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

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Abstract

Introduction

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

Background

In symbiotic legume nodules, endosymbiotic rhizobia (bacteroids) fix atmospheric N\textsubscript{2}, an ATP-dependent catalytic process yielding stoichiometric ammonium and hydrogen gas (H\textsubscript{2}). While in most legume nodules this H\textsubscript{2} is quantitatively evolved, which loss drains metabolic energy, certain bacteroid strains employ uptake hydrogenase activity and thus evolve little or no H\textsubscript{2}. Rather, endogenous H\textsubscript{2} is efficiently respired at the expense of O\textsubscript{2}, driving oxidative phosphorylation, recouping ATP used for H\textsubscript{2} production, and increasing the efficiency of symbiotic nodule N\textsubscript{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\textsubscript{2} and thus are fully competent for endogenous H\textsubscript{2} recycling. Whereas, nodules formed with A. caulinodans exo-, endo-hydrogenase double-mutants evolve endogenous H\textsubscript{2} quantitatively and thus suffer complete loss of H\textsubscript{2} recycling capability. More generally, from bioinformatic analyses, diazotrophic microaerophiles, including rhizobia, which respire H\textsubscript{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\textsubscript{2} produced by N\textsubscript{2} fixation. Thus, H\textsubscript{2} recycling by symbiotic legume nodules may involve multiple respiratory hydrogenases.
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$_2$ [22]. 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 II 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 a 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 asceptically 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 nodule-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.
From bioinformatic analyses (Table 2), orthologous hyq* operons encoding endo-hydrogenase are generally present in N₂ fixing microaerophiles able to recycle endogenous H₂. 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, H₂ 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 H₂ 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 N₂ fixation in microaerophilic (non-fermentative) α-proteobacteria. Nevertheless, in every N₂ fixing microaerophile with both exo- and endo-hydrogenases, these gene-sets, as well as the nif genes encoding N₂ 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 H₂ 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 H₂ in diazotrophic culture (data not presented). Accordingly, these exo- and endo-hydrogenase gene-sets seem fully autonomous.

<table>
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<th>H₂ recycling proficient:</th>
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<th>diazotrophy</th>
<th>hupSL*genes</th>
<th>hyq* operon</th>
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<td>A2C4361-4355</td>
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<td>–</td>
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<td>–</td>
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<tr>
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Table 2. N₂-fixing microaerophilic α-proteobacteria carrying orthologous hup*/hyp* (exo-hydrogenase) and hyq* (endo-hydrogenase) genes.

Discussion

Among legume-Rhizobium symbioses, H₂ 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 H₂ in Glycine max (soy) nodules [21], [22]. Many subsequent studies with H₂ recycling legume nodules all presumed uptake hydrogenase activity as 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 H₂ produced by N₂ fixation.

Whereas, early on the investigative timeline, B. japonicum single mutants unable to be cultured autotrophically on endogenous H₂ yet still able to recycle endogenous H₂ in soy nodules were identified [26]. As these strains showed induction of uptake hydrogenase activity in cultures shifted to O₂ limitation (≤11 μM DOT), they were perhaps understandably considered transcriptional control mutants hypersensitive to O₂. 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 Hyg endo-hydrogenase. In A. cauliformans, hyq operon expression requires NifA as transactivator [17], and the pniA* promoter is in turn strongly transactivated by Fnr, which process requires physiological O2 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 (N2 as sole N-source) cultures, A. cauliformans exo-hydrogenase knockout mutants grow as wild-type, whereas endo-hydrogenase knockout mutants exhibit slow growth [17]. Are exo- and endo-hydrogenase H2 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*/hup* (exo-hydrogenase) and hyq* (endo-hydrogenase) gene-sets are then strongly transcribed [17], [25], [29]. Because hup mutants suffer loss of chemoaotrophy with exogenous H2 as energy substrate [17], [26], exo-hydrogenase kinetic behavior may constitute simple diffusion control. Because hyq mutants do not adversely impact chemoaotrophy with exogenous H2, 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 mm−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 108 cc−1, whereas in determine S. rostrata nodules, bacteroid densities approach 1011 cc−1, the latter obviously more conducive to endogenous H2 recycling under simple diffusion control. Notwithstanding, given their apparent co-selection in N2 fixing micoaerophilic α-proteobacteria capable of H2 recycling, exo- and endo-hydrogenases likely possess additional, distinctive functionalities yet to be elucidated.

Methods

Bacterial strains and culture conditions

Azorhizobium cauliformans 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. cauliformans 61305R, a 57100 derivative carrying an is50R 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 hupAS2 allele in which the (upstream) hupS translation initiation codon is fused in-frame to a synthetic 21bp linker sequence fused in-frame to the (downstream) hupL termination codon. Similarly, haploid endo-hydrogenase mutants each carry a hyqAR17 allele, in which the hyqRBCFEGI operon has been replaced by a deletion allele comprising the hyqR initiation codon fused in-frame to the 21bp 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. cauliformans 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. H2 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


