* or *nifN*, again indicating that biosynthesis of FeFe-co can take place in the absence of the NifEN scaffold (33). However, there are other nitrogenase-like sequences encoded in the *R. capsulatus* genome, which could potentially perform this function (23). In contrast, the increase of *vnfENX* expression in iron-only conditions in *A. vinelandii* (14), coupled with the absence of diazotrophic growth in double mutants lacking *nifEN* and *vnfE* (13), strongly supports a role for the VnfEN scaffold in FeFe nitrogenase maturation in this organism. Although it is difficult to rationalize the discrepancy between our results and the results observed in *A. vinelandii*, it is important to emphasize that the strictly aerobic lifestyle of *Azotobacter* is very different from the facultative anaerobe *E. coli*, which can only support nitrogen fixation under anaerobic conditions. These differences in physiology may also account for our observation that the *anfOR* genes, which are located downstream of *anfHDGK* and are essential for nitrogen but not acetylene reduction in *A. vinelandii*, are not required for nitrogen reduction by the FeFe nitrogenase expressed in *E. coli*.

The engineering of *anf* genes in a model nondiazotroph also has distinct advantages for analyzing the biochemical properties of alternative nitrogenases and their interactions with different cofactors. Our results suggest that the minimal *anf* system does not enable synthesis of FeMo-co; hence, the FeFe nitrogenase produced under these conditions is not responsive to the presence of Mo. Although, ethane production from acetylene is a hallmark of alternative nitrogenases (3), we did not observe significant levels of ethane production by the minimal *anf* system.

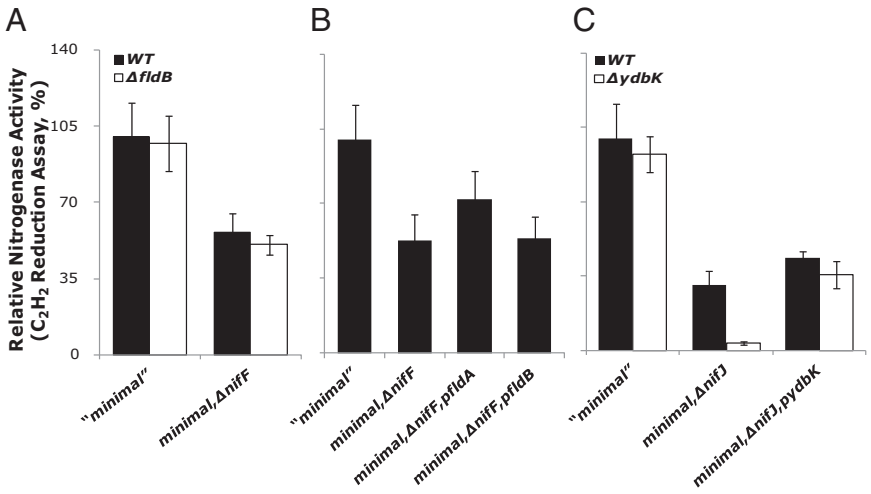

Fig. 5. Genetic analysis of *nifF* and *nifJ* orthologs in *E. coli*. Comparison of the C_2H_2 reduction activity of the minimal FeFe nitrogenase and its *nifF*/*nifJ* deletion derivatives in different genetic backgrounds. (*A*) Nitrogenase activity observed from the minimal FeFe system (pKU7815) and its *nifF* deletion derivative (pKU7823, marked as minimal, Δ*nifF*) in WT (JM109) and its *fldB* deletion derivative (represented by the solid and open bars), respectively. (*B*) Nitrogenase activity of the minimal FeFe system and its *nifF* deletion derivative (marked as minimal, Δ*nifF*) and its *nifF* deletion derivative with additional *fldA* or *fldB* carried on a plasmid (pKU7824, marked as minimal, Δ*nifF*, *pfldA*, and pKU7825, marked as minimal, Δ*nifF*, *pfldB*, respectively). (*C*) Nitrogenase activity observed from the minimal FeFe system, its *nifJ* deletion derivative (pKU7826, marked as minimal, Δ*nifJ*) and its *nifJ* deletion derivative with an additional plasmid carrying the *ydbK* gene (pKU7827, marked as minimal, Δ*nifJ*, *pydbK*) in WT (solid bars) and the *ydbK* deletion strain (open bars).

The ratio of ethane/ethylene produced by FeFe nitrogenase is dependent upon electron flux through the nitrogenase component proteins (32), which may be limiting in the engineered system. However, when biosynthesis of FeMo-co was enabled by addition of *nifENX* to the minimal Anf system, formation of ethane as a product of acetylene reduction was significantly enhanced in the presence of Mo. This property has been previously characterized in both *A. vinelandii* and *R. capsulatus* as the "molybdenum effect," in which FeMo-co can be inserted into the AnfDGK apoprotein instead of FeFe-co (32, 43–45). The increased formation of ethane under these conditions is thought to arise from the altered polypeptide environment surrounding the cofactor. In contrast, the addition of *vnfENX* to the minimal system did not increase ethane production in the presence of either Mo or V. Although little is known about V transport in *E. coli*, this result is consistent with previous studies in *A. vinelandii*, whereby V addition did not enhance ethane production by the *anf* system (46). The absence of a significant "molybdenum effect" when *vnfENX* was added to the minimal *anf* system may suggest either that VnfEN provides a less effective scaffold for FeMo-co biosynthesis than NifEN or that VnfX is relatively inefficient at transferring FeMo-co from VnfEN to AnfDGK.

Our results provide strong evidence that NifM, a potential peptidyl-prolyl isomerase required for maturation of the Fe protein of nitrogenase (36, 47), is not required for synthesis of active AnfH in *E. coli*. Seven conserved proline residues in NifH are considered to be potential substrates for NifM (15), although these residues are also conserved in AnfH (Fig. S1). The discrepancy between our findings and the findings observed in *A. vinelandii*, where NifM is apparently needed for all three nitrogenases, can perhaps be rationalized by the complexity of the gene regulatory circuits in *Azotobacter*, because AnfA, the transcriptional activator of the *anf* genes, requires the presence of either NifH or VnfH for its activity (46). Because the molybdenum (NifH) and vanadium (VnfH) Fe proteins require NifM for maturation, the absence of *nifM* will inactivate AnfA, thus preventing expression of the Anf structural genes. This intricate regulatory circuit has been bypassed in *E. coli*, thus enabling us to analyze requirements readily for AnfH activity.

There is considerable interest in the synthetic manipulation of nitrogen fixation genes, with the ultimate aim of engineering diazotrophic eukaryotes, particularly crop plants. A crucial step toward this goal is to define the minimal number of genes required for nitrogenase activity and to interface these studies with the identification of accessory components required to support nitrogen fixation (2). Engineering minimal gene clusters is an important goal of synthetic biology. Recently, it was shown that an operon comprising nine genes derived from *Paenibacillus* sp. WLY78 is sufficient to express active molybdenum nitrogenase in *E. coli* (20). However, the minimal FeFe nitrogenase system described here has certain advantages, notably the simpler mode of FeFe-co biosynthesis and, in particular, the absence of a requirement for Mo, because this element is most probably limiting in plant organelles that lack molybdoenzymes.

Materials and Methods

Construction of Bacterial Strains. Bacterial strains used in this study are listed in Table S1. The *ppiC*, *ppiD*, *surA*, *fldB*, and *ydbK* mutant alleles were moved into strain JM109 by P1 bacteriophage transduction from the Coli Genetic Stock Center (CGSC) strains JW3748-3, JW0431-1, JW0052-1, JW2863-2, and JW1372-1, respectively. The P1 phage lysates of donor CGSC strains were incubated with the recipient strain JM109 (pKD46-*recA*) in stationary phase for about 20 min at 37 °C, and 1 mL of LB containing 125 mM Na-citrate was then added to the mixture. After further incubation for 40 min at 37 °C, the mixture was plated on Kan^R plates containing 20 mM Na-citrate. The JM109 mutations were confirmed by PCR, and the PCR products were verified by DNA sequencing. The Kan^R cassette was deleted by homologous recombination at the flippase recognition target site (48). The *ppiD*/*surA* double mutant was constructed by further P1 transduction based on the *surA* single mutant.

Growth Medium and Chemicals. LB broth and M9 medium were used for growth of *E. coli*, prepared as previously described (49). Nitrogen-deficient media used in this study contained 10.4 g/L of Na_2HPO_4, 3.4 g/L of KH_2PO_4, 26 mg/L of $CaCl_2{\cdot}2H_2O$, 30 mg/L of $MgSO_4$, 0.3 mg/L of $MnSO_4$, 36 mg/L of ferric citrate, 10 mg/L of *p*-aminobenzoic acid, 5 mg/L of biotin, and 2% (wt/vol) glucose, with 10 mM glutamate as the nitrogen source. Trace molybdenum in the medium was removed by using Mo-starved *A. vinelandii* cells as described (50). When necessary, 30 μM Na_3VO_4 or 30 μM Na_2MoO_4 was added to the nitrogen-deficient medium. Antibiotics were added in the following concentration: 50 μg/mL ampicillin, 25 μg/mL chloram-

phenicol, 10 μg/mL tetracycline, and 25 μg/mL kanamycin. The medium for *A. vinelandii* was Mo-deficient Burk's medium, as described previously (6).

Construction of Recombinant Plasmids. Plasmids used in this study are listed in Table S1. Plasmid pKU7017 is a pACYC184 derivative containing all seven σ^{54}-dependent *nif* operons from *K. oxytoca* constructed with the BioBrick interface (21). For construction of pKU7801, the *anfHDGKOR* ORFs were amplified from the genome of *A. vinelandii*, and the resultant fragment was inserted into the *pBR322M-*P_{nifH} plasmid, resulting in plasmid pKU7800. The [P_{nifH}-*anfHDGKOR*] cassette derived from pKU7800 was used to replace the *nifHDKTY* operon in pKU7017, using the unique SnaBI restriction site. Whole-operon deletion derivatives of pKU7801, including the *nifBQ*, *nifUSVWZM*, *nifENX*, *nifF*, and *nifJ* deletions, were constructed by direct removal of the operons using the unique restriction sites flanking each transcription unit (21). To facilitate construction of multiple gene deletion mutants in pKU7801, deletions were first constructed on plasmids carrying a single *nif* operon, namely, *pBR322M-nifBQ*, *pBR322M-nifUSVWZM*, and *pBR322M-*[P_{nifH}-*anfHDGKOR*], which were then used to replace the WT operons. As an example, *pBR322M-nifQ* was obtained by deletion of *nifB* in *pBR322M-nifBQ* by an optimized PCR-based method (51). The NheI fragment containing the *nifB* deletion was then excised from the *pBR322M-nifQ* plasmid and used to replace the *nifBQ* operon in the pKU7806 (a *nifENX* deletion derivative of pKU7801), resulting in the multiple gene deletion mutant Δ*nifENX/B* shown in Fig. 2*A* and Table S1. For construction of the "minimal" FeFe system with additional *KpnifENX*, *AvnifENX*, and *AvvnfENX,* the BioBrick parts of the *KpnifENX*, *AvnifENX*, and *AvvnfENX* operons were excised with XbaI/SpeI and integrated into the XbaI site of pKU7815. For construction of plasmids carrying *fldA*, *fldB*, or *ydbK*, the respective genes were amplified from the genome of *E. coli* MG1655 flanked with XbaI/SpeI restriction sites and inserted into the appropriate vector (pKU7823 or pK7826).

Acetylene Reduction Assay. The C_2H_2 reduction method was used to assay nitrogenase activity as previously described (19). Recombinant *E. coli* JM109 strains were initially grown overnight in M9 medium and then diluted into 2 mL of nitrogenase activity assay medium in 25-mL sealed tubes (supplemented with appropriate antibiotics) to a final OD_{600} of ∼0.4. Air in the tube was repeatedly evacuated and flushed with argon. After incubation at 30 °C for 6–8 h, 2 mL of C_2H_2 was injected, and the gas phase was analyzed ∼16 h later with a Shimadzu GC-2014 gas chromatograph. Data presented are mean values based on at least three replicate cultures.

$^{15}N_2$ Incorporation Assay. Strains were grown overnight in M9 medium, centrifuged, and resuspended to an OD_{600} of ∼0.4 in 50 mL of nitrogen-deficient medium containing 2 mM glutamate in a 100-mL serum bottle. The serum bottles were filled with N_2 gas; 8 mL of gas was then removed, and 5 mL of $^{15}N_2$ (99%$^+$; Shanghai Engineering Research Center for Stable Isotopes) gas was injected. After 72 h of incubation at 30 °C, the cultures were collected and freeze-dried, ground, weighed, and sealed into tin capsules. Isotope ratios are expressed as $\delta^{15}N$, whose values are a linear transform of the isotope ratios $^{15}N/^{14}N$, representing the per mille difference between the isotope ratios in a sample and in atmospheric N_2 (52). Data presented are mean values based on at least two replicate cultures.

Western Blotting. Proteins were applied to a 5–15% (wt/vol) gradient SDS polyacrylamide gel and then analyzed by immunoblotting with a 1:5,000 dilution of NifH rabbit polyclonal antibody. The antiserum against NifH (which cross-reacts with AnfH) was a gift from Jilun Li (China Agriculture University, Beijing). Antibody–antigen complexes were visualized with alkaline phosphatase conjugated to goat anti-rabbit IgG. For Western blot analysis, samples were taken just after testing nitrogenase activity, with either 5 μg or 15 μg of total protein (or supernatant after sonication) loaded for NifH and AnfH, respectively.

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