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Functional Genomic Analysis of Three Nitrogenase Isozymes in the Photosynthetic Bacterium *Rhodopseudomonas palustris*‡

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The photosynthetic bacterium *Rhodopseudomonas palustris* is one of just a few prokaryotes described so far that has *vnf* and *anf* genes for alternative vanadium cofactor (V) and iron cofactor (Fe) nitrogenases in addition to *nif* genes for a molybdenum cofactor (Mo) nitrogenase. Transcriptome data indicated that the 32 genes in the *nif* gene cluster, but not the *anf* or *vnf* genes, were induced in wild-type and Mo nitrogenase-expressing strains grown under nitrogen-fixing conditions in Mo-containing medium. Strains that were unable to express a functional Mo nitrogenase due to mutations in Mo nitrogenase structural genes synthesized functional V and Fe nitrogenases and expressed *vnf* and *anf* genes in nitrogen-fixing growth media that contained Mo and V at concentrations far in excess of those that repress alternative nitrogenase gene expression in other bacteria. Thus, not only does *R. palustris* have multiple enzymatic options for nitrogen fixation, but in contrast to reports on other nitrogen-fixing bacteria, the expression of its alternative nitrogenases is not repressed by transition metals. Between 95 and 295 genes that are not directly associated with nitrogenase synthesis and assembly were induced under nitrogen-fixing conditions, depending on which nitrogenase was being used by *R. palustris*. Genes for nitrogen acquisition were expressed at particularly high levels during alternative nitrogenase-dependent growth. This suggests that alternative nitrogenase-expressing cells are relatively starved for nitrogen and raises the possibility that fixed nitrogen availability may be the primary signal that controls the synthesis of the V and Fe nitrogenases.

Nitrogenases convert nitrogen gas to ammonia with the concomitant obligate production of hydrogen (8). This difficult reaction requires large amounts of ATP and reductant. *Rhodopseudomonas palustris* is a purple facultatively photosynthetic bacterium that is an attractive organism to develop as a biocatalyst for hydrogen production by means of nitrogen fixation because it can generate ATP from light and reductant from acetate and green plant-derived aromatic compounds to drive this process (1, 13). It should be possible to configure bioreactors wherein *R. palustris* cultures illuminated by sunlight degrade agricultural waste and generate hydrogen as a product of nitrogen fixation.

A striking feature of the genome of *R. palustris* CGA009 is that it has genes predicted to encode three different nitrogenases, each with a different transition metal-containing cofactor at its active site (19) (Fig. 1). Most nitrogen-fixing bacteria encode only a molybdenum nitrogenase (Mo nitrogenase). Some bacteria, including the purple nonsulfur phototrophs *Rhodobacter capsulatus* and *Rhodospirillum rubrum* as well as various cyanobacteria and *Clostridium pasteurianum*, encode, in addition, either an iron nitrogenase (Fe nitrogenase) or a vanadium nitrogenase (V nitrogenase) (5, 23). Alternative nitrogenases have been proposed to serve as a route for nitrogen fixation in situations where molybdenum is limited in the environment. Only the obligately aerobic heterotroph *Azotobacter vinelandii* has been shown to have three different functional nitrogenases, although genes for all three nitrogenases are also present in *Methanosarcina acetovorans* (9).

Mo nitrogenases consist of two components, designated the dinitrogenase reductase (encoded by *nifH*) and the dinitrogenase or MoFe protein (encoded by *nifDK*) (8). The MoFe protein has an iron molybdenum cofactor at its active site. Reduced NifH serves as the electron donor to the MoFe protein, which contains the site of nitrogen reduction. The electron transfer reaction from NifH to NifDK is accompanied by the hydrolysis of ATP. Nitrogen is progressively reduced at the active site to produce partially reduced intermediates until, finally, ammonium is formed and released along with hydrogen. Alternative V and Fe nitrogenases are comprised of homologous VnfHDK and AnfHDK subunits. These enzymes also include VnfG and AnfG subunits as additional structural components of the dinitrogenase (23). Many accessory proteins participate in the synthesis of the transition metal cofactors and in the assembly of nitrogenases (Fig. 1). Some of the cofactor synthesis and assembly proteins encoded by the *nif* gene cluster participate in alternative nitrogenase cofactor synthesis and assembly (30).

Here we present work demonstrating that *R. palustris* can express three functional nitrogenase isozymes. Wild-type cells expressed nitrogenase isozymes in a hierarchy according to

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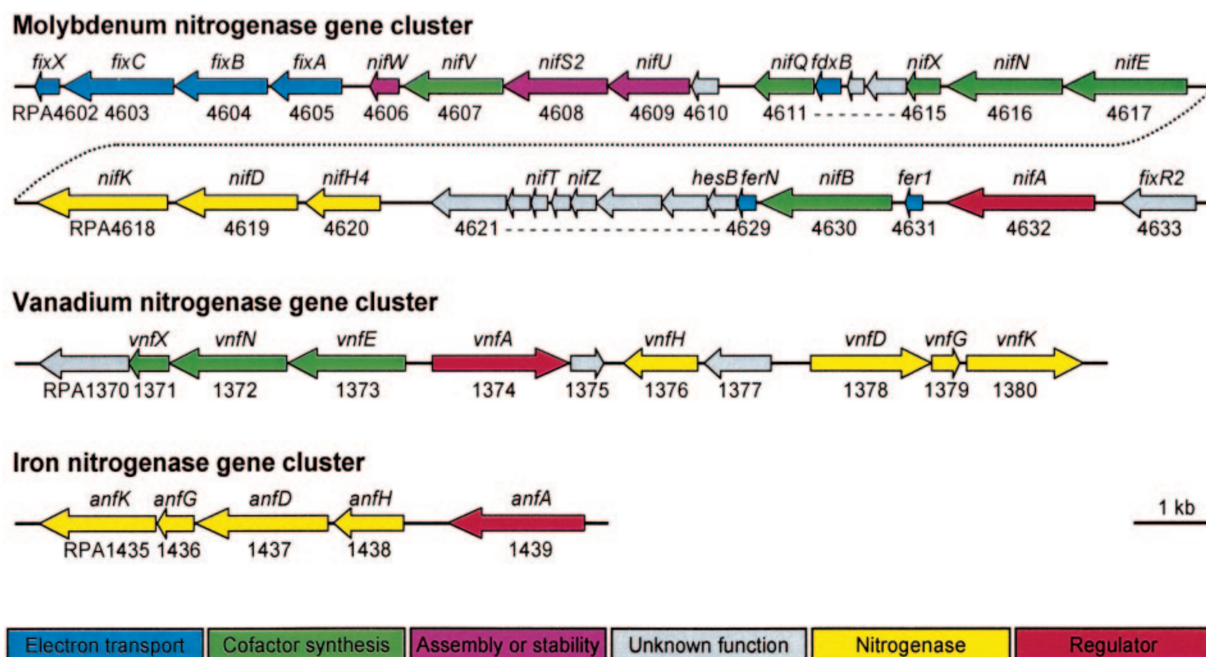

FIG. 1. Organization of Mo, V, and Fe nitrogenase gene clusters in *R. palustris*. Gene functions are annotated according to Rubio and Ludden (30).

metal availability, with Mo nitrogenase expressed in preference to V and Fe nitrogenases when Mo was present and V nitrogenase expressed in preference to Fe nitrogenase when Mo was absent and V was present. Specific metal-responsive regulators have been shown to repress alternative nitrogenase gene expression in some bacteria (18), and this is the generally accepted mechanism for the differential regulation of nitrogenase isozymes (18, 28). There is one report that is not consistent with this mechanism in which convincing evidence is presented that Mo does not directly repress Fe nitrogenase synthesis in *R. rubrum* (20). This observation was never further investigated, though. We present evidence here that *R. palustris* resembles *R. rubrum* in that it synthesizes its alternative nitrogenases in the presence of Mo in situations where it is unable to express a functional Mo nitrogenase. We hypothesize based on transcriptome data that alternative nitrogenase gene expression occurs in *R. palustris* in response to increasing levels of fixed nitrogen starvation rather than in direct response to transition metals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used for this study are listed in Table 1. *R. palustris* strains were grown anaerobically in light in defined mineral medium containing ammonium sulfate and 10 mM succinate at 30°C (non-nitrogen-fixing medium) (17). Nitrogen-fixing medium was the same medium without added ammonium sulfate. Cells were grown in sealed tubes with a nitrogen gas headspace. Mo-depleted growth medium was prepared by treating 10-fold-concentrated nitrogen-fixing medium with 8-hydroxyquinoline (8-HQ) at pH 3.5, followed by extraction with dichloromethane (33). The pH was then raised to 7.2 with a solution of ultrapure NaOH (Fluka MicroSelect). The trace element solution used in the growth medium was prepared without Mo salt but was not treated with 8-HQ. All glassware was soaked in a solution of 1% Count-OFF (New England Nuclear) and 10 mM EDTA for 24 h and then washed with deionized H_2O (Millipore system) (34). The concentrations of Mo and V in growth media were determined by inductively coupled plasma mass spectrometry (ICP-MS; Varian UltraMass 700) at the University of Iowa Hygienic Laboratory. Mo was added to 8-HQ-treated medium as Na_2MoO_4, and V was added as VCl_3.

Nitrogenase activity and hydrogen production measurement. Nitrogenase activity was measured by the acetylene reduction assay (2). Assays were carried out in sealed tubes that had been flushed with argon gas and autoclaved. Cultures in the mid-logarithmic phase of growth and 2% acetylene (final concentration) were injected into the tubes and incubated in light at 30°C. Gas-phase samples (100 μl) were withdrawn with a Hamilton sample lock syringe at intervals, and ethylene and ethane were measured with a Hewlett Packard model 5890 series II gas chromatograph fitted with a flame ionization detector and a Porapak N column (80/100 mesh, 1/8 in. by 6 ft). The temperatures of the injector, detector, and oven were 50°C, 150°C, and 40°C, respectively. Nitrogen gas was the carrier, at a flow rate of 40 ml/min. Hydrogen was measured with a thermal conductivity detector and a Molecular Sieve-13X column (80/100 mesh, 1/4 in. by 8 ft). The temperatures of the injector, detector, and oven were 100°C, 100°C, and 50°C, respectively. Nitrogen gas was the carrier, at a flow rate of 40 ml/min.

Construction of *R. palustris* mutant strains and transcriptional fusions. In-frame deletions of the *nifH*, *vnfH*, *anfH*, and *anfA* genes were generated by overlap extension PCR (14) as described previously (12). PCR primers and recombinant plasmids are described in Table 1. Broad-host-range plasmids were mobilized from *Escherichia coli* S17-1 into *R. palustris* CGA009 by conjugation, and double recombinant strains were selected as previously described (6). Sucrose-resistant and gentamicin-sensitive colonies were screened by colony PCR and sequencing to validate the expected chromosomal in-frame deletion mutations. A *nifD*::Tn*5* mutant was identified from an *R. palustris* mini-Tn*5*-*lacZ1* mutant library (11) that had been screened for strains defective in nitrogen fixation. This mutant was used as a starting strain for the construction of a Δ*nifH nifD*::Tn*5* Δ*anfA* strain. Plasmids containing promoter-*lacZ* fusions were constructed as previously described (7). The primers used to amplify promoter fragments are given in Table 1. β-Galactosidase activities were measured as described previously (7).

Whole-genome microarray construction. An *R. palustris* whole-genome microarray was constructed by using the same procedures as those for a *Shewanella oneidensis* whole-genome microarray (10). PCR primers for 4,508 of 4,836 predicted open reading frames (ORFs) of the *R. palustris* chromosome were designed using PRIMEGENS software (36). Each gene was amplified 16 times in parallel in 100-μl reaction mixtures in 96-well plates. All of the amplified products (16 by 100 μl) were pooled and purified using a Biomek F/X automated workstation (Beckman). All of the amplified products were analyzed in 1.5%

TABLE 1. Bacterial strains, plasmids, and primers used for this study

Strain, plasmid, or primer	Genotype, phenotype, or sequence of primer[a] (5′ to 3′)	Reference, origin, or description
R. palustris strains		
CGA009	Wild-type strain; spontaneous Cm^r derivative of CGA001	17
CGA750	Δ*nifH* (792 bp deleted from *nifH* gene)	This study
CGA751	Δ*vnfH* (816 bp deleted from *vnfH* gene)	This study
CGA752	Δ*anfH* (753 bp deleted from *anfH* gene)	This study
CGA753	Δ*vnfH* Δ*anfH*; Mo nitrogenase strain	This study
CGA755	Δ*nifH* Δ*vnfH*; Fe nitrogenase strain	This study
CGA756	Δ*nifH* Δ*vnfH* Δ*anfH*	This study
CGA761	Δ*nifH* Δ*vnfH* Δ*anfA*	This study
CGA762	Δ*nifH nifD*::Tn*5*	This study
CGA766	Δ*nifH nifD*::Tn*5* Δ*anfA*; V nitrogenase strain	This study
CGA770	*nifD*::Tn*5*	This study
CGA780	Δ*anfA* (1,491 bp deleted from *anfA* gene)	This study
E. coli strains		
DH5α	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA relA1* φ80d*lacZ*Δ*M15*	Gibco-BRL
S17-1	*thi pro hsdR hsdM$^+$ recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7)	32
S17-1 λpir	S17-1 lysogenized with λpir phage	3
Plasmids		
pJQ200KS	Gm^r; *sacB*; mobilizable suicide vector	29
pUC19	Ap^r; high-copy-number cloning vector	37
pHRP309	Gm^r lncQ; *lacZ* transcriptional fusion vector	27
pHRP311	Gm^r Sm^r Sp^r; negative control (cassette from cohort vector inserted into pHRP309)	27
pHRP316	Ap^r Sm^r Sp^r; cohort cloning vector for use with pHRP309	27
pUTmini-Tn*5 lacZ1*	Ap^r Km^r; delivery plasmid for mini-Tn*5 lacZ1*	3
pUC-Δ*nifH*	Ap^r; in-frame deletion of *nifH* gene (constructed by PCR and cloned into BamHI/XbaI sites of pUC19)	This study
pUC-Δ*vnfH*	Ap^r; in-frame deletion of *vnfH* gene (constructed by PCR and cloned into BamHI/XbaI sites of pUC19)	This study
pUC-Δ*anfH*	Ap^r; in-frame deletion of *anfH* gene (constructed by PCR and cloned into BamHI/XbaI sites of pUC19)	This study
pUC-Δ*anfA*	Ap^r; in-frame deletion of *anfA* gene (constructed by PCR and cloned into SalI/XbaI sites of pUC19)	This study
pJQ-Δ*nifH*	Gm^r; BamHI/XbaI fragment of pUC-Δ*nifH* cloned into pJQ200KS	This study
pJQ-Δ*vnfH*	Gm^r; BamHI/XbaI fragment of pUC-Δ*vnfH* cloned into pJQ200KS	This study
pJQ-Δ*anfH*	Gm^r; BamHI/XbaI fragment of pUC-Δ*anfH* cloned into pJQ200KS	This study
pJQ-Δ*anfA*	Gm^r; SalI/XbaI fragment of pUC-Δ*anfA* cloned into pJQ200KS	This study
pHRP316-P*fer1*	Ap^r Sm^r Sp^r; *fer1* gene promoter region in pHRP316	This study
pHRP309-P*fer1*	Gm^r Sm^r Sp^r; *fer1-lacZ* transcriptional fusion in pHRP309	This study
pHRP316-P*fixA*	Ap^r Sm^r Sp^r; *fixA* gene promoter region in pHRP316	This study
pHRP309-P*fixA*	Gm^r Sm^r Sp^r; *fixA-lacZ* transcriptional fusion in pHRP309	This study
pHRP316-P*rpa1377*	Ap^r Sm^r Sp^r; *rpa1377* gene promoter region in pHRP316	This study
pHRP309-P*rpa1377*	Gm^r Sm^r Sp^r; *rpa1377-lacZ* transcriptional fusion in pHRP309	This study
Primers		
U*nifH*-BamHI	CGGGATCCTGGTGTCCGACAGCGACTATGTCG	*nifH* upstream primer
D*nifH*-XbaI	GCTCTAGAGGCCCATCTCCTCGAGCAGGATGCGC	*nifH* downstream primer
nifH-delF	GGCAAGGGCGGCATCGGCAAGCTGCAGGCGCTCGCCGAACTGCAGGCC	*nifH* in-frame deletion forward primer
nifH-delR	GGCCTGCAGTTCGGCGAGCGCCTGCAGCTTGCCGATGCCGCCCTTGCC	*nifH* in-frame deletion reverse primer
U*vnfH*-BamHI	CGGGATCCGACGCCCGAGCAACACTTTTCCTCC	*vnfH* upstream primer
D*vnfH*-XbaI	GCTCTAGAAGCCAAGGTCGAGGCGGTCGAATACG	*vnfH* downstream primer
vnfH-delF	GGTAAAGGCGGAATCGGCAAGCTGCAGGAAGCCGCCAAGGCGGCGGCC	*vnfH* in-frame deletion forward primer
vnfH-delR	CGCCGCCGCCTTGGCGGCTTCCTGCAGCTTGCCGATTCCGCCTTTACC	*vnfH* in-frame deletion reverse primer
U*anfH*-BamHI	CGGGATCCGCGAGCTGGAGAACGTGATCGAGCG	*anfH* upstream primer
D*anfH*-XbaI	GCTCTAGATGCCCATCCGGGTGAAGTAGTCGAGC	*anfH* downstream primer
anfH-delF	GGCAAGGGTGGCATCGGCAAGCTGCAGATGGTCGTGAAGTACGGCCTG	*anfH* in-frame deletion forward primer

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or primer	Genotype, phenotype, or sequence of primer[a] (5′ to 3′)	Reference, origin, or description
anfH-delR	CAGGCCGTACTTCACGACCATCTGCAGCTTGCCGATGCCACCCTTGCC	*anfH* in-frame deletion reverse primer
U*anfA*-SalI	ACGCGTCGACCGGCGCATAAACCGGCCTGATCTTTGCG	*anfA* upstream primer
D*anfA*-XbaI	GCTCTAGATCGCGGTGATGACGCCG	*anfA* downstream primer
anfA-delFApaI	GACGCCATGTCCAAAGGCCTCGGGCCCCTGACGCGGCGAATGCTCGGC	*anfA* in-frame deletion forward primer
anfA-delRApaI	GCCGAGCATTCGCCGCGTCAGGGGCCCGAGGCCTTTGGACATGGCGTC	*anfA* in-frame deletion reverse primer
fer1-BamHI	CGGGATCCACGCATTCTCGGCCTCAC	*fer1* promoter forward primer
fer1-SmaI	TCCCCCGGGATAGGTGCCGCGCTTCAT	*fer1* promoter reverse primer
fixA-BamHI	CGGGATCCGATCAACGGGTGTTCAAGGT	*fixA* promoter forward primer
fixA-EcoRI	CGGAATTCGCATGATCGTGTTGGTGAC	*fixA* promoter reverse primer
*rpa*1377-SmaI	TCCCCCGGGAGGGGAGGAAAAGTGTTG	*rpa*1377 promoter forward primer
*rpa*1377-BamHI	CGGGATCCGGAATGTCCTTGTCGCACTT	*rpa*1377 promoter reverse primer
LacZinternal	AAAACTTTCAGTGCCGCCAGC	Arbitrary PCR
LacZout	CAGCATTTTCTCTGGCTC	Arbitrary PCR and screening of transcriptional fusions

[a] Restriction sites are underlined.

agarose gels and were considered correct if the PCRs amplified single products of the expected sizes. Of the 4,508 total predicted genes, 4,350 ORFs were correctly amplified, which represents approximately 90% of the genome. Specific 50-mer oligonucleotide probes (385 ORFs) were synthesized for the genes that were not amplified. In total, the PCR amplicons and oligonucleotide probes represented 98% of the total predicted gene content of *R. palustris*. The purified PCR products were diluted in 50% dimethyl sulfoxide to a minimum concentration of 50 ng/μl. Probes were then printed onto Corning UltraGAPS coated slides (Corning, NY) with a BioRobotics Microgrid II printer (Genomic Solutions, Ann Arbor, MI) as recommended by the manufacturer and were UV cross-linked to the surfaces of slides (Stratagene UV cross-linker; Stratagene, La Jolla, CA). Each slide was printed with probes representing two copies of the genome.

RNA isolation. For RNA isolation, *R. palustris* strains were subcultured at least twice after initial inoculation from a plate. Cells were grown to the mid-logarithmic phase of growth in mineral medium containing ammonium sulfate or in nitrogen-fixing medium, chilled in an ice-water bath, and harvested by centrifugation. The cells were then frozen in liquid nitrogen and stored at −80°C for RNA isolation at a later time. Thawed cells were disrupted by bead beating, and RNAs were purified with RNeasy mini kits (QIAGEN), including DNase treatment on the columns, as described previously (12). The quality of RNA (integrity and DNA contamination) was determined with an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA) and by reverse transcription (RT)-PCR using the 16S rRNA-targeted primer set of 27F (*Escherichia coli* positions 8 to 27) and 519R (positions 536 to 519) (25).

Microarray experiments and data analysis. Fluorescently labeled cDNAs were prepared by the direct incorporation of either Cy3-dCTP or Cy5-dCTP (Amersham Biosciences) during first-strand RT reactions. The labeling efficiency was calculated by measuring the absorbance at 260 nm, 550 nm (Cy3 incorporation), and 650 nm (Cy5 incorporation). Prior to performing hybridizations, the array slides were incubated at 65°C for 45 min in prehybridization buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.3% sodium dodecyl sulfate [SDS], and 1% bovine serum albumin). The two labeled cDNA samples to be compared were mixed, and 20× SSC (final concentration, 3×), 10 mg/ml salmon sperm DNA (Invitrogen; final concentration, 0.8 mg/ml), and 5% SDS (final concentration, 0.3%) were added to the sample. This hybridization mixture was divided into two tubes and incubated at 95°C for 3 min, and the mixture in each tube was applied to prehybridized slides that had been covered with Lifterslips (Erie Scientific Company, Portsmouth, NH). The slides were assembled with hybridization chambers (Corning, Corning, NY) and submerged in a 65°C water bath. After 14 to 16 h of hybridization, the slides were washed with 1× SSC and 0.2% SDS for 5 min, 0.1× SSC and 0.2% SDS for 5 min, and 0.1× SSC for 5 min. The slides were then dried by centrifugation (200 × *g* for 5 min) and scanned with a ScanArray 4000XL scanner (Perkin-Elmer, Boston, MA). Images (Cy3 and Cy5) were captured as TIFF files and were analyzed with the image processing software ImaGene, version 5.6 (BioDiscovery, Inc., El Segundo, CA). The median intensity of each spot was used for further analysis. Intensity values for portions of array slides that had not been printed with DNA were used as negative controls. The average plus 2 standard deviations of the intensity values of the negative control was calculated for each slide and used as the threshold for data filtering (26).

The software package lcDNA was used for data normalization and assessments of the statistical confidence intervals of gene expression (15, 35). Duplicate calibration experiments and three comparative experiments using RNAs from three separately grown cultures (three biological replicates), with duplicate slides for each (10 slides in total), were used to generate each data set. Genes whose ratios (nitrogen-fixing conditions/non-nitrogen-fixing conditions) were ≥2 and whose scores were <0.025 (26) were considered expressed at higher levels under nitrogen-fixing conditions. Genes whose ratios were ≤0.5 and whose scores were >0.975 (26) were considered expressed at lower levels under nitrogen-fixing conditions. The microarray data have been deposited at http://www.ncbi.nlm.nih.gov/geo under accession number GSE3030.

RESULTS

R. palustris **encodes three functional nitrogenase isozymes.** Nitrogen fixation was tested by measuring growth with nitrogen gas as a sole nitrogen source and by assaying acetylene reduction. A diagnostic feature of V and Fe nitrogenases is that they catalyze the production of ethane (C_2H_6) along with ethylene (C_2H_4) as a product of acetylene reduction, whereas Mo nitrogenases reduce acetylene exclusively to ethylene (23). *R. palustris* wild-type cells grow well with nitrogen gas as a sole nitrogen source, and we concluded that this is mediated by a Mo nitrogenase because ethylene was generated as the sole product in acetylene reduction assays. To determine whether the gene clusters annotated as encoding V and Fe nitrogenases

TABLE 2. Growth rate, nitrogenase activity, and hydrogen production from various *R. palustris* strains in the presence or absence of vanadium in nitrogen-fixing medium[d]

Genotype	Type of nitrogenase expressed	Parameter for nitrogen-fixing medium				Parameter for nitrogen-fixing medium supplemented with 10 μM vanadium			
		Growth rate[a]	Nitrogenase activity[b]	C_2H_6/C_2H_4 ratio	H_2 production[c]	Growth rate[a]	Nitrogenase activity[b]	C_2H_6/C_2H_4 ratio	H_2 production[c]
Wild type	Mo	12.9 (1.0)	95.9 (23.8)	–	33 (9)	14.5 (2.4)	91.7 (17.6)	–	38 (16)
Δ*vnfH* Δ*anfH*	Mo	13.5 (0.1)	107 (19.0)	–	34 (4)	14.3 (0.3)	92.0 (12.3)	–	30 (9)
Δ*nifH nifD*::Tn*5*	V and Fe	25.4 (0.1)	3.6 (1.6)	0.056	ND	24.9 (0.1)	12.8 (2.9)	0.015	ND
Δ*nifH nifD*::Tn*5* Δ*anfA*	V	NG	ND	ND	ND	19.1 (0.6)	8.6 (0.9)	0.017	51 (4)
Δ*nifH* Δ*vnfH*	Fe	23.6 (1.2)	1.4 (0.2)	0.061	161 (17)	25.1 (1.0)	1.2 (0.2)	0.052	140 (30)
Δ*nifH* Δ*vnfH* Δ*anfA*	None	NG	ND	ND	ND	NG	ND	ND	ND
Δ*nifH* Δ*vnfH* Δ*anfH*	None	NG	ND	ND	ND	NG	ND	ND	ND

[a] Doubling time in h.
[b] Nitrogenase activity in nmol C_2H_4 formed/min/mg protein.
[c] Hydrogen production in μmol/mg protein. Data were obtained from exponentially growing cultures (optical density at 660 nm, 0.25 to 0.5).
[d] Data are averages of three or more measurements, and standard deviations are shown in parentheses. NG, no growth; ND, not determined; −, no ethane produced.

encode functional nitrogenase isozymes and to characterize the Mo nitrogenase, we constructed strains mutated in various combinations of the *nif*, *vnf*, and *anf* genes. Δ*vnfH* Δ*anfH*, Δ*nifH nifD*::Tn*5* Δ*anfA*, and Δ*nifH* Δ*vnfH* mutants grew with nitrogen gas as a sole source of nitrogen, and each reduced acetylene to ethylene. AnfA is predicted to encode a regulator necessary for the expression of *anf* genes. A triple Δ*nifH* Δ*vnfH* Δ*anfA* mutant did not grow under nitrogen-fixing conditions (Table 2).

The Δ*vnfH* Δ*anfH* strain had the characteristics of a Mo nitrogenase-expressing strain. It had relatively high rates of acetylene reduction and failed to reduce acetylene to ethane. Its growth rate matched that of wild-type cells (Table 2). The standard growth medium that we used to cultivate *R. palustris* included about 15 nM Mo, as determined by ICP-MS. The Δ*vnfH* Δ*anfH* strain failed to grow in medium that had been extracted with 8-HQ to remove Mo to undetectable levels (<0.1 ppb). The addition of Mo to the 8-HQ-extracted medium to a final concentration of 15 nM restored growth and nitrogenase activity. The Δ*nifH nifD*::Tn*5* Δ*anfA* strain had the expected characteristics of a V nitrogenase-expressing strain. Our standard nitrogen-fixing growth medium, which lacks added V and has an undetectable level of V (<0.1 ppb), as measured by ICP-MS, failed to support the growth of this strain. However, the addition of exogenous V to a final concentration of 10 μM allowed the Δ*nifH nifD*::Tn*5* Δ*anfA* strain to grow and to reduce acetylene to ethane and ethylene at ratios of about 0.015 ethane to 1 ethylene. A Δ*nifH* Δ*vnfH* strain had the phenotype of an Fe nitrogenase-expressing strain. It had low rates of acetylene reduction and produced ethane and ethylene at a ratio of about 0.055 (Table 2).

We characterized a Δ*nifH nifD*::Tn*5* strain and concluded that it could express both alternative nitrogenase isozymes depending on V availability. It had the characteristics of an Fe nitrogenase-expressing strain when grown in medium lacking V and of a V nitrogenase-expressing strain when grown in medium supplemented with V (Table 2). Similarly, we found that wild-type cells grown in metal-depleted (8-HQ-extracted) medium expressed Mo nitrogenase when the medium was supplemented with Mo, V nitrogenase when the medium was supplemented with V, and Fe nitrogenase when the medium remained unsupplemented with metals (data not shown).

V and Fe nitrogenases from other bacteria produce relatively more hydrogen and less ammonia than the traditional Mo nitrogenases that are synthesized by all nitrogen-fixing bacteria (5, 23). *R. palustris* strains expressing each of the nitrogenase isozymes produced hydrogen. The V and Fe nitrogenases catalyzed the production of twofold and fourfold more hydrogen per unit of biomass formed during growth, respectively, than the Mo nitrogenase (Table 2). *R. palustris* strain CGA009 has a frameshift in the *hupV* gene, encoding a regulatory protein necessary for the expression of a functional uptake hydrogenase (19). This renders strain CGA009 unable to recycle hydrogen produced by means of its nitrogenases.

Transcriptome profiles of strains expressing a single nitrogenase isozyme. Transcriptome profiles of *R. palustris* strains grown in nitrogen-fixing medium were compared with those of strains grown in the same medium supplemented with ammonium sulfate, a condition that blocks nitrogenase activity in *R. palustris*, as evidenced by the observation that cells do not produce hydrogen or reduce acetylene when grown in the presence of ammonium. As expected based on our physiological studies, wild-type cells expressed genes in the *nif* cluster (RPA4602 to RPA4633) at as much as 300-fold higher levels under nitrogen-fixing conditions. Genes in the *vnf* and *anf* clusters were expressed at only very low levels or not at all (Table 3). This confirms that the wild-type strain synthesizes only its Mo nitrogenase when grown in our standard nitrogen-fixing growth medium. The gene expression profile of wild-type cells grown in nitrogen-fixing medium supplemented with 10 μM V was similar to that of wild-type cells grown in the same medium without added V (Table 3). The Δ*vnfH* Δ*anfH* (Mo nitrogenase-expressing) strain had a gene expression profile similar to that of wild-type cells (Table 3).

Most of the *nif* genes and all of the *vnf* and *anf* genes were expressed at a high level in the Δ*nifH* Δ*vnfH* (Fe nitrogenase-expressing) strain grown under nitrogen-fixing conditions, even though this strain was unable to synthesize functional Mo or V nitrogenase due to *nifH* and *vnfH* structural gene mutations. Also, most *nif* genes were expressed in the Δ*nifH nifD*::Tn*5* Δ*anfA* strain, in spite of the fact that this strain synthesized only an active V nitrogenase. Several genes (RPA4611 to RPA4618) situated downstream of *nifD* were not expressed by the Δ*nifH nifD*::Tn*5* Δ*anfA* strain. This most likely reflects polar effects of the mini-Tn*5* insertion in *nifD* on other genes in its transcriptional unit. The observation that the Δ*nifH*

TABLE 3. Expression of the three nitrogenase gene clusters in different nitrogenase-expressing strains

RPA no.	Gene name	Annotation	Avg expression ratio (N_2-fixing/non-N_2-fixing)[a]				
			Wild type	Wild type (plus V)	Mo strain	V strain (plus V)	Fe strain
Vanadium nitrogenase gene cluster							
1370	RPA1370	Nitrogen-fixing NifU, C-terminal	1.5	ND	1.8	200	140
1371	*vnfX*	V nitrogenase protein	ND	ND	ND	280	150
1372	*vnfN*	Nitrogenase cofactor synthesis protein	ND	ND	ND	67	14
1373	*vnfE*	Nitrogenase cofactor synthesis protein	4.6	2.6	4.6	250	200
1374	*vnfA*	V nitrogenase transcriptional regulator	2.5	2.4	3.0	78	31
1375	RPA1375	Hypothetical protein	1.1	1.0	1.1	36	6.2
1376	*vnfH*	Nitrogenase Fe protein	1.1	1.5	Mutated	54	Mutated
1377	RPA1377	Possible glyoxalase	ND	ND	2.5	210	44
1378	*vnfD*	Nitrogenase V-Fe protein, alpha chain	ND	ND	ND	930	140
1379	*vnfG*	V dinitrogenase, delta subunit	ND	ND	ND	490	44
1380	*vnfK*	Nitrogenase V-Fe protein, beta chain	ND	ND	ND	710	110
Iron nitrogenase gene cluster							
1435	*anfK*	Alternative nitrogenase 3, beta chain	1.0	0.8	0.9	2.8	94
1436	*anfG*	Dinitrogenase 3, delta subunit	ND	ND	ND	ND	1000
1437	*anfD*	Nitrogenase Mo-Fe protein, alpha chain	1.2	0.7	1.0	9.4	400
1438	*anfH*	Nitrogenase Fe protein	NA	NA	Mutated	NA	NA
1439	*anfA*	Fe nitrogenase transcriptional regulator	0.8	0.9	0.8	Mutated	69
Molybdenum nitrogenase gene cluster							
4602	*fixX*	Ferredoxin-like protein	50	42	53	190	110
4603	*fixC*	Nitrogen fixation protein	59	44	30	250	180
4604	*fixB*	Electron transfer flavoprotein, alpha chain	22	16	14	70	63
4605	*fixA*	Electron transfer flavoprotein, beta chain	51	69	39	120	180
4606	*nifW*	Nitrogenase stabilizer	15	14	9.3	16	22
4607	*nifV*	Homocitrate synthase	130	130	120	340	440
4608	*nifS2*	Nitrogenase cofactor synthesis protein	34	41	24	140	84
4609	*nifU*	NifU protein	250	140	140	380	440
4610	RPA4610	Protein of unknown function	33	53	29	310	51
4611	*nifQ*	Nitrogen fixation protein	32	110	34	ND	4.1
4612	*fdxB*	Ferredoxin 2[4Fe-4S] III	14	23	13	1.4	1.9
4613	RPA4613	DUF683	48	71	52	ND	16
4614	RPA4614	DUF269	76	64	72	1.5	41
4615	*nifX*	Nitrogenase Mo-Fe protein	130	160	120	ND	27
4616	*nifN*	Nitrogenase reductase-associated ferredoxin	12	17	9.3	ND	3.6
4617	*nifE*	Nitrogenase Mo cofactor synthesis protein	81	54	96	1.6	44
4618	*nifK*	Nitrogenase Mo-Fe protein, beta chain	310	180	210	1.2	220
4619	*nifD*	Nitrogenase Mo-Fe protein, alpha chain	330	180	230	Mutated	260
4620	*nifH4*	Nitrogenase Fe protein	NA	NA	NA	Mutated	Mutated
4621	RPA4621	Conserved hypothetical protein	53	44	60	97	110
4622	RPA4622	Hypothetical protein	6.7	8.5	4.6	24	17
4623	*nifT*	Conserved hypothetical protein	24	110	22	280	62
4624	RPA4624	Hypothetical protein	NA	NA	NA	NA	NA
4625	*nifZ*	NifZ domain	96	74	78	240	210
4626	RPA4626	Protein of unknown function	120	70	97	260	290
4627	RPA4627	Conserved hypothetical protein	16	16	18	49	53
4628	*hesB*	Protein of unknown function	80	64	70	280	110
4629	*ferN*	Ferredoxin 2[4Fe-4S]	8.9	4.8	4.5	26	15
4630	*nifB*	Nitrogen fixation protein	150	75	120	530	200
4631	*fer1*	Ferredoxin 2[4Fe-4S]	65	ND	56	230	89
4632	*nifA*	Mo/Fe nitrogenase transcriptional regulator	1.8	2.0	2.0	3.7	3.0
4633	*fixR2*	Short-chain dehydrogenase	9.5	5.1	9.2	52	12

[a] Mo strain, V strain, and Fe strain refer to the Mo nitrogenase-only strain (Δ*vnfH* Δ*anfH*), the V nitrogenase-only strain (Δ*nifH nifD*::Tn5 Δ*anfA*), and the Fe nitrogenase-only strain (Δ*nifH* Δ*vnfH*), respectively. "Plus V" indicates that 10 μM VCl_3 was added to the growth medium. ND, not determined due to low signal intensities; NA, genes were not on the array.

nifD::Tn*5* Δ*anfA* strain expressed the *anf* genes at only very low levels is consistent with the prediction that AnfA controls *anf* gene expression. Plasmids carrying transcriptional fusions of *lacZ* to the promoter regions of two different *nif* genes (*fer-1* and *fixA*) and one *vnf* gene (RPA1377) were constructed and moved into several *R. palustris* strains. The relative levels of expression of these genes, as determined by their β-galactosidase activities, in cells that were grown with nitrogen gas or with ammonium as a nitrogen source were in general agreement with our transcriptome data (Table 4).

The transition metals Mo and V do not repress the synthesis of alternative nitrogenases in *R. palustris*. In the experiments described above, *R. palustris* expressed active Fe and V nitrogenases when grown in nitrogen-fixing medium that included

TABLE 4. Expression of promoter-*lacZ* fusion plasmids under nitrogen-fixing or non-nitrogen-fixing conditions[a]

Background	P*fer1-'lacZ* expression		P*fixA-'lacZ* expression		PRPA 1377-*lacZ* promoter expression		pHRP311 expression	
	N_2-fixing	Non-N_2-fixing	N_2-fixing	Non-N_2-fixing	N_2-fixing	Non-N_2-fixing	N_2-fixing	Non-N_2-fixing
Wild-type strain	9,600 (700)	470 (10)	1,500 (210)	30 (5)	4 (1)	5 (1)	110 (3)	70 (10)
Mo strain	6,900 (1,300)	310 (130)	5,900 (1,400)	340 (10)	70 (10)	20 (8)	1,000 (100)	900 (200)
V strain	ND	ND	ND	ND	830 (70)	100 (10)	280 (20)	100 (20)

[a] Units of β-galactosidase are in nmol product/min/mg protein. Data are averages of three or more measurements, and standard deviations are shown in parentheses. Mo strain and V strain refer to the Mo nitrogenase-only strain (Δ*vnfH* Δ*anfH*) and the V nitrogenase-only strain (Δ*nifH nifD*::Tn5 Δ*anfA*), respectively. pHRP311 is the control vector with no promoter insert. ND, not determined.

about 15 nM molybdate salt. This was surprising because Mo has been reported to repress the synthesis of alternative nitrogenases. Mo at concentrations of >10 nM, for example, prevented the diazotrophic growth of an *R. capsulatus nifHDK* mutant and the expression of its Fe nitrogenase activity (31). We thought it possible that the relatively small amount of Mo present in our nitrogen-fixing growth medium might only partially block the expression and activity of the Fe and V nitrogenases. However, the addition of Mo to concentrations as high as 100 μM did not substantially affect the growth rates of or rates of acetylene reduction by the Fe and V nitrogenase-expressing strains (Table 5 and data not shown). Also, the addition of V at a concentration of 100 μM did not block Fe nitrogenase expression, in contrast to what has been observed in *A. vinelandii* (16; data not shown).

We grew *R. palustris* in medium that had been extracted with 8-HQ to check whether the removal of Mo might derepress Fe nitrogenase or V nitrogenase activity further from what was detected in nitrogen-fixing medium. The 8-HQ-treated medium contained undetectable amounts of Mo and V (<0.1 ppb for both Mo and V), and the Fe nitrogenase-expressing strain reduced acetylene at about the same rate as that when grown in untreated medium (data not shown). Further addition of Mo or V to the metal-depleted medium did not result in reduced rates of acetylene reduction by this strain. In all these experiments, the cells produced relatively high levels of ethane, indicative of an active Fe nitrogenase. Similar results were obtained with the V nitrogenase-expressing strain. The removal of all Mo did not substantially affect the rates of acetylene reduction, and the addition of Mo to concentrations as high as 100 μM did not depress the rates of acetylene reduction (Table 5).

TABLE 5. Effect of metal ion addition on vanadium nitrogenase synthesis and activity[a]

Metal ion (concn [μM])	Parameter for nitrogen-fixing medium		Parameter for metal-depleted nitrogen-fixing medium	
	Nitrogenase activity	C_2H_6/C_2H_4 ratio	Nitrogenase activity	C_2H_6/C_2H_4 ratio
V (10)	8.2 (1.1)	0.019	5.9 (1.1)	0.013
V (100)	9.2 (2.0)	0.019	ND	ND
V (10) and Mo (10)	8.6 (0.9)	0.021	3.7 (1.0)	0.020
V (10) and Mo (100)	6.0 (1.1)	0.021	4.8 (1.0)	0.019

[a] *R. palustris* strain CGA766 (Δ*nifH nifD*::Tn5 Δ*anfA*) was tested. Data are averages of three or more measurements, and standard deviations are shown in parentheses. ND, not determined.

Many nitrogen acquisition genes are coordinately regulated with the *anf* and *vnf* gene sets. Our findings show that metal ion availability per se does not control the expression of *vnf* and *anf* genes in *R. palustris*. Instead, this species synthesizes V and Fe nitrogenases in situations where it is unable to synthesize a functional Mo nitrogenase regardless of the amount of Mo that is present. The three nitrogenases might be regulated such that they are each synthesized at different thresholds of fixed nitrogen or redox availability rather than by direct metal ion repression. We examined our transcriptome data to further investigate this possibility. About twice as many genes were expressed at twofold or higher levels in the V nitrogenase (324 genes)- and Fe nitrogenase (369 genes)-expressing strains than in the wild-type (164 genes) and Mo nitrogenase-expressing (185 genes) strains under nitrogen-fixing conditions (see Table S1 in the supplemental material). Among these were about 20 genes that are located immediately adjacent to the *vnf* genes on the *R. palustris* chromosome and 4 genes that are located immediately adjacent to the *anf* genes (Table 6). Genes or groups of genes unlinked to nitrogenase gene clusters that were expressed at fivefold or higher levels in one or more strains are listed in Table 7. The gene or operon functions can be loosely grouped into the following four categories related to nitrogen fixation: oxygen stress, reductant supply, iron acquisition, and nitrogen acquisition. Hypothetical and conserved hypothetical genes are also listed. Of these categories, a significant proportion of genes involved in nitrogen acquisition were expressed at higher levels in V or Fe nitrogenase-expressing strains than in Mo nitrogenase-expressing strains (Table 7). One explanation for this is that cells express genes involved in fixed nitrogen acquisition at higher levels to try to compensate for the fixed nitrogen limitation that occurs during V or Fe nitrogenase-dependent growth.

DISCUSSION

The *R. palustris* genome sequence has enabled a comprehensive analysis of gene expression during nitrogen fixation by a single bacterial species expressing three nitrogenase isozymes. In this study, transcriptome data were valuable for defining the full extent of the *nif*, *vnf*, and *anf* gene clusters and for identifying genes that are coordinately expressed with a particular nitrogenase. As has been observed in other bacteria, metal ion availability dictated the type of nitrogenase isozyme expressed by wild-type *R. palustris*. In contrast to what has been concluded for many other bacteria, however, our results indicate that the repression of gene expression by Mo or V is not

TABLE 6. Expression of genes adjacent to vanadium and iron nitrogenase gene clusters

RPA no.	Gene name	Annotation	Avg expression ratio (N_2-fixing/non-N_2-fixing)[a]			
			Wild type	Mo strain	V strain (plus V)	Fe strain
Genes adjacent to vanadium nitrogenase gene cluster						
1381	RPA1381	Hypothetical protein	1.3	1.0	50	40
1382	RPA1382	Nitrogen fixation-related protein	0.7	0.8	170	43
1383	RPA1383	Putative transcriptional regulator, ModE family	1.0	1.0	5.5	3.3
1384	RPA1384	Putative vanadate permease, component of ATP-dependent uptake system	0.8	0.8	83	98
1385	RPA1385	Putative vanadate transport system, substrate-binding protein	0.8	ND	460	230
1386	RPA1386	Putative ATP-binding component of ABC vanadate transporter	1.1	0.8	160	71
1387	RPA1387	Conserved unknown protein	ND	ND	240	32
1388	RPA1388	Hypothetical protein	0.7	0.6	11.2	1.4
1389	RPA1389	Conserved hypothetical protein	ND	ND	150	33
1390	RPA1390	Conserved hypothetical protein	ND	ND	67	25
1391	RPA1391	Possible ABC transporter ATP-binding protein	0.8	0.9	12	1.4
1392	RPA1392	Nitroreductase family proteins	ND	ND	180	49
1393	RPA1393	Conserved hypothetical protein	1.9	1.9	28	38
1394	RPA1394	Transcriptional regulator, GntR family with aminotransferase domain	1.2	1.4	8.0	12
1395	RPA1395	Possible trehalose/maltose binding protein	ND	ND	82	120
1396	RPA1396	Possible multiple sugar transport system permease protein	0.7	0.9	18	36
1397	RPA1397	Putative sugar ABC transporter, permease protein	1.0	ND	30	43
1398	RPA1398	Putative sugar ABC transporter, ATP-binding protein	ND	ND	5.5	3.7
1399	RPA1399	Putative transport system ATP-binding protein	ND	1.1	2.9	2.1
1400	RPA1400	Possible glutamine amidotransferase	ND	ND	5.7	15
1401	*glnAIII*	Putative glutamine synthetase III	1.2	0.9	11	13
Genes adjacent to iron nitrogenase gene cluster						
1431	*draT1*	Putative NAD^+ ADP-ribosyltransferase	ND	ND	ND	65
1432	RPA1432	Putative nitroimidazole resistance protein	ND	ND	ND	290
1433	RPA1433	Conserved hypothetical protein	ND	0.8	1.7	180
1434	RPA1434	Conserved hypothetical protein	ND	ND	5.0	230
1440	RPA1440	Conserved hypothetical protein	1.0	ND	8.3	4.5

[a] Mo strain, V strain, and Fe strain refer to the Mo nitrogenase-only strain (Δ*vnfH* Δ*anfH*), the V nitrogenase-only strain (Δ*nifH nifD*::Tn5 Δ*anfA*), and the Fe nitrogenase-only strain (Δ*nifH* Δ*vnfH*), respectively. "Plus V" indicates that 10 μM VCl_3 was added to the growth medium. ND, not determined due to low signal intensities.

the mechanism that underlies the hierarchical expression of nitrogenases in *R. palustris*. We reasoned that transcriptome data might point to physiological signals governing the differential expression of the three nitrogenase isozymes. A surprisingly large number of genes that are not obviously involved in nitrogenase synthesis were expressed under nitrogen-fixing conditions. Some of these genes are located directly adjacent to the *anf* or *vnf* gene cluster (Table 6). The *anf*-associated genes RPA1431, RPA1433, and RPA1434 are homologous to genes located adjacent to the *R. rubrum anf* gene cluster (https://maple.lsd.ornl.gov/microbial/rrub/). RPA1431 encodes a dinitrogen reductase ADP-ribosyltransferase (DRAT) predicted to be involved in inactivating dinitrogenase reductase by ADP-ribosylation in response to darkness or ammonia (21). *R. palustris* and *R. rubrum* each have a second DRAT gene that is paired with a dinitrogenase reductase-activating glycohydrolase gene elsewhere on their chromosomes. The *vnf*-associated genes RPA1381 to -1386 are homologous to genes that are located adjacent to the *A. vinelandii vnf* gene cluster (https://maple.lsd.ornl.gov/microbial/avin/). Among these is a set of ABC transport genes that likely encode a vanadate permease. The other highly expressed *vnf*-associated genes have no obvious functions. Two exceptions are genes for a glutamine amidotransferase (RPA1400) and a glutamine synthetase (*glnAIII*; RPA1401) that were expressed at about 10-fold higher levels under nitrogen-fixing conditions in the V and Fe nitrogenase-expressing strains but were not differentially expressed in Mo nitrogenase-expressing strains.

A large number of genes or groups of genes unlinked to nitrogenase gene clusters were also expressed at elevated levels under nitrogen-fixing conditions, and we grouped these into four physiological categories (Table 7). One of the categories is oxygen stress. Nitrogenases have metal ion clusters that render these enzymes extremely sensitive to oxygen and reactive oxygen species. This may explain why we saw elevated expression in all strains of genes encoding cytochrome aa_3 oxidase and catalase, proteins that consume oxygen or detoxify toxic oxygen species. We also cannot exclude the possibility that cells were exposed to small amounts of oxygen during harvesting and breakage and that as a consequence of their high metal content, nitrogenase-containing cell extracts generated reactive oxygen species that in turn led to the elevated expression of oxygen stress-related genes. All nitrogenases require Fe, and several Fe acquisition genes were expressed at equivalent elevated levels in strains expressing each type of nitrogenase isozyme. Since nitrogenase activity demands large amounts of reductant, it is also logical that ferredoxin and flavodoxin genes (RPA1927 and -1928 and RPA2116 and -2117) were expressed at high levels. The Fe nitrogenase is the most reductant demanding of the nitrogenases. The observation that the NAD-dependent formate dehydrogenase genes (RPA0732 to RPA0736) were expressed at relatively high lev-

TABLE 7. Genes or groups of genes unlinked to nitrogenase gene clusters that were expressed at fivefold or higher levels in one or more strains under nitrogen-fixing conditions[b]

RPA or operon no.	Gene name	Avg expression ratio (N_2-fixing/non-N_2-fixing)[a]				Functional category	Function
		Wild type	Mo strain	V strain (plus V)	Fe strain		
0274–0275	*glnK2-amtB2*	30	31	67	31	Nitrogen acquisition	PII protein-ammonium transport
0429	*katG*	1.6	1.9	5.6	3.5	Oxygen stress	Catalase
0732–0736	*fdsA-fdsG*	1.0	1.1	1.0	5.1	Reductant supply	NAD-dependent formate dehydrogenase
0758–0762		4.9	4.4	24	15	Nitrogen acquisition	Oligopeptide transport
0832–0836	*coxA-coxG*	6.9	8.8	3.6	8.1	Oxygen stress	Cytochrome aa_3 oxidase
0984	*glnA4*	2.1	2.1	12	7.6	Nitrogen acquisition	Glutamine synthetase
1001		2.0	ND	5.2	2.6	Conserved hypothetical	
1471–1473		2.9	2.9	11	6.1	Nitrogen acquisition	Dipeptide transport
1927–1928		90	52	140	120	Reductant supply	Hypothetical/ferredoxin
2112–2114	*nrtABC*	4.3	3.6	24	9.7	Nitrogen acquisition	Nitrate transport
2115	*cynS*	2.7	2.9	17	9.0	Nitrogen acquisition	Cyanate lyase
2116–2117		4.5	5.7	5.8	5.3	Reductant supply	Hypothetical/flavodoxin
2124–2128		11	8.1	7.4	17	Iron acquisition	TonB-dependent iron transport
2156		7.8	3.1	26	8.9	Hypothetical	
2333–2338		3.4	2.4	4.3	7.9	Unknown protein[c]	
2377		5.9	3.8	5.3	3.8	Conserved hypothetical	
2378		13	7.5	14	7.3	Iron acquisition	TonB-dependent iron receptor
2380		2.8	2.1	10	2.5	Iron acquisition	TonB-dependent iron receptor
2382–2390		7.8	4.6	5.7	5.7	Iron acquisition	Iron transport and siderophore biosynthesis
2409–2410		3.3	3.3	11	7.5	Nitrogen acquisition	Urea binding protein and regulator
2464–2469	*sufBCDS*	2.9	2.1	5.5	6.3	Oxygen stress	Iron cluster formation during oxygen stress
2497–2500		2.2	5.6	24	6.8	Nitrogen acquisition	Amide transporter and amidase
2518–2519		1.5	1.7	5.8	3.0	Hypothetical	
3011		5.4	2.0	3.2	2.1	Unknown protein	
3201		5.6	3.1	24	19	Nitrogen acquisition	Formate/nitrate transport
3211–3214		2.6	2.0	5.7	5.8	Hypothetical	
3308–3309		4.5	4.0	2.6	6.1	Unknown protein	
3481		2.7	ND	5.1	2.6	Hypothetical	
3665–3669		9.0	7.7	10	5.1	Nitrogen acquisition	Urea transport
3819		89	81	160	240	Hypothetical	
4145	*nirK2*	7.6	9.1	1.0	2.0	Nitrogen acquisition	Dissimilatory nitrite reductase
4209	*glnAII*	35	43	84	37	Nitrogen acquisition	Glutamine synthetase
4538–4539		1.0	1.1	2.2	7.4	Hypothetical	
4634		2.5	2.1	7.6	4.1	Hypothetical	
4713		4.3	3.3	24	7.3	Hypothetical	
4714		21	14	38	40	Hypothetical	
4827		13	8.7	35	12	Conserved hypothetical	

[a] Mo strain, V strain, and Fe strain refer to the Mo nitrogenase-only strain (Δ*vnfH* Δ*anfH*), the V nitrogenase-only strain (Δ*nifH nifD*::Tn5 Δ*anfA*), and the Fe nitrogenase-only strain (Δ*nifH* Δ*vnfH*), respectively. "Plus V" indicates that 10 μM VCl_3 was added to the growth medium. ND, not determined due to low signal intensities.

[b] RPAs forming putative operons are grouped, and the values shown represent the gene with the highest average expression ratio.

[c] Unknown proteins are proteins of hypothetical function that have been detected in *R. palustris* by proteomic techniques.

els in the Fe nitrogenase-expressing strain may reflect the fact that this strain is seeking more reducing power from various sources. Many genes involved in nitrogen acquisition were expressed at higher levels in cells growing under nitrogen-fixing conditions. These included a glutamine synthetase encoded by RPA0984 and genes for a number of transport systems for nitrogenous compounds, including transporters for dipeptides (RPA1471 to -1473), oligopeptides (RPA0758 to -0762), nitrate (RPA3201), urea (RPA2409 to -2410), and amides (RPA2497 to -2500). Genes for enzymes that function to access fixed nitrogen, including amidase and cyanate lyase, were also expressed at elevated levels. Of the gene categories depicted in Table 7, only those involved in fixed nitrogen acquisition were more highly expressed in both V nitrogenase- and Fe nitrogenase-expressing strains than in Mo nitrogenase-expressing strains. These data and growth rate data (Table 2) suggest that V and Fe nitrogenase-expressing cells are starved for nitrogen compared to Mo nitrogenase-expressing cells, and this suggests that fixed nitrogen availability may be a key physiological signal that mediates the differential expression of the nitrogenase isozymes.

R. palustris encodes a set of nitrogen signal transduction proteins that overlap with those known to control nitrogen fixation in other purple nonsulfur bacteria, nitrogen-fixing symbionts, and *A. vinelandii* in response to the intracellular nitrogen status (4, 22, 24, 38). It is possible that these proteins control transcription of the regulatory genes *nifA*, *vnfA*, and *anfA* in a hierarchy in response to various levels of fixed nitrogen starvation. Transcription of the *anfA* and *vnfA* genes does seem to be an important point of control for V and Fe nitrogenase expression considering that when cells were shifted to nitrogen-fixing conditions, *vnfA* was expressed at 78-fold higher levels in the V nitrogenase-expressing strain and *anfA* was expressed at 69-fold-higher levels in the Fe nitrogenase-

expressing strain. *R. palustris* regulator and signal transduction proteins that could be encoded by one or more of the hypothetical genes that were induced under nitrogen-fixing conditions (Table 7) may also control *anf* and *vnf* gene expression. The intracellular redox status, oxygen availability, and intracellular energy status are other signals that might influence alternative nitrogenase gene expression.

A thorough understanding of the regulation of nitrogenase expression and activity will be helpful in the design of strategies to metabolically engineer *R. palustris* to produce hydrogen. It has been speculated that nitrogenases are essentially hydrogenase enzymes that have been modified by evolutionary pressures to reduce the triple bond of nitrogen as well as to reduce protons to hydrogen gas. The alternative V and Fe nitrogenases have good potential as catalysts for hydrogen production because they produce relatively more hydrogen and less ammonia than the traditional Mo nitrogenases that are synthesized by all nitrogen-fixing bacteria. Although the *R. palustris* alternative nitrogenases were less active than Mo nitrogenase, and accordingly produced hydrogen at a lower rate, these enzymes may nevertheless be superior hydrogen-producing catalysts in some situations.

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