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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.

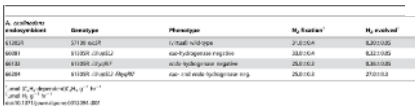

Table 1. N_2 fixation and H_2 recycling in *S. rostrata–A. caulinodans* stem nodules.
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Additional excised nodules from these *S. rostrata* plants were simultaneously tested under air for H_2 evolution activity using gas chromatography coupled to a reducing-compound 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.

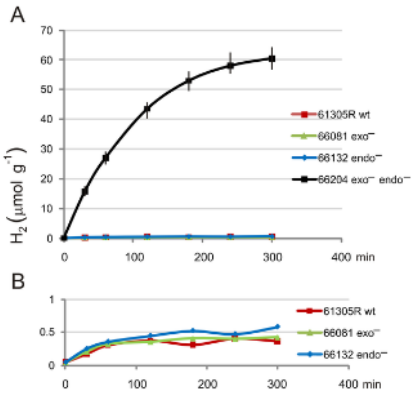

Figure 1. Hydrogen (H_2) evolution by excised *S. rostrata* stem nodules elicited by indicated *A. caulinodans* strains as endosymbiont.
(A) 70 µmol scale; (B) expanded ordinate, 1 µmol scale; evolved H_2 measured as µmol g^{-1} (fresh biomass).
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N_2 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 *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 diazotrophic culture (data not presented). Accordingly, these *exo*- and *endo*-hydrogenase gene-sets seem fully autonomous.

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*	–	–	–

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

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 (≤11 µ*M* 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.

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