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Symbiotic Legume Nodules Employ Both Rhizobial *Exo*- and *Endo*-Hydrogenases to Recycle Hydrogen Produced by Nitrogen Fixation

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Abstract

Background: In symbiotic legume nodules, endosymbiotic rhizobia (bacteroids) fix atmospheric N_2, an ATP-dependent catalytic process yielding stoichiometric ammonium and hydrogen gas (H_2). While in most legume nodules this H_2 is quantitatively evolved, which loss drains metabolic energy, certain bacteroid strains employ uptake hydrogenase activity and thus evolve little or no H_2. Rather, endogenous H_2 is efficiently respired at the expense of O_2, driving oxidative phosphorylation, recouping ATP used for H_2 production, and increasing the efficiency of symbiotic nodule N_2 fixation. In many ensuing investigations since its discovery as a physiological process, bacteroid uptake hydrogenase activity has been presumed a single entity.

Methodology/Principal Findings: *Azorhizobium caulinodans*, the nodule endosymbiont of *Sesbania rostrata* stems and roots, possesses both orthodox respiratory (*exo*-)hydrogenase and novel (*endo*-)hydrogenase activities. These two respiratory hydrogenases are structurally quite distinct and encoded by disparate, unlinked gene-sets. As shown here, in *S. rostrata* symbiotic nodules, haploid *A. caulinodans* bacteroids carrying single knockout alleles in either *exo*- or-*endo*-hydrogenase structural genes, like the wild-type parent, evolve no detectable H_2 and thus are fully competent for endogenous H_2 recycling. Whereas, nodules formed with *A. caulinodans exo*-, *endo*-hydrogenase double-mutants evolve endogenous H_2 quantitatively and thus suffer complete loss of H_2 recycling capability. More generally, from bioinformatic analyses, diazotrophic microaerophiles, including rhizobia, which respire H_2 may carry both *exo*- and *endo*-hydrogenase gene-sets.

Conclusions/Significance: In symbiotic *S. rostrata* nodules, *A. caulinodans* bacteroids can use either respiratory hydrogenase to recycle endogenous H_2 produced by N_2 fixation. Thus, H_2 recycling by symbiotic legume nodules may involve multiple respiratory hydrogenases.

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Introduction

Legume root and stem nodules fix atmospheric dinitrogen (N_2) yielding anabolic-N, which augments growth and reproduction of host plants. In these nodules, the biochemical conversion of N_2 to ammonium is owed to endosymbiotic rhizobia (bacteroids) who carry the N_2 fixation genes encoding the dinitrogenase complex. Whether N_2 fixation occurs in legume nodules [1] or in pure cultures of diazotrophic (able to use N_2 as sole N-source) bacteria [2], hydrogen gas (H_2) is then co-produced. From subsequent mechanistic studies of dinitrogenase activity, H_2 co-production is both stoichiometric and requires 2 ATP per H_2 formed [3–4]. Yet in agronomic surveys, many legume nodules typically evolve H_2 at high levels, and such H_2 evolution rates correlate with N_2 fixation rates [5]. However, in certain symbiotic legume nodules, bacteroids avidly fix N_2 yet reproducibly evolve little or no H_2 [1]. As this endogenous H_2 production consumes metabolic energy, H_2 recycling, which recoups that energy, allows increased efficiency of N_2 fixation and, in principle, increased plant biomass yields [6–7]. This symbiotic nodule H_2 recycling capability correlates with specific bacteroid strains, although host legume cultivars also contribute to H_2 recycling and yield [8,9]. Indeed, in biochemical assays, bacteroids isolated from H_2 recycling (non-evolving) nodules show high levels of respiratory uptake hydrogenase activity [10,11].

H_2 recycling during N_2 fixation was first observed with the aerobe *Azotobacter vinelandii*, a diazotroph but not a legume symbiont. In pure culture, *A. vinelandii* induces a particulate (respiratory) hydrogenase activity which oxidizes H_2 at the expense of and tolerant of O_2 [2]. In following studies with legume nodule bacteroids, such uptake hydrogenase activity was also affirmed [10]. In the ensuing forty years, hydrogenases, extensively studied, have proven both biochemically diverse and broadly distributed across bacteria and archaea [12]. Among aerobes and microaerophiles able to use H_2 as energy source, uptake hydrogenase activities are typically classified as group I: heterodimeric,

globular, hydrophilic proteins carrying a heteronuclear Ni,Fe-catalytic center; group I hydrogenases are generally O_2 tolerant [12]. In cellular terms, the group I, Ni,Fe uptake hydrogenases are tightly associated with respiratory membranes via integral diheme *b*-type cytochromes, required for physiological activity [13,14] [Bernhard]. As the group I cell membrane-peripheral complexes face the periplasm, or cell exterior [15], they may be termed *exo*-hydrogenases.

Azorhizobium caulinodans, a microaerophilic α-*proteobacterium* originally isolated as nodule endosymbiont of the host legume *Sesbania rostrata*, is capable of N_2 fixation both *in planta* and in pure diazotrophic culture [16]. Recently, we discovered in *A. caulinodans* a second, novel respiratory hydrogenase encoded by the seven-gene *hyq* operon [17]. The inferred Hyq hydrogenase includes six different structural proteins, including a heterodimeric Ni,Fe-catalytic center hydrogenase conserved with group I enzymes. From bioinformatic analyses, the remaining four Hyq proteins are all membrane-integral [17]. Because all six Hyq hydrogenase subunits are NADH:quinone oxidoreductase (respiratory complex I) homologs [18], the Hyq complex is classified with the reversible group IV hydrogenases [12]. Given structural and functional homology to respiratory complex I [18], the Ni,Fe-catalytic center heterodimers of group IV complexes associated with respiratory membranes presumably face the cell-interior and thus may be termed *endo*-hydrogenases.

Results

In symbiotic legume nodules both *exo*- and *endo*-hydrogenases recycle H_2 produced by N_2 fixation

To assess physiological roles for both bacteroid Hup *exo*- and Hyq *endo*-hydrogenases in symbiotic legume nodules fixing N_2 and recycling H_2, *A. caulinodans* haploid derivatives carrying precise (to the nucleotide pair) in-frame deletions of *hup* and *hyq* structural genes encoding the conserved catalytic subunits of, respectively, *exo*- and *endo*-hydrogenases were constructed and verified by nucleotide sequencing of mutant loci [17]. Specifically, *A. caulinodans exo*-hydrogenase null mutants carried in-frame, precise, complete *hupSL* deletions; *endo*-hydrogenase null mutants comprised precise, complete *hyqRBCEFGI* operon deletions. As well, haploid recombinant double-mutants carrying both *exo*- and *endo*-hydrogenase null alleles were also constructed (Methods). Pure *A. caulinodans* cultures were used to inoculate both stems and roots of *S. rostrata* seedlings aseptically germinated and individually cultivated under N-limitation (Methods). In *S. rostrata*, symbiotic nodules are developmentally determinate, not meristematic. While both stem- and root-nodules subsequently developed on inoculated plants only, as they are invariably absent on uninoculated plants, stem nodules were chosen for further study. Three week-old and five week-old determinate stem nodules were excised from inoculated plants and individually tested for N_2 fixation activity, assaying acetylene-dependent ethylene production by gas chromatography with flame ionization detection (Methods). Excised stem nodules all showed similar (±15%) high levels of acetylene reduction activity when normalized per fresh nodule biomass (Table 1). Accordingly, all *A. caulinodans* strains tested were assigned both nodulation-competent (Nod^+) and N_2 fixation-competent (Fix^+) phenotypes.

Additional excised nodules from these *S. rostrata* plants were simultaneously tested under air for H_2 evolution activity using gas chromatography coupled to a reducing-compound photometric detector (Methods). In kinetic studies with excised nodules elicited by *A. caulinodans* strains 61305R (parental), 66081 (*exo*-hydrogenase mutant) and 66132 (*endo*-hydrogenase mutant), H_2 evolution was nonexistent (Figs. 1a,b). Whereas, nodules elicited by double (*exo*- and *endo*-hydrogenase) mutant 66204 evolved H_2 at very high rates (Fig. 1a) comparable to those measured for acetylene reduction (Table 1). Thus, H_2 evolution by double-mutant 66204-elicited nodules was quantitatively owed to N_2 fixation (dinitrogenase) activity. Results with five week-old determinate nodules from additional *S. rostrata* plants entirely corroborated results with three week-old nodules (data not presented). Pure bacterial cultures were reestablished from aseptically crushed nodules and strain identities verified by nucleotide sequencing of *hup* and *hyq* loci (Methods). In conclusion, *A. caulinodans* bacteroids in *S. rostrata* nodules employ both *exo*- and *endo*-hydrogenases to recycle endogenous H_2 produced by N_2 fixation. Moreover, H_2 recycling is quantitative, entirely accounting for N_2 fixation activities. Yet as measured by H_2 evolution rates, bacteroid *exo*- and *endo*-hydrogenase are interchangeable and individually are fully competent to handle endogenous H_2 recycling in symbiotic *S. rostrata* nodules.

N_2 fixing, microaerophilic α-proteobacteria able to recycle H_2 carry *exo*- and *endo*-hydrogenase gene-sets

From bioinformatic analyses (Table 2), orthologous hyq^+ operons encoding *endo*-hydrogenase are generally present in N_2 fixing microaerophiles able to recycle endogenous H_2. These strains include both free-living diazotrophs as well as certain rhizobia, such as *B. japonicum*, the endosymbiont of *Glycine max* (soy). In *Rhizobium leguminosarum*, a metastable species with several descendant biovars each with genomes comprised of variable multipartite replicons, H_2 recycling capability in symbiotic legume nodules varies among strains. As well, both the hup^+/hyp^+ (*exo*-hydrogenase) and the hyq^+ (*endo*-hydrogenase) gene-sets are also variables [9,19,20]. Yet other diverse rhizobia (*e.g. Sinorhizobium meliloti* 1021; *Mesorhizobium loti* MAFF303099; *Rhizobium etli* CFN42; *Rhizobium sp.* NGR234) all incapable of H_2 recycling in symbiotic legume nodules, completely lack both hup^+/hyp^+ and

Table 1. N_2 fixation and H_2 recycling in *S. rostrata–A. caulinodans* stem nodules.

A. caulinodans endosymbiont	Genotype	Phenotype	N_2 fixation[†]	H_2 evolved[‡]
61305R	57100 *nic5R*	(virtual) wild-type	31.0±0.4	0.30±0.05
66081	61305R *ΔhupSL2*	*exo*-hydrogenase negative	33.0±0.4	0.32±0.05
66132	61305R *ΔhyqRI7*	*endo*-hydrogenase negative	25.0±0.3	0.36±0.05
66204	61305R *ΔhupSL2 ΔhyqRI7*	*exo*- and *endo*-hydrogenase neg.	25.0±0.3	27.0±0.3

[†]μmol (C_2H_2-dependent)C_2H_4 g^{-1} hr^{-1}
[‡]μmol H_2 g^{-1} hr^{-1}
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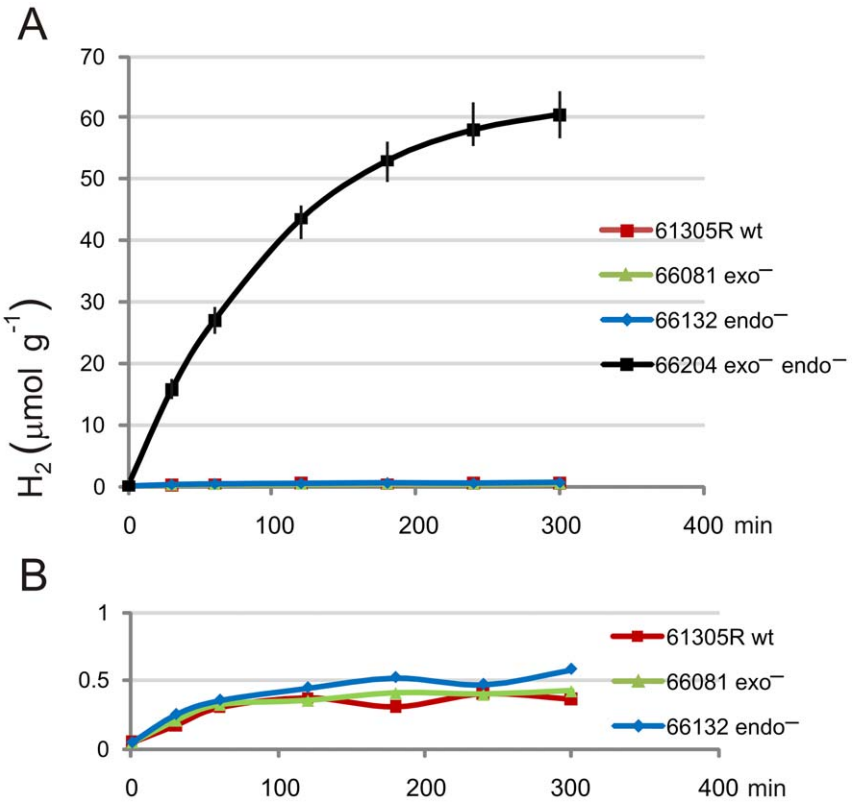

Figure 1. Hydrogen (H_2) evolution by excised *S. rostrata* stem nodules elicited by indicated *A. caulinodans* strains as endosymbiont. (A) 70 µmol scale; (B) expanded ordinate, 1 µmol scale; evolved H_2 measured as µmol g^{-1} (fresh biomass).
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hyq$^+$ gene-sets (Table 2). As the *hyq*$^+$ operon is also absent from anaerobic (fermentative) diazotrophs, fully aerobic diazotrophs (*e.g. Azotobacter spp.*), and non-diazotrophs generally, Hyq *endo*-hydrogenase seems co-selected with N_2 fixation in microaerophilic (non-fermentative) α-proteobacteria. Nevertheless, in every N_2 fixing microaerophile with both *exo*- and *endo*-hydrogenases, these gene-sets, as well the *nif* genes encoding N_2 fixation activities are all unlinked (Table 2). Moreover, *A. caulinodans* haploid strains carrying complete (20-gene) *hup*$^+$/*hyp*$^+$ (including *hupSL*$^+$) operon deletions entirely lacking *exo*-hydrogenase and ancillary activities, nevertheless retain full H_2 recycling activity both in pure cultures and in *S. rostrata* stem nodules. As well, *Rhodocista centenaria* (aka *Rhodospirillum centenum*) SW, which possesses the *hyq*$^+$ operon but not the *hup*$^+$/*hyp*$^+$ operon (Table 2), completely recycles H_2 in

Table 2. N_2-fixing microaerophilic α-proteobacteria carrying orthologous *hup*$^+$/*hyp*$^+$ (*exo*-hydrogenase) and *hyq*$^+$ (*endo*-hydrogenase) genes.

H_2 recycling proficient:	**legume host**	**diazotrophy**	***hupSL*$^+$*genes***	***hyq*$^+$ *operon***
Azorhizobium caulinodans ORS571	*Sesbania rostrata*	+	AZC0598-0599	AZC4361-4355
Beijerinckia indica ATCC 9039	–	+	BIND1150-1151	BIND2473-2479
Bradyrhizobium japonicum USDA110	*Glycine max*	–	BLR1720-1721	BLR6338-6344
Rhodocista centenaria SW	–	+	–	RC11420-1415
Rhodopseudomonas palustris BisB5	–	+	RPD1162-1163	RPD3855-3850
Xanthobacter autotrophicus PY2	–	+	XAUT2173-2174	XAUT0165-0171
H_2 recycling deficient:				
Sinorhizobium meliloti 1021	*Medicago sativa*	–	–	–
Mesorhizobium loti MAFF303099	*Lotus japonicus*	–	–	–
Rhizobium etli CFN42	*Phaseolus vulgaris*	–	–	–
Rhizobium sp. NGR234	*Vigna unguiculata*	–	–	–

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diazotrophic culture (data not presented). Accordingly, these *exo*- and *endo*-hydrogenase gene-sets seem fully autonomous.

Discussion

Among legume-*Rhizobium* symbioses, H_2 recycling was first reported in *Pisum sativum* (garden pea) nodules elicited by specific *Rhizobium leguminosarum* bv. *viciae* strains [2]. Genetic studies were subsequently undertaken with [*Brady*]*Rhizobium japonicum* strains able to recycle H_2 in *Glycine max* (soy) nodules [21,22]. Many subsequent studies with H_2 recycling legume nodules all presumed uptake hydrogenase activity a single entity. These studies include combined genetic and physiological analyses which might have challenged this assertion. For the case of *A. caulinodans*, single mutants W58, U58 as well as *hupSL* impaired strain ORS571.2 all were reported to suffer substantial to complete loss of uptake hydrogenase activity [23,24,25]. Such conclusions are incompatible with the present finding: *A. caulinodans* employs two structurally and functionally distinct, genetically-independent, respiratory hydrogenases to recycle endogenous H_2 produced by N_2 fixation.

Whereas, early on the investigative timeline, *B. japonicum* single mutants unable to be cultured autotrophically on exogenous H_2 yet still able to recycle endogenous H_2 in soy nodules were identified [26]. As these strains showed induction of uptake hydrogenase activity in cultures shifted to O_2 limitation ($\leq$11 μM DOT), they were perhaps understandably considered transcriptional control mutants hypersensitive to O_2. With the benefit of hindsight, this phenotype is precisely that expected of true loss-of-function point mutants affecting *hup* operon structural genes encoding Hup *exo*-hydrogenase activity, were the observed limiting-DOT uptake hydrogenase activity in fact owed to Hyq *endo*-hydrogenase. In *A. caulinodans*, *hyq* operon expression requires NifA as transactivator [17], and the p*nifA*$^+$ promoter is in turn strongly transactivated by Fnr, which process requires physiological O_2 limitation in diazotrophic culture [27]. In principle, both *exo*- and *endo*-hydrogenase gene-sets, despite being encoded at disparate loci in all organisms identified, might nevertheless share a common genetic predisposition, allowing strategic single mutations to convey dual loss-of-function. However, as strains carrying complete *hyq* operon deletions still possess wild-type Hup *exo*-hydrogenase activity, and *vice versa*, evidence for any genetic, post-transcriptional interaction or interdependence between the two gene-sets is entirely lacking.

As shown previously, in pure diazotrophic (N_2 as sole N-source) cultures, *A. caulinodans exo*-hydrogenase knockout mutants grow as wild-type, whereas *endo*-hydrogenase knockout mutants exhibit slow growth [17]. Are *exo*- and *endo*-hydrogenase H_2 recycling efficiencies in pure culture and in legume nodules then demonstrably different? Or, do diazotrophic phenotypes imply additional *endo*-hydrogenase function(s), *e.g.* chemiosmotic work associated with membrane ion translocation [28] not undertaken by *exo*-hydrogenase? Obviously, effective *exo*- and *endo*-hydrogenase cellular concentrations and/or distributions might be dissimilar in legume nodules and in pure diazotrophic cultures, even though both *hup*$^+$/*hyp*$^+$ (*exo*-hydrogenase) and *hyq*$^+$ (*endo*-hydrogenase) gene-sets are then strongly transcribed [17,25,29]. Because *hup* mutants suffer loss of chemoautotrophy with exogenous H_2 as energy substrate [17,26], *exo*-hydrogenase kinetic behavior may constitute simple diffusion control. Because *hyq* mutants do not adversely impact chemoautotrophy with exogenous H_2, *endo*-hydrogenase kinetic behavior might not constitute simple diffusion control. A critical test of this hypothesis is still lacking. Diazotrophic liquid batch cultures typically employ constant sparging with relatively high gas-phase exhaust rates (0.5 min^{-1}), complicating kinetic behavior and analysis of cellular processes with gaseous substrate(s) subject to simple diffusion control. In such pure liquid diazotrophic batch cultures bacterial densities typically reach 10^8 cc^{-1}, whereas in determinate *S. rostrata* nodules, bacteroid densities approach 10^{11} cc^{-1}, the latter obviously more conducive to endogenous H_2 recycling under simple diffusion control. Notwithstanding, given their apparent co-selection in N_2 fixing micoaerophilic α-proteobacteria capable of H_2 recycling, *exo*- and *endo*-hydrogenases likely possess additional, distinctive functionalities yet to be elucidated.

Methods

Bacterial strains and culture conditions

Azorhizobium caulinodans ORS571 wild-type (strain 57100), originally isolated from *Sesbania rostrata* stem-nodules [16], was cultured as previously described [30]. As 57100 wild-type is a pyridine nucleotide auxotroph, to serve as 'virtual' wild-type, all experiments reported here employ *A. caulinodans* 61305R, a 57100 derivative carrying an IS*50*R insertion in the (catabolic) nicotinate hydroxylase structural gene. Precise, in-frame deletion mutants were constructed by a 'crossover PCR' method [31]. Haploid *exo*-hydrogenase knockout mutants each carry a *hup*Δ*SL2* allele in which the (upstream) *hupS* translation initiation codon is fused in-frame to a synthetic 21np linker sequence fused in-frame to the (downstream) *hupL* termination codon. Similarly, haploid *endo*-hydrogenase mutants each carry a *hyq*Δ*RI7* allele, in which the *hyqRBCEFGI* operon has been replaced by a deletion allele comprising the *hyqR* initiation codon fused in-frame to the 21np linker sequence fused in-frame to the *hyqI* termination codon. After gene replacement, haploid strains carrying deletion alleles were verified by PCR and DNA sequencing analyses [17].

Sesbania rostrata nodulation tests

S. rostrata plants were germinated, cultivated aseptically, and stem inoculated with pure *A. caulinodans* strain cultures as described [16]. Either three or five weeks post-inoculation, stem nodules were detached, weighed, individually placed in septated vials. Dinitrogenase activity was assayed kinetically by acetylene reduction [32] and product ethylene was measured by gas chromatography with flame-ionization detection. H_2 evolution was assayed kinetically and measured by gas chromatography with reducing compound photometer detection (RCP1; Peak Laboratories LLC, Mountain View, CA.), both at atmospheric pressure and 29°C [33]. Enzymatic activities are expressed per gram nodule fresh-biomass at 29°C.

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Author Contributions

Conceived and designed the experiments: RL. Performed the experiments: CC NR JM. Analyzed the data: CC NR JM DC RL. Contributed reagents/materials/analysis tools: RL. Wrote the paper: RL.

References

1. Phelps AS, Wilson PW (1941) Occurrence of hydrogenase in nitrogen-fixing organisms. Proc Soc Exp Biol 47: 473–476.

2. Hyndman LA, Burris RH, Wilson PW (1953) Properties of hydrogenase from *Azotobacter vinelandii*. J Bacteriol 65: 522–531.

3. Thorneley RNF, Lowe DJ (1985) in *Molybdenum Enzymes*; Spiro TG, ed. New York: Wiley-Interscience. pp 221–284.
4. Burgess BK, Lowe DJ (1996) Mechanism of molybdenum nitrogenase. Chem Rev 96: 2983–3011.
5. Schubert KR, Evans HJ (1976) Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc Natl Acad Sci USA 73: 1207–1211.
6. Albrecht SL, Maier RJ, Hanus FJ, Russell SA, Emerich DW, et al. (1979) Hydrogenase in *Rhizobium japonicum* increases nitrogen fixation by nodulated soybeans. Science 203: 1255–1257.
7. Eisbrenner G, Evans HJ (1983) Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Ann Rev Plant Physiol 34: 105–136.
8. Bedmar EJ, Phillips DA (1984) *Pisum sativum* cultivar effects on hydrogen metabolism in *Rhizobium*. Can J Bot 62: 1682–1686.
9. López M, Carbonero V, Cabrera E, Ruiz-Argüeso T (1983) Effects of host on the expression of the H_2-uptake hydrogenase of *Rhizobium* in legume nodules. Plant Sci Lett 9: 191–199.
10. Dixon ROD (1972) Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch Mikrobiol 85: 193–201.
11. Dixon ROD (1968) Hydrogenase in pea root nodule bacteroids. Arch Mikrobiol 62: 272–283.
12. Vignais PM, Billoud B, Meyer J (2001) Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 25: 455–501.
13. Cauvin B, Colbeau C, Vignais PM (1991) The hydrogenase structural operon in *Rhodobacter capsulatus* contains a third gene, *hupM*, necessary for the formation of a physiologically competent hydrogenase. Mol Microbiol 5: 2519–2527.
14. Bernhard M, Benelli B, Hochkoeppler A, Zannoni D, Friedrich B (2004) Functional and structural role of the cytochrome *b* subunit of the membrane-bound hydrogenase complex of *Alcaligenes eutrophus*. Eur J Biochem 16: 179–186.
15. Burgdorf T, Lenz O, Buhrke T, van der Linden E, Jones AK, et al. (2005) [NiFe]-hydrogenases of *Ralstonia eutropha* H16: modular enzymes for oxygen-tolerant biological hydrogen oxidation. J Mol Microbiol Biotechnol 10: 181–196.
16. Dreyfus BL, Dommergues YR (1981) Nitrogen fixing nodules induced by *Rhizobium* on strains of the tropical legume *Sesbania rostrata*. FEMS Microbiol Lett 10: 313–317.
17. Ng G, Tom CGS, Park AS, Zenad L, Ludwig RA (2009) A novel *endo*-hydrogenase activity recycles hydrogen produced by nitrogen fixation. PLoS ONE 4(3): e4695. doi:101371/journalpone0004695.
18. Sazanov LA, Hinchcliffe P (2006) Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science 311: 1430–1436 (2006).
19. Ruiz-Argüeso T, Hanus J, Evans HJ (1978) Hydrogen production and uptake by pea nodules as affected by strains of Rhizobium leguminosarum. Arch Microbiol 116: 113–118.
20. Nelson LM, Grosskopf E, Tichy HV, Lotz W (1985) Characterization of *hup*-specific DNA in *Rhizobium leguminosarum* strains of different origin. FEMS Microbiol Lett 30: 53–58.
21. Lepo JE, Hickok RE, Cantrell MA, Russell SA, Evans HJ (1981) Revertible hydrogen uptake deficient mutants of *Rhizobium japonicum*. J Bacteriol 146: 614–620.
22. Drevon JJ, Frazier L, Russell SA, Evans HJ (1982) Respiratory and nitrogenase activities of soybean nodules formed by hydrogen uptake negative (Hup^-) mutant and revertant strains of [Brady]*Rhizobium japonicum* characterized by protein patterns. Plant Physiol 70: 1341–1346.
23. De Vries W, Ras J, Stam H, van Vlerken MMA, Hilgert U, et al. (1988) Isolation and characterization of hydrogenase-negative mutants of *Azorhizobium caulinodans* ORS571. Arch Microbiol 150: 595–599.
24. Boogerd FC, Ferdinandy-Van Vlerken M, Mawadza C, Pronk AF, et al. (1994) Nitrogen fixation and hydrogen metabolism in relation to the dissolved oxygen tension in chemostat cultures of the wild type and a hydrogenase-negative mutant of *Azorhizobium caulinodans*. App Env Microbiol 60: 1859–1864.
25. Baginsky C, Palacios JM, Imperial J, Ruiz-Argüeso T, Brito B (2004) Molecular and functional characterization of the *Azorhizobium caulinodans* ORS571 hydrogenase gene cluster. FEMS Microbiol Lett 237: 399–405.
26. Maier RJ, Merberg DM (1982) [Brady]*Rhizobium japonicum* mutants that are hypersensitive to repression of H_2 uptake by oxygen. J Bacteriol 150: 161–167.
27. Loroch AI, Nguyen B, Ludwig RA (1995) FixLJK and NtrBC signals interactively regulate *Azorhizobium nifA* transcription via overlapping promoters. J Bacteriol 177: 7210–7221.
28. Ohnishi T, Salerno JC (2005) Conformation-driven and semiquinone-gated proton-pump mechanism in the NADH-ubiquinone oxidoreductase (complex I). FEBS Lett 579: 4555–4561.
29. Brito B, Martinez M, Fernandez M, Rey L, Babrera E, et al. (1997) Hydrogenase genes from *Rhizobium leguminosarum* bv *viciae* are controlled by the nitrogen fixation regulatory protein NifA. Proc Natl Acad Sci USA 94: 6019–6024.
30. Donald RGK, Nees D, Raymond CK, Loroch AI, Ludwig RA (1986) Three genomic loci encode [Azo]*Rhizobium sp* ORS571 N_2 fixation genes. J Bacteriol 165: 72–81.
31. Link AJ, Phillips D, Church GM (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol 179: 6228–6237.
32. Burris RH, Arp DJ, Benson DR, Emerich DW, Hageman RV, et al. (1980) The biochemistry of nitrogenase, *in* Stewart WDP, Gallon JR, eds. *Nitrogen Fixation*, London: Academic Press. pp 37–54.
33. Vreman HJ, Mahoney JJ, Van Kessel AL, Stevenson DK (1988) Carboxyhemoglobin as measured by gas chromatography and with the IL282 and 482CO-oximeters. Clin Chem 34: 2562–2566.