Light-driven carbon dioxide reduction to methane by nitrogenase in a photosynthetic bacterium

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Nitrogenase is an ATP-requiring enzyme capable of carrying out multielectron reductions of inert molecules. A purified remodeled nitrogenase containing two amino acid substitutions near the site of its FeMo cofactor was recently described as having the capacity to reduce carbon dioxide (CO_2) to methane (CH_4). Here, we developed the anoxygenic phototroph, *Rhodopseudomonas palustris*, as a biocatalyst capable of light-driven CO_2 reduction to CH_4 in vivo using this remodeled nitrogenase. Conversion of CO_2 to CH_4 by *R. palustris* required constitutive expression of nitrogenase, which was achieved by using a variant of the transcription factor NifA that is able to activate expression of nitrogenase under all growth conditions. Also, light was required for generation of ATP by cyclic photophosphorylation. CH_4 production by *R. palustris* could be controlled by manipulating the distribution of electrons and energy available to nitrogenase. This work shows the feasibility of using microbes to generate hydrocarbons from CO_2 in one enzymatic step using light energy.

nitrogenase | *Rhodopseudomonas* | bioenergy | methane | engineered bacterium

An essential process for life and an important step in the biogeochemical nitrogen cycle is nitrogen fixation by nitrogenase, in which nitrogen gas (N_2) is converted to ammonia (NH_3) (1). The difficult reduction of N_2 to two NH_3 occurs at an FeMoS cluster called FeMo cofactor in Mo-dependent nitrogenase in a reaction that requires ATP hydrolysis and dihydrogen production as shown in **1**

$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i. \quad [1]$$

Nitrogenase deprived of access to N_2 but provided with a source of electrons produces H_2 exclusively. Also, the ability of nitrogenase to carry out the multielectron reduction of an inert molecule is not limited to reduction of N_2. This enzyme can also reduce other molecules with double and triple bonds, including carbon-containing compounds (reviewed in ref. 2). Recently, we found that a remodeled nitrogenase with substitutions in two key amino acids near the FeMo cofactor is capable of reducing carbon dioxide (CO_2) to methane (CH_4) in vitro. This enzyme did not retain its ability to reduce N_2 but was active in H_2 production (3). It was unclear if the remodeled nitrogenase gene could confer to bacteria the ability to reduce CO_2 to CH_4.

Here, we describe a biocatalyst capable of generating the energy-rich hydrocarbon CH_4 by reduction of CO_2 using a remodeled nitrogenase. Development of this biocatalyst required selection of an appropriate microbial host, because large amounts of cellular reductant and ATP are used by nitrogenase, and as a consequence, this energetically expensive enzyme is repressed by both transcriptional and posttranslational regulatory mechanisms when an alternative nitrogen source, like ammonium, is available (4). Use of nitrogenase to generate a product not used by the organism would require overcoming these regulatory constraints to achieve expression of active enzyme, while at the same time providing cells with ammonium for growth. We reasoned that the anoxygenic photosynthetic bacterium *Rhodopseudomonas palustris* would be a good chassis for studying the remodeled nitrogenase in the context of a biological system, because it can fix nitrogen, and an activating mutation in *nifA*, encoding the transcription activator of nitrogenase genes, has been identified that can bypass the regulatory networks that repress nitrogenase (5–7). Also, *R. palustris* can generate the considerable amount of ATP needed for the activity of a remodeled nitrogenase from light by cyclic photophosphorylation.

We found that *R. palustris* reduced CO_2 to CH_4 using a remodeled nitrogenase with NifD^{V75AH201Q} amino acid substitutions when the enzyme was expressed in a genetic background that allows for its constitutive production and cells were incubated in light. We also found that *R. palustris* is a tractable system that allows control of CH_4 production by manipulating the distribution of electrons and energy available to nitrogenase. In nature, the eight-electron reduction of CO_2 to CH_4 requires a multistep metabolic pathway found only in methanogenic archaea (8). The results presented here describe a one-step route to biological hydrocarbon production.

Results

A variant of the FeMo-containing nitrogenase protein, NifD, from *Azotobacter vinelandii* containing amino acid substitutions V70A and H195Q reduces CO_2 to CH_4 and also produces H_2 in vitro, but it is unable to reduce N_2 to NH_3 (3). Nucleotide substitutions to generate homologous substitutions of residues in *R. palustris* NifD (V75A and H201Q) were introduced into the *nifD* coding sequence by homologous recombination. The *R. palustris*

Significance

One of the most important life-sustaining metabolisms that results from a net reduction reaction is the conversion of nitrogen gas to ammonia by nitrogenase in a process known as nitrogen fixation. A nitrogenase variant has been described that converts carbon dioxide to methane in vitro. Here, we expressed this variant in an engineered strain of the photosynthetic bacterium *Rhodopseudomonas palustris* and showed biological light-driven methane production. Its ability to reduce carbon dioxide at ambient temperature and pressure with a single enzyme and energy provided by light makes the methane-producing strain of *R. palustris* an excellent starting point to understand how a biological system can marshal resources to produce an energy-rich hydrocarbon in one enzymatic step.

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Table 1. CH_4 and H_2 production by *R. palustris* NifA* strains expressing either a WT nitrogenase or a remodeled nitrogenase

Genotype	Growth rate (h)	H_2 production (μmol/mg total protein)	CH_4 production (nmol/mg total protein)
nifA nifD*^{WT}	11.6 (0.1)	36.2 (6.2)	ND
nifA nifD*^{V75AH201Q}	12.7 (0.9)	55.1 (3.2)	30.1 (2.0)

Data are the average of three or more experiments, with the SDs shown in parentheses. Cells were grown with 20 mM acetate and 10 mM $NaHCO_3$ and incubated in light. ND, not detected.

nifD^{V75AH201Q} allele was integrated into the chromosome of an *R. palustris* strain CGA009 *nifA** mutant that expresses Mo nitrogenase constitutively, even in the presence of ammonium (5, 7). *R. palustris* can also synthesize alternative V or Fe nitrogenase isozymes, but these isozymes are not expressed in the NifA* strain grown with ammonium (5, 9). The NifA* strain expressing the remodeled nitrogenase produced CH_4 when grown with acetate as a carbon source and HCO_3^- as a source of CO_2 (Table 1). The growth rate of this strain was similar to that of its parent strain expressing WT nitrogenase. Both strains also produced comparable levels of H_2 (Table 1). *R. palustris* CGA009 is able to produce H_2 only through the activity of its nitrogenase, and thus, H_2 production is a measure of nitrogenase activity (10). The *nifA* nifD*^{V75AH201Q} strain produced almost 2,000 times more H_2 than CH_4 (Fig. 1 and Table 1). A similarly high ratio of H_2 to CH_4 produced was observed in vitro with purified remodeled nitrogenase (3).

To verify that the remodeled nitrogenase was directly responsible for CH_4 production by *R. palustris*, the product profile of the enzyme was examined. Remodeled enzyme purified from *R. palustris* converted CO_2 to CH_4 (1.95 nmol CH_4/nmol MoFe protein). No CH_4 production was detected for purified WT nitrogenase. In addition, nongrowing cells of the *R. palustris nifA* nifD*^{V75AH201Q} strain supplied with thiosulfate as an electron donor and $H^{13}CO_3^-$ as a source of CO_2 produced a product that, when analyzed by gas chromatography - mass spectrometry (GC-MS), had an *m/z* of 17 and a retention time that correlates with $^{13}CH_4$. A peak with an *m/z* of 16 and a retention time corresponding to CH_4 occurred when $H^{12}CO_3^-$ was provided. Almost 90% of the CH_4 produced was from CO_2 reduction (Fig. 2). These data confirm that the CH_4 produced by *R. palustris* cells comes directly from CO_2. As expected, the remodeled nitrogenase purified from *R. palustris* was unable to reduce N_2 to NH_3, but it reduced acetylene (C_2H_2) to ethylene (C_2H_4) (Fig. S1). These results indicate that *R. palustris* functions to catalyze the production of CH_4 and H_2 as shown in Fig. 3. Electrons derived from the oxidation of either an organic carbon source (acetate) or an inorganic compound (thiosulfate) are used to reduce CO_2 to CH_4. HCO_3^- acquired from outside the cell is converted to intracellular CO_2 by carbonic anhydrase (11). Some CO_2 is also generated from acetate metabolism.

A common method used to divert cellular resources to microbial biocatalysts is to use nongrowing cells. Such cells are spared the imperative of carrying out biosynthetic reactions required for growth, which frees up electrons derived from the oxidation of external electron donors for use in reduction reactions (12). To test if diversion of electrons from biosynthesis leads to more CH_4 production by the remodeled nitrogenase, the cells of the *R. palustris nifA* nifD*^{V75AH201Q} strain grown to midlogarithmic phase were harvested, resuspended in nitrogen-free medium, and tested for their ability to produce CH_4. As shown in Fig. 4*A*, nongrowing cells produced almost 20 times more CH_4 than growing cells.

R. palustris has the Calvin Bassham Benson (CBB) cycle for CO_2 fixation, and it is known that nitrogenase competes with the CBB cycle for electrons (7, 13). Cells in which CBB cycle activity has been genetically disrupted produce more hydrogen, because more reducing equivalents are available to nitrogenase (7, 13). We found that *R. palustris nifA* nifD*^{V75AH201Q} *ΔcbbMLS* cells, which had both sets of Rubisco genes deleted, produced twice as much CH_4 as cells with an intact CBB cycle (Fig. 4*B*).

When nongrowing cells of the *nifA* nifD*^{V75AH201Q} strain were incubated in the dark, no detectable CH_4 was produced (Fig. 5). This result was expected, because light is required for ATP production, and ATP is required for nitrogenase activity. As shown in Fig. 5, the amount and rate of CH_4 production correlated with light intensity, and more CH_4 was produced under high light intensities than under low light intensities. The exception was at the two highest light intensities: 30 and 60 μmol photons/m^2 per second, where the amount and rate of CH_4 production were not significantly different. This result likely reflects that ATP is not the limiting parameter for CH_4 production under higher light intensities.

The results shown in Fig. 4 indicate that CH_4 production can be modulated by controlling the electron flux to nitrogenase. In *R. palustris*, electrons are generated during oxidation of organic carbon sources, and we have found that the organic electron donor provided influences the amount of electrons available to nitrogenase (13). The substrate oxidation state, the route that the substrate takes to generate biosynthetic precursors, and the amount of CBB cycle flux that occurs on a given substrate all influence the amount of electrons available to nitrogenase in *R. palustris* (13). When we grew *R. palustris nifA* nifD*^{V75AH201Q} cells on different carbon sources to determine how different electron donors may influence CH_4 production, we found that, with the

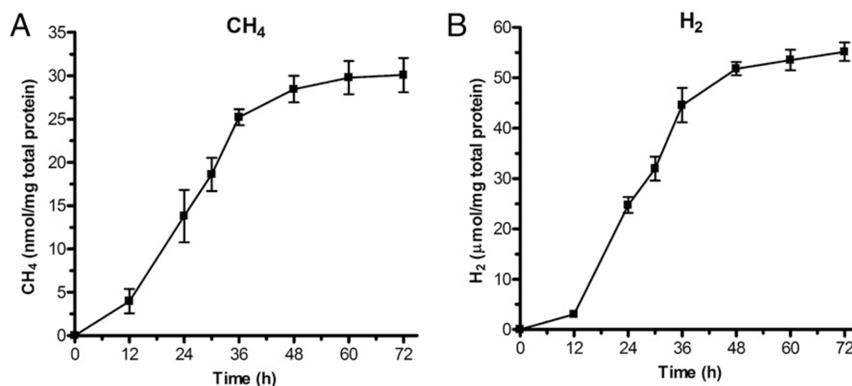

Fig. 1. An engineered strain of *R. palustris* expressing a remodeled nitrogenase produces CH_4. *R. palustris* NifA* cells expressing a remodeled nitrogenase produce (*A*) CH_4 and (*B*) H_2 during growth. These data are the average of three independent experiments, and error bars represent SDs. Cells were grown with 20 mM acetate and 10 mM $NaHCO_3$ and incubated in light.

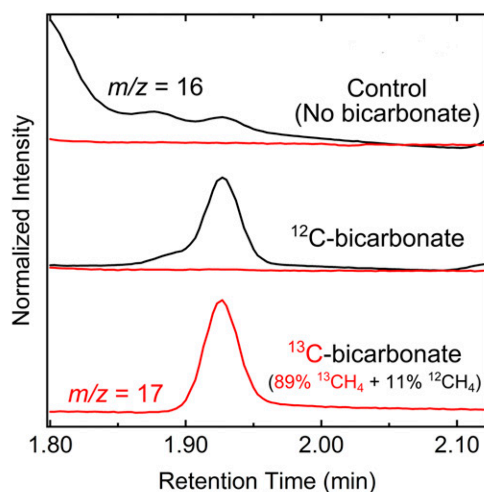

Fig. 2. CH_4 produced by *R. palustris* NifA* cells expressing a remodeled nitrogenase is caused by CO_2 reduction by the remodeled nitrogenase. GC-MS confirmation of CH_4 production from CO_2 reduction by *R. palustris* NifA* cells expressing remodeled nitrogenase. The black traces are the GC-MS data for monitoring $^{12}CH_4$ ($m/z = 16$), and the red traces are the data for monitoring $^{13}CH_4$ ($m/z = 17$). Thiosulfate was supplied as an electron donor for the reaction.

exception of fumarate and succinate, the amount of CH_4 produced was similar, regardless of the type of organic carbon source used (Fig. 6). *R. palustris* expressing remodeled nitrogenase produced H_2 as well as CH_4 under all conditions that we tested. The amount of H_2 that cells produced was always on the order of 1,000–7,000 times greater than that of CH_4.

Discussion

R. palustris has clear advantages as a system to develop the use of a remodeled nitrogenase in synthetic biology. One is that active remodeled nitrogenase can be produced constitutively. This feature is important, because it allows *R. palustris* to grow with ammonium as a nitrogen source and use an energetically expensive enzyme to produce a product that it cannot use. The other major advantage to using *R. palustris* is that it can meet the high ATP requirement for CH_4 production by nitrogenase by using cyclic photophosphorylation (Fig. 3). We have shown that the amount and rate of CH_4 production are dependent on the light intensity provided (Fig. 5). The simplest explanation for this correlation is that decreased light intensity results in a change in the energy status of the cell, so that less ATP is available for nitrogenase. However, this effect on CH_4 production could be more complex, because cellular energy levels may affect posttranslational modification of nitrogenase (14).

CH_4 production can be improved by diverting electrons away from biosynthesis or the CBB cycle (Fig. 4), and maximal CH_4 production will likely involve manipulating both of these parameters. *R. palustris* can maintain a nongrowing but metabolically active state for months as long as it has access to light and electrons (15). It can obtain these electrons from both organic and inorganic donors and even use an electric current from an electrode as an electron donor (13, 16, 17). Substrate conversion efficiencies of 86% have been shown using an inorganic electron donor, much higher than the 60% conversion efficiency on an organic electron donor (16). This ability sets up the possibility of exploiting nongrowing *R. palustris nifA* nifD^{V75AH201Q}* in microbial electrolysis cells (MECs), where electrons are provided by a cathode and used to generate CH_4. Generation of a chemical product at the cathode has both environmental and monetary benefits that increase the potential of MECs in biofuel generation (18).

Despite an ability to increase CH_4 production by diverting electrons to nitrogenase, maximizing CH_4 production using a biocatalyst like *R. palustris* will ultimately require altering the ratio of CH_4 to H_2 produced by the remodeled nitrogenase. The *nifA** strain used in this study has a defect in its uptake hydrogenase (10). One way to recycle the reductant used to make hydrogen would be to restore the uptake hydrogenase. Additionally, increasing the ratio of the Fe protein to the MoFe protein has been shown to favor CH_4 production, resulting in a reduction in the ratio of H_2 to CH_4 produced (3). A similar approach could be taken by overexpressing the Fe protein in *R. palustris nifA* nifD^{V75AH201Q}*.

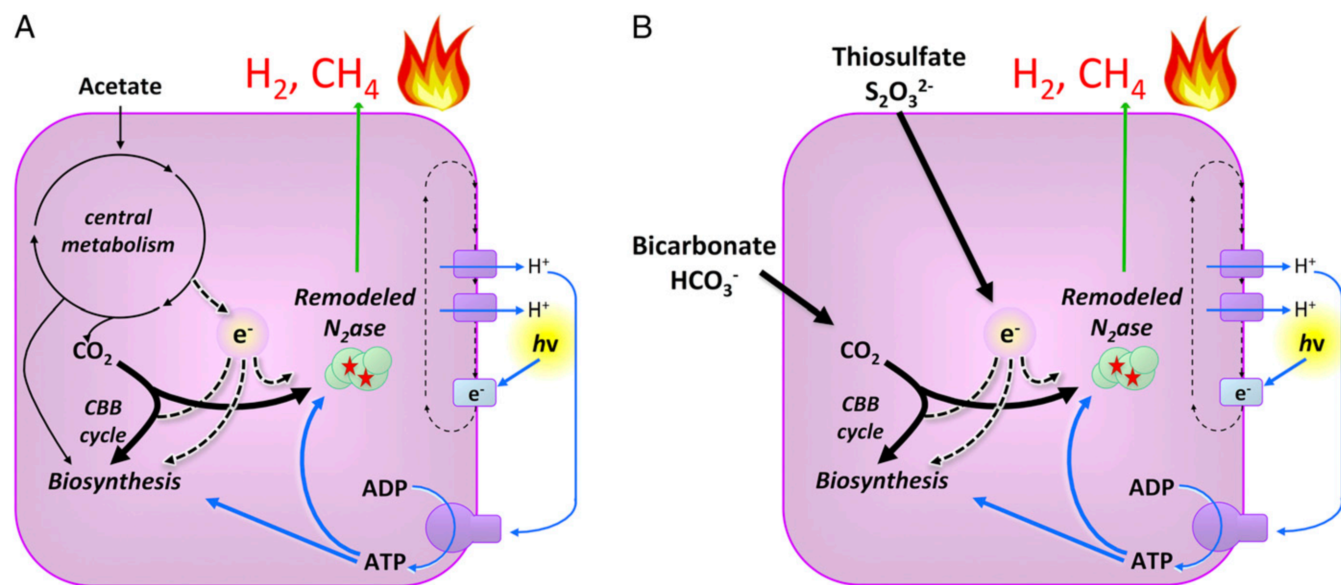

Fig. 3. Metabolic route of CH_4 production by an engineered strain of *R. palustris* expressing a remodeled nitrogenase. ATP is produced by cyclic photophosphorylation, in which electrons energized by light are cycled through a proton-pumping electron transport chain rather than transferred to a terminal electron acceptor. Electrons are generated by oxidation of (*A*) organic compounds or (*B*) inorganic compounds, such as thiosulfate. CO_2 from bicarbonate or generated from acetate oxidation is converted by remodeled nitrogenase to CH_4 and the CBB cycle to cell material.

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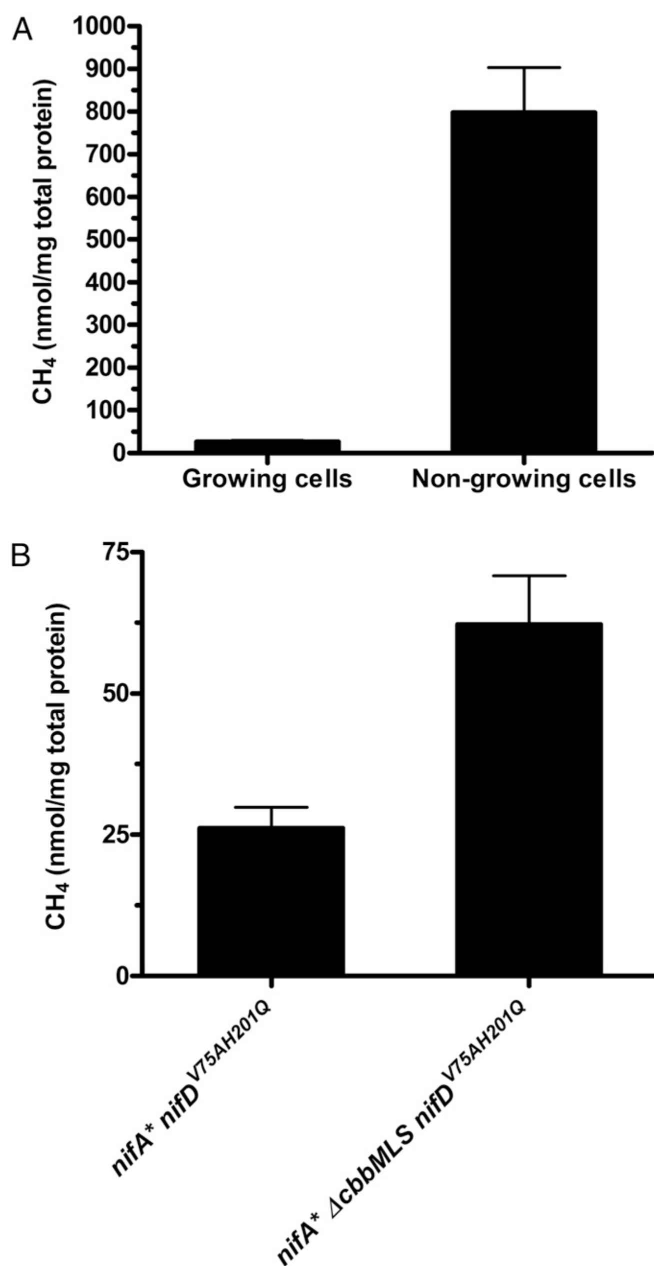

Fig. 4. Diverting more electrons to the remodeled nitrogenase results in more CH_4 production by *R. palustris* whole cells. Diversion of electrons away from (*A*) biosynthesis using nongrowing cells or (*B*) the CBB cycle through genetic mutation (Δ*cbbMLS*) results in more CH_4 production by *R. palustris* NifA* cells expressing a remodeled nitrogenase. These data are the average of three or more independent experiments, and the error bars represent SDs.

Future studies will focus on manipulating light, electron flux, and nitrogenase itself to improve CO_2 reduction to CH_4. *R. palustris* can also synthesize alternative V or Fe nitrogenase isozymes (9). These isozymes could allow for even greater expansion of the catalytic repertoire of nitrogenase and further our understanding of how to produce energy-rich hydrocarbons in one step.

Materials and Methods

Reagents, Bacteria, and Culture Methods. All reagents were obtained from Sigma-Aldrich or Fisher Scientific and used without additional purification. Gases (argon, dinitrogen, hydrogen, CH_4, ethane, C_2H_2, and C_2H_4) were purchased from Air Liquide or Gasco. Manipulation of proteins and buffers was done in septum-sealed serum vials under an argon atmosphere or on a Schlenk line. All gas transfers were made using gas-tight syringes. All *R. palustris* strains were grown on photosynthetic medium (PM) as previously described (5–7, 19). The parent *R. palustris* strain, CGA009, used in all experiments is defective in expression of its uptake hydrogenase enzyme, and thus, hydrogen production by this strain can be used as a direct measure of nitrogenase activity. All cultures were initially grown anaerobically with 30 μmol photons/m^2 per s from a 60-W incandescent light bulb (General Electric) and then, diluted into fresh PM medium. This light intensity supports the maximum growth rate of *R. palustris*. Where appropriate, organic acids and alcohols were added at a final concentration of 40 mM carbon, and $NaHCO_3$ was added at a final concentration of 10 mM. Light intensity was altered using a 15-W incandescent light bulb controlled by a dimmer switch to provide light intensities of 3 and 1 μmol photons/m^2 per second, and light intensity of 60 μmol photons/m^2 per second was obtained by using a 200-W incandescent light bulb. We generated suspensions of nongrowing cells by washing midexponential-phase cultures with nitrogen-free PM (NFM) and flushing the headspace with argon gas after degassing the medium. Additional culture methods and growth conditions can be found in *SI Materials and Methods*.

Genetic Manipulation of *R. palustris*. All strains and plasmids used are listed in Table S1. Plasmids were mobilized into *R. palustris* CGA676 or CGA679 by conjugation with *Escherichia coli* S17-1, and double–cross-over events for allelic exchange were achieved using a selection and screening strategy as described previously (5). Details of strain and plasmid construction for this study can be found in *SI Materials and Methods*.

Protein Purification. Cell extracts from *R. palustris* cells were prepared by using a French pressure cell operated at 1,500 lb/in^2 in a degassed 50 mM Tris·HCl buffer (pH 8.0) with 2 mM sodium dithionite under Ar. His-tagged MoFe proteins were purified by an immobilized metal affinity chelation chromatography protocol (20), with minor modifications. Protein concentrations were determined by the Biuret assay using BSA as the standard. The purities of these proteins were determined based on SDS/PAGE analysis with Coommasie staining.

CO_2 Reduction Assays. CO_2 reduction assays were conducted in 9.4-mL serum vials containing an assay buffer consisting of an MgATP regeneration system (15 mM $MgCl_2$, 90 mM phosphocreatine, 15 mM ATP, 0.6 mg/mL creatine phosphokinase, 1.2 mg/mL BSA) and 12 mM sodium dithionite in 100 mM 4-morpholinepropanesulfonic acid sodium salt (Mops) buffer at pH 7.0. After solutions were made anaerobic, 0.45 atm CO_2 was added, and the gas and liquid phases were allowed to equilibrate for ∼20 min. MoFe protein was then added, the vials were ventilated to atmospheric pressure, and the

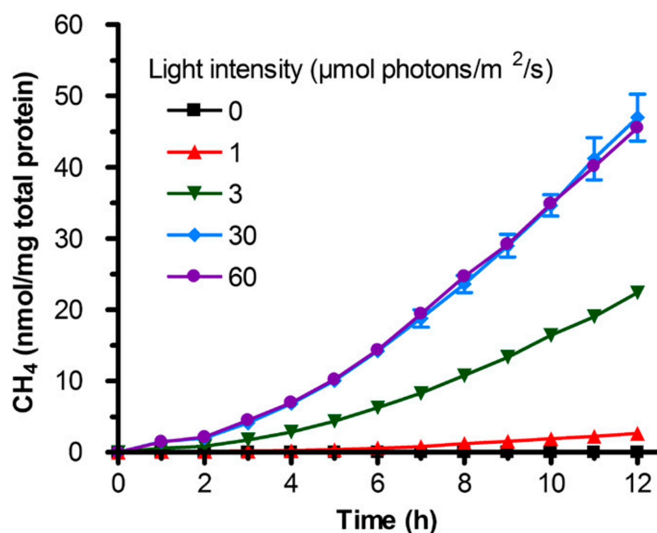

Fig. 5. CH_4 production by *R. palustris* NifA* cells expressing a remodeled nitrogenase is a light-driven process that can be controlled by light intensity. Altering the light intensity provided to cells alters the amount of CH_4 produced. These data are the average of three or more independent experiments, and the error bars represent SDs.

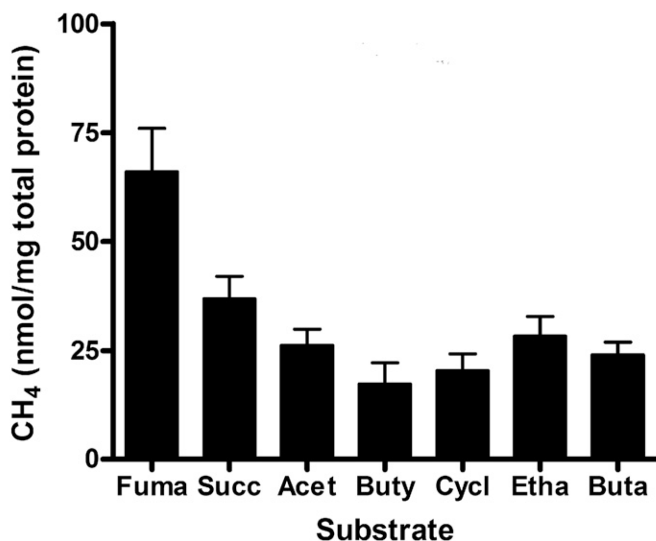

Fig. 6. CH_4 produced by *R. palustris* NifA* cells expressing remodeled nitrogenase is affected by the organic compound supplied as a growth substrate. CH_4 production by cells grown on different substrates. Organic acids and alcohols were added at a final concentration of 40 mM carbon. The data shown are the average of five or more independent experiments, and the error bars represent SDs. Acet, acetate; Buta, butanol; Buty, butyrate; Cycl, cyclohexane carboxylate; Etha, ethanol; Fuma, fumarate; Succ, succinate.

reaction was initiated by the addition of Fe protein. Reactions were conducted at 30 °C for 60 min and then quenched by the addition of 700 μL 400 mM EDTA (pH 8.0). Quantification of CH_4 was done according to a published protocol (3).

Dinitrogen, C_2H_2, and Proton Reduction Assays. Reduction assays were conducted in 9.4-mL serum vials containing an assay buffer consisting of an MgATP regeneration system (6.7 mM $MgCl_2$, 30 mM phosphocreatine, 5 mM ATP, 0.2 mg/mL creatine phosphokinase, 1.2 mg/mL BSA) and 12 mM sodium dithionite in 100 mM Mops buffer at pH 7.0. After solutions were made anaerobic, the headspace gases in the reaction vials were adjusted to proper partial pressures of different gases for different substrates (1 atm N_2 for N_2, 0.1 atm C_2H_2 and 0.9 atm Ar for C_2H_2 reduction, and 1 atm Ar for proton reduction). This step was followed by the addition of the MoFe protein. After the vials were ventilated to atmospheric pressure, the reactions were initiated by the addition of the Fe protein. Reactions were conducted at 30 °C for 8 min and then, quenched by the addition of 300 μL 400 mM EDTA (pH 8.0). The products (NH_3, H_2, and C_2H_4) from different substrate reduction assays were quantified according to published methods with minor modifications (3, 21, 22).

H_2 and CH_4 Measurements from *R. palustris* Cells. Gas-phase samples (50 μL) were withdrawn with a Hamilton sample lock syringe from the culture vial headspace at intervals, and hydrogen was measured with a Shimadzu GC-2014 Gas Chromatograph as previously described (5, 7, 12, 13). Total protein concentrations were determined using the Bio-Rad Protein Assay Kit. Ethane was used as an internal standard to quantify CH_4; 200 μL ethane (0.08% or 0.8%; 1 atm) was placed into a 27-mL anaerobic tube with 10 mL culture and then, shaken gently to distribute the gases uniformly. A 100-μL gas sample was taken from the headspace of the tube for gas chromatography - flame ionization (GC-FID) analysis using a Hamilton Sample Lock Syringe. The separation of CH_4 was performed using a stainless steel 80/100 Porapak N Column (1.8 m × 1/8 in × 2.1 mm i.d.) purchased from Sigma-Aldrich. The column temperature was held at 60 °C, and the injector and detector temperatures were set to 85 °C and 150 °C, respectively. Argon was used as the carrier gas at a rate of 35 mL/min.

GC-MS Analysis for in Vivo CO_2 Reduction to CH_4 by *R. palustris*. GC-MS analysis was conducted to confirm the production of CH_4 from CO_2 reduction using a Shimadzu GC-2010 Gas Chromatograph equipped with a programmed temperature vaporizing injector and a Shimadzu GCMS-QP2010S Mass Spectrometer by using $^{12/13}C$-enriched $NaHCO_3$ as the CO_2 source for *R. palustris* V75A/H201Q mutant. Additional details can be found in *SI Materials and Methods*.

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