A Novel Endo-Hydrogenase Activity Recycles Hydrogen Produced by Nitrogen Fixation

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Abstract

Background: Nitrogen (N₂) fixation also yields hydrogen (H₂) at 1:1 stoichiometric amounts. In aerobic diazotrophic (able to grow on N₂ as sole N-source) bacteria, orthodox respiratory hupSL-encoded hydrogenase activity, associated with the cell membrane but facing the periplasm (exo-hydrogenase), has nevertheless been presumed responsible for recycling such endogenous hydrogen.

Methods and Findings: As shown here, for Azorhizobium caulinodans diazotrophic cultures open to the atmosphere, exo-hydrogenase activity is of no consequence to hydrogen recycling. In a bioinformatic analysis, a novel seven-gene A. caulinodans hyq operon was identified. By analogy, Hyq hydrogenase is also integral to the cell membrane, but its active site faces the cytoplasm (endo-hydrogenase). An A. caulinodans in-frame hyq operon deletion mutant, constructed by “crossover PCR”, showed markedly decreased growth rates in diazotrophic cultures; normal growth was restored with added ammonium—as expected of an H₂-recycling mutant phenotype. Using A. caulinodans hyq merodiploid strains expressing β-glucuronidase as promoter-reporter, the hyq operon proved strongly and specifically induced in diazotrophic culture; as well, hyq operon induction required the NIFA transcriptional activator. Therefore, the hyq operon is constitutive of the nif regulon.

Conclusions: Representative of aerobic N₂-fixing and H₂-recycling α-proteobacteria, A. caulinodans possesses two respiratory Ni,Fe-hydrogenases: HupSL exo-hydrogenase activity drives exogenous H₂ respiration, and Hyq endo-hydrogenase activity recycles endogenous H₂, specifically that produced by N₂ fixation. To benefit human civilization, H₂ has generated considerable interest as potential renewable energy source as its makings are ubiquitous and its combustion yields no greenhouse gases. As such, the reversible, group-4 Ni,Fe-hydrogenases, such as the A. caulinodans Hyq endo-hydrogenase, offer promise as biocatalytic agents for H₂ production and/or consumption.

Introduction

Azorhizobium caulinodans is an obligate oxidative, microaerophilic bacterium originally isolated from stem- and root-nodules of the legume host plant Sesbania rostrata [1]. In legume nodules, endosymbiotic rhizobia, including A. caulinodans, fix atmospheric dinitrogen (N₂) yielding ammonium as utilizable N-source for the host plant. Unlike typical rhizobia which fix N₂ only endosymbiotically, A. caulinodans is also diazotrophic (able to grow on N₂ as N-source in pure culture). Both processes are owed to molybdenum-containing (Mo) dinitrogenase, an α₂β₂-tetrameric protein complex catalyzing directed electron-transfer. Metabolic electrons are tapped from pyruvate oxidation [2] and singly transmitted via flavo- and FeS-proteins ultimately to the Mo-dinitrogenase catalytic center, its iron-molybdenum cofactor (FeMo-co); the enzyme complex effectively operates an 8-electron reductive cycle of recursive single electron transfers [3,4]. At the FeMo-co center, the first two arriving electrons combine with hydrogen-ions to yield a molecule of H₂. The bound H₂ is then displaced by N₂, and the subsequent six, arriving electrons, together with hydrogen-ions, now reduce N₂ to yield two molecules of ammonium as co-product:

\[
\text{N}_2 + (10)\text{H}^+ + (8)e^- \rightarrow (2)\text{NH}_4^+ + \text{H}_2
\]

In vivo, H₂ yields (relative to 1:1 in vitro stoichiometry) may further increase as a function of Mo-dinitrogenase turnover [5]. As the substrate N₂ triple-bond is highly unreactive, the dinitrogenase catalytic cycle is kinetically limiting as an in vivo biochemical standard process. To accelerate catalysis and render such thermodynamically favorable, Mo-dinitrogenase is both one-electron reduced and energetically charged by homodimeric dinitrogenase reductase, which harbors a bridging 4Fe-4S-center and two ATP binding sites, one per subunit. During single-
electron transfer from dinitrogen reductase to Mo-dinitrogenase, 2 ATP hydrolyze to yield 2 ADP and 2 orthophosphate (Pi). Thus, in the 8-electron dinitrogen complex catalytic cycle:

\[
(16)\text{ATP} + (16)\text{H}_2\text{O} \rightarrow (16)\text{ADP} + (16)\text{Pi}
\]

earning Mo-dinitrogen complex activity distinction as the most ATP-conservative metabolic reaction on a per substrate basis [5].

Notably, \textit{A. caulinodans} diazotrophic cultures, as with other aerobic diazotrophic bacteria, do not evolve significant \textit{H}_2. Rather, \textit{H}_2 produced by Mo-dinitrogenase complex activity is efficiently recycled as respiratory electron donor to \textit{O}_2 (as preferred electron-acceptor), thus recouping by oxidative phosphorylation ATP invested in \textit{H}_2 production as part of the dinitrogenase catalytic cycle:

\[
\text{H}_2 + (1/2)\text{O}_2 + (4)\text{ADP},\text{Pi} \rightarrow \text{H}_2\text{O} + (4)\text{ATP}
\]

which represents some 25\% of total ATP invested in \textit{N}_2 fixation [6].

\textit{H}_2 production has long been associated with \textit{N}_2 fixation in pure diazotrophic cultures of both fermentative and oxidative bacteria as well as endosymbiotic rhizobia in legume nodules [7]. Endogenous \textit{H}_2 recycling, both in diazotrophic bacterial cultures [8] as well as in certain symbiotic nodules, among those, garden pea [9], has been presumed owed to a respiratory Ni,Fe-hydrogenase activity highly conserved among disparate aerobic diazotrophic bacteria [10,11]. As studied in archetypal aerobic bacteria such as \textit{Ralstonia eutropha}, orthodox respiratory hydrogenase is a heterodimeric protein comprising a bimetallic Ni,Fe-catalytic subunit and a 4Fe-4S-center subunit, which complex with a tetraaza-heme \textit{b}-type cytochrome, linking Ni,Fe-hydrogenase \textit{H}_2-oxidizing activity to cellular respiration and oxidative phosphorylation [12].

To the contrary, as we report here for \textit{A. caulinodans} diazotrophic cultures open to the environment, endogenous \textit{H}_2 is not recycled via orthodox respiratory \textit{exo}-hydrogenase activity but instead via a novel respiratory \textit{endo}-hydrogenase complex, presumably reflecting the need to sequester endogenous \textit{H}_2 by metabolic channeling.

Results

\textit{A. caulinodans} \textit{exo}-hydrogenase deletion mutants lose chemotrophy but retain diazotrophy

To study the metabolic role of the orthodox respiratory \textit{exo}-hydrogenase activity for \textit{H}_2 recycling in diazotrophic culture, \textit{A. caulinodans} haploid strain 66081 carrying an in-frame \textit{hup\textit{ΔSL2}} allele (Table 1), a result of perfect gene-replacement, was constructed by “crossover PCR” mutagenesis [13] (Fig. 1; Methods). To verify its \textit{hupSL} deletion genotype, strain 66081 genomic DNA served as template for diagnostic PCR analysis. Using haploid genomic oligodeoxynucleotides \textit{HupSL-Prox} and \textit{HupSL-Dist} (Table 1; Fig. 1) as primer-pair, a single, novel 2.3 kbp DNA fragment was amplified from the 66081 genome, as template, and then sequenced on both strands. Strain 66081 therefore carries the in-frame \textit{hup\textit{ΔSL2}} allele, arisen by perfect gene-replacement. When tested in chemotrophotropic liquid batch cultures (Methods), strains 61305R and 66081(\textit{hup\textit{ΔSL2}}) both proved fully proficient (able to grow on \textit{N}_2 as sole N-source; \textit{Nif} phenotype), in comparison to \textit{Nif} – strains 60035R(\textit{nifD}) and 60035R(\textit{nifD})+, both deficient [14]. Strain 66081(\textit{hup\textit{ΔSL2}}) also grew as wild-type when plated on solid, defined medium lacking added-N, thus requiring use of atmospheric \textit{N}_2 (Methods). Therefore, orthodox respiratory \textit{HupSL exo}-hydrogenase activity was not material to growth, nor, by presumption, endogenous \textit{H}_2 recycling in \textit{A. caulinodans} diazotrophic cultures.

![Bioinformatic identification of a novel respiratory endo-hydrogenase gene-cluster](image)

\textbf{Table 1.} Bacterial strains and oligodeoxynucleotide primers employed.

| Strain            | Plasmids                                                                 |
|-------------------|--------------------------------------------------------------------------|
| \textit{Azorhizobium caulinodans} | pSUP202\textsuperscript{p} pSUP202\textsuperscript{p} |
A. caulinozdans hup locus

Figure 1. A. caulinozdans hup genetic locus and physical map: creation of in-frame translational fusion deletions. The top line represents the genetic map of the 20-gene hup polycistronic operon spanning 21 kbp. The second line represents an expanded physical map of hupSL DNA indicating positions of and (5′−3′) polarity for synthetic A, B, C, and D oligodeoxynucleotide primers of genome-identical sequence used in two, separate PCR reactions to generate DNA fragments A→B and C→D. The third line indicates a follow-up PCR reaction in which DNA fragments A→B and C→D were mixed, thermally denatured, allowed to partially renature, and used as combination PCR template/primer. As synthetic primers B and C share a complementary 21 bp extension sequence (angled line), the A→B Watson and C→D Crick strands (and vice versa) may partially reanneal via this 21 bp linker sequence in the third line, when such occurs, the resulting, partially-reannealed A→B(21 bp)C→D spliced DNA fragment which carries 5′-overhangs and free 3′-ends on both strands is a template for the thermostable DNA polymerase elongation reaction, producing a finished A→D duplex fragment which may then be further amplified by PCR in the presence of added A and D primers. As verified by DNA sequencing analysis, finished, amplified A→D duplex fragments carry a genetic crossover which fuses (via the 21 bp linker sequence) in-frame the “stop” codon of the proximal hupS gene to the “start” codon of the distal hupL gene. Primers A and D may be extended with genome non-complementary elements to facilitate molecular cloning of resulting A→D fragments (Table 1). In vivo using homologous genetic recombination, wild-type loci are then exchanged for recombinant A→D crossover DNA fragments, which yield in-frame, translational deletion alleles of target genes of interest (Methods). doi:10.1371/journal.pone.0004695.g001

identified, and localized to the same polycistronic operon carrying the hupSL genes, were the hupUV genes encoding a cytoplasmic sensory hydrogenase activity [15]. From both nucleotide and protein multiple sequence alignments, the A. caulinozdans hupUV genes proved orthologs of the R. eutropha hoxBC genes, which encode a sensory Ni,Fe-hydrogenase coupled to the HoxJ histidine kinase; in R. eutropha, this soluble HoxBCJ complex senses H2 availability, transactivates hox genes in response to H2 and is necessarily present only at very low catalytic activity on a per cell basis [16]. Thus, we broadened the hydrogenase search to unlinked loci, initially without benefit of an A. caulinozdans genomic sequence. Using the BLAT algorithm [17] to search an (~8 Mbp total) A. caulinozdans ORS571 shotgun genome sequence dataset (generously provided by B. A. Roe, unpublished results), we assembled several contigs spanning an ~8 kbp genomic sequence, unlinked to the hup operon, but showing homology to Ni,Fe-hydrogenase genes (Fig. 1). From these genome-contigs, we designed synthetic oligodeoxynucleotide primers, carried out PCR amplification and nucleotide sequencing, and assembled the complete sequence for a novel, tightly organized, seven-gene hyqRBCEF operon (GenBank accession: FJ378904; Fig. 2). In the presumed hyq operon, the distal hyqGI genes encode a canonical heterodimeric Ni,Fe-hydrogenase. The hyqBCEF genes all specify integral-membrane proteins orthologous to the Escherichia coli hyf genes, whose syntax in labeling the A. caulinozdans hyq genes, including hyqGI, has thus been conserved. (Note the A. caulinozdans hyq operon however lacks both E. coli hyfA and hyfD genes.) The E. coli hyf operon encodes hydrogenase-4 [18], an integral-membrane complex representative of the H2-evolving or group-4 hydrogenases, previously identified in and restricted to anaerobic bacteria [11].
As well, the group-4 integral-membrane hydrogenases include multiple subunits homologous to those of respiratory NADH : quinone dehydrogenase (NADH-DH), an integral membrane complex whose active site for NADH oxidation faces the cytoplasm [11,18,19]. Such homology extends to both NADH-DH L0 (integral-membrane) and L1 (membrane-associated) subcomplex proteins. By analogy to NADH-DH, and to conceptually distinguish the two A. caulinodans cell membrane-associated, respiratory hydrogenases, we therefore denote the presumed HyqBCEFGI complex as endo-hydrogenase and the HupSL+ cyt b complex as exo-hydrogenase, so as to distinguish relative orientations of substrate oxidative sites: the former presumably facing the cell interior (cytoplasm), the latter facing the cell exterior (periplasm). As the A. caulinodans genome encodes bona fide respiratory complex I in an unlinked 15-gene operon (AZC_1667 to AZC_1681) [20], the hyq genes and their encoded endo-hydrogenase complex, while similar, are indeed structurally and functionally distinct.

Figure 2. A. caulinodans hyq genetic locus and physical map: creation of in-frame, translational fusion deletions. The main line represents the genetic map of the 7-gene hyq polycistronic operon (7.4 kbp coding DNA). Superimposed above is the ~300 np sequence upstream of the hyqR start (ATG) codon, presumably comprising the hyq control region, which includes a canonical NIFBox element, facilitating binding of the NIF transcriptional activator, adjacent to a s54F-box element, allowing initiation by RNA polymerase-s54F complex (see Results). For additional details, please refer to Fig 1.

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A. caulinodans Hyq endo-hydrogenase activity facilitates growth in diazotrophic cultures open to the environment

To test metabolic role(s) for the presumptive Hyq endo-hydrogenase complex, an A. caulinodans hyq operon deletion (hyqAR7) allele was constructed using crossover PCR, in which the hyqR start- and hyqI stop-codons were fused in-frame by a 21 bp linker sequence (Fig. 2; Methods). In both strain 61305R(virtual wild-type) and 66081(hupD SL2), the wild-type hyq+ operon was then swapped for this hyqAR7 allele by homologous recombination (Methods). As with strain 66081, the resulting haploid strain 66132 was verified by combined PCR and DNA sequencing analyses. Using haploid genomic Hyq-Prox and Hyq-Dist oligodeoxynucleotides (Table 1; Fig. 2) as primer-pair and strain 66132 genomic DNA as template, a single 2.2 kbp DNA fragment was amplified by PCR and then sequenced on both strands using either Hyq-Prox or Hyq-Dist as DNA sequencing primers. Accordingly, strain 66132 proved a true hyqAR7 haploid arisen by perfect gene-replacement. Similarly,
starting with strain 66081(hupASL2), strain 66203 proved a true double-mutant haploid, carrying both hupASL2 and hyqAR7 alleles.

Growth kinetics of four haploid strains, 61305R and its descendants 66081(hupASL2), 66132(hyqAR7) and 66203(hupASL2, hyqAR7) were analyzed in liquid batch cultures with defined media. For all strains, batch cultures were started in defined medium with 40 mM succinate added as sole C- and energy-source, and 0.5 mM ammonium added as N-source. All starter cultures grew exponentially up to viable cell counts of \( \sim 1 \times 10^8 \text{ ml}^{-1} \) at which growth arrested due to ammonium limitation (as when 5 mM ammonium was then added, exponential growth rapidly resumed for at least two additional cell doublings). These N-limited, static cultures were one-thousandfold diluted into the same defined growth medium with or without added 5 mM ammonium, placed in sealed 30 ml vials, sealed and subcultured with continuous sparging (6 ml min\(^{-1}\)) using a defined gas mixture (2% O\(_2\), 5% CO\(_2\), bal. N\(_2\)). Samples were periodically withdrawn and plated on rich medium for viable cell counts (Methods). Whether in the presence and absence of added ammonium, strains 61305R and 66081(hupASL2) grew similarly (Table 2). In contrast, while both strains 66132(hyqAR7) and 66203(hupASL2, hyqAR7) grew similarly in the presence of added ammonium, cell doubling-times slowed 21% in the absence of ammonium, relative to strains 61305R and 66081 (Table 2).

These growth experiments were repeated, except that cultures were sparged with an H\(_2\)-enriched defined gas mixture (2% O\(_2\),5% CO\(_2\), bal. N\(_2\)). Samples were periodically withdrawn and plated on rich medium for viable cell counts (Methods). Whether in the presence and absence of added ammonium, strains 61305R and 66081(hupASL2) grew similarly (Table 2). In contrast, while both strains 66132(hyqAR7) and 66203(hupASL2, hyqAR7) grew similarly in the presence of added ammonium, cell doubling-times slowed 21% in the absence of ammonium, relative to strains 61305R and 66081 (Table 2).

Therefore, while cell bioenergetic role(s) for Hyq might also be membrane proton-motive and/or electrogenic, translocating multiple ions such as H\(^+\), K\(^+\) and/or Na\(^+\) (Discussion).

In contrast, strain 66081(hupASL2) showed only a slight increase in growth rate in diazotrophic culture, and strain 66203(hupASL2, hyqAR7) showed no detectable increase in growth rate, both in response to added 20% H\(_2\) (Table 2). Therefore, in A. caulinodans, exogenous H\(_2\)-driven respiration is essentially run by HupSL endo-hydrogenase activity, marginally augmented by Hyq endo-hydrogenase activity. In contrast, for diazotrophic cultures open to the atmosphere, endogenous H\(_2\) (produced by Mo-dinitrogenase activity) was exclusively recycled by Hyq endo-hydrogenase activity. In enclosed cultures, or when liquid batch diazotrophic cultures open to the atmosphere became sufficiently dense near saturation (>1 \times 10^8 cells ml\(^{-1}\)) some amount of endogenous H\(_2\) recycling by HupSL exo-hydrogenase activity was detected (data not presented).

The A. caulinodans hyq operon is strongly and specifically induced in diazotrophic cultures

To assess growth conditions in which the hyq operon was genetically expressed, A. caulinodans strains using β-glucuronidase activity to report hyq operon transcription were constructed. The E. coli uidA gene, encoding β-glucuronidase, was amplified by PCR and, using standard in vitro molecular cloning techniques, the resulting 1.8 kbp uidA\(^{\text{b}}\) coding sequence was inserted in-frame into the 21 bp crossover linker sequence of pHyqAR7 yielding plasmid pHyqRU5 (Table 1; Methods). Derived from both A. caulinodans 57100 and 60107(hyqR) as parent, hyq merodiploid strains 66205 and 66210, both carrying an upstream in-frame fusion hyqR:uidA\(^{\text{b}}\) hyqAR7 operon in tandem with the downstream hyq operon, were isolated and verified by both PCR and DNA sequencing analyses (Methods). Merodiploid hyq reporter strain 66205 was first tested for bacterial colony appearance on solid defined media supplemented with X-Gluc (Methods) as chromogenic β-glucuronidase substrate. When inoculated onto defined medium also supplemented with 5 mM ammonium and cultured in fully aerobic conditions, strain 66205 colonies were white, lacking any evidence of β-glucuronidase activity. When the same petri plates were incubated under a reduced O\(_2\) atmosphere (2% O\(_2\), 5% CO\(_2\), bal. N\(_2\)), strain 66205 colonies appeared light-blue, or partially induced. When 0.5 mM L-glutamine was added to solid culture medium, 66205 colonies were again completely white when incubated under 2% O\(_2\) indicating the hyq operon was strongly repressed. When 66205 was cultured diazotrophically (in the absence of added ammonium and L-glutamine) under reduced O\(_2\) colonies were dark blue, indicative of strong hyq operon expression.

To obtain quantitative data for both strains 66205 and 66210, liquid batch cultures were pre-grown aerobically in defined medium with 0.5 mM ammonium as N-source to cell titers of \( \sim 1 \times 10^8 \text{ ml}^{-1} \) (at which available ammonium was exhausted) and physiologically shifted to diazotrophic culture conditions (Methods) for 12 hr at 29°C. Cells were then harvested and β-glucuronidase specific activities were measured in cell-free extracts (Methods). These results (Table 3) corroborated visual inspections of bacterial plate cultures supplemented with chromogenic X-Gluc. The hyq operon was specifically and strongly expressed in diazotrophic culture but strongly repressed either in the presence of added ammonium under air or in the presence of added 0.5 mM L-glutamine under reduced O\(_2\). As strain 66210 was only weakly induced (Table 3), hyq operon induction specifically required NIFA transcriptional activation.

When the presumed hyq operon control region (immediately upstream from the hyqR coding sequence) was analyzed, the

| Strain | %O\(_2\), %CO\(_2\), bal. N\(_2\) atmosphere (hr) |
|--------|---------------------------------------------|
|        | **N-source** | **<5 mM NH\(_4\)\(^{-}\)** | **atm~20% H\(_2\)** |
|        | **t\(_0\)** | **t\(_2\)** | **t\(_0\)** | **t\(_2\)** |
| 61305R | 2.3 (1.0)\(^{a}\) | 7.2 (1.0)\(^{a}\) | 4.2 (1.0)\(^{a}\) |
| 66081 hupSSL | 2.3 (1.0 ± 0.04) | 7.2 (1.0 ± 0.03) | 6.8 (0.62 ± 0.02) |
| 66132 hyqAR7 | 2.4 (0.96 ± 0.03) | 8.8 (0.82 ± 0.02) | 5.0 (0.84 ± 0.03) |
| 66203 hupSSL hyqAR7 | 2.4 (0.95 ± 0.05) | 8.8 (0.80 ± 0.04) | 8.8 (0.48 ± 0.02) |

\(^{a}\) doubling-time; representative single experiment.

\(^{b}\) doubling-time relative to wild-type (w); multiple experiments.

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physiological conditions and so have been termed H2-evolving, hydrogenases are active under anaerobic, strictly fermentative dehydrogenase activity [23,24]. In all cases these group-4 hydrogenases (hydrogenase-4), encoded by the operon, seem coupled to yet another fermentative formate oxidizing formate to CO2 and reducing 2H\textsuperscript+ ions to yield H2 (gas), all as fermentative end-products. Thus, the operon is constituent of the group-4 hydrogenase activity is presumably proton-motive, energy-conserving [18] and thus likely drives aerobic respiration. From multiple protein alignments including sequences identified in the four aerobic bacteria (A. caulinodans, B. japonicum, R. leguminosarum, X. autotrophicus), together with the E. coli HygGI proteins, the endo-hydrogenase peripheral HygG large-subunit carries the Ni,Fe-hydrogenase catalytic center and the HygI small-subunit carries the (N2) proximal 4Fe–4S center as likely electron-donors to membrane-bound quinoines.

Given this inferred organization and integral-membrane orientation of the Hyq endo-hydrogenase complex (for which we as yet lack direct experimental evidence), one implication is obvious: the Hyq endo-hydrogenase might physically interact with Mo-dinitrogenase so as to channel evolved H2 as substrate for membrane-driven respiration and oxidative phosphorylation. Coupled respiration would allow quantitative recovery of ATP consumed by Mo-dinitrogenase complex activity in H2 evolution during strictly fermentative metabolism, nevertheless remain capable of H2 oxidation, albeit slowly [11]. Because Mo-dinitrogenase complex activity has exceedingly slow in vivo turnover (<10 sec\textsuperscript–1), any directly coupled Hyq endo-hydrogenase H2 oxidizing activity might operate at correspondingly very slow rates in vivo.

H2-oxidative endo-hydrogenase activity necessitates that H\textsuperscript+ ions be membrane-translocated else deplete the cell membrane proton-motive force. Any endo-hydrogenase driven, vector H\textsuperscript+ translocation, an energy-requiring process, would be necessarily slow by comparison with exo-hydrogenase activity, uncoupled from H2 translocation, and thus relatively fast. (In the latter case, as H\textsuperscript+ ions are produced external to the cell membrane, they in principle contribute directly to the cell membrane proton-motive force.)

Table 3. A caulinodans hyq operon expression, PhyqR β-glucuronidase reporter activity.

| Strain | Atmosphere | N-source(s) | N\textsubscript{2} only | +5 m M NH\textsubscript{4}\textsuperscript{+} | +5 m M NH\textsubscript{4}\textsuperscript{+} (atm = 78%) | +05 m M L-glutamine |
|--------|------------|-------------|---------------------|----------------|---------------------|---------------------|
| 66205  | 2% O\textsubscript{2} | 1410±200 | 220±35 | <10 | |
| 66210  | 21% O\textsubscript{2} | 40±10 | 40±10 | <10 | |

*Inmol 5-bromo-4-chloro-3-indole min\textsuperscript–1 mg protein\textsuperscript–1.

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Discussion

In rhizobia, obligate oxidative bacteria, orthodox respiratory Ni,Fe-hydrogenase is encoded by contiguous hupSL genes. In other obligate oxidative bacteria, orthologous gene assignments are variant, e.g. hoxKG in R. eutropha [12]. Notably, the Ni,Fe-catalytic center of this conserved respiratory hydrogenase complex is periplasmic-oriented, i.e. exo-hydrogenase. Indeed, orthologous rhizobial HupS and R. eutropha HoxK encoded FeS-center subunits possess a periplasmic export (RRxFxK) signal peptide motif [11]. Typical of H2-recycling rhizobia, the A. caulinodans exo-hydrogenase encoding genomic locus comprises a 21 kbp contiguous set of highly-conserved genes, among them hupSL [15] (Fig. 1). This respiratory hydrogenase activity is obviously adapted for use of exogenous H2.

Archetypal for the group-4 hydrogenases is E. coli hydrogenase-3, encoded by hygGE. This heterodimeric Ni,Fe-hydrogenase anchors an integral-membrane formate–hydrogen lyase complex, oxidizing formate to CO2 and reducing 2H\textsuperscript+ to H2, all cell-internal, under strict, fermentative conditions [19]. In E. coli, a second group-4 hydrogenase (hydrogenase-4), encoded by the hyf operon, seems coupled to yet another fermentative formate dehydrogenase activity [18]. In Rhodospirillum rubrum a distinct, but related, group-4 hydrogenase activity is coupled to CO2-dehydrogenase activity [23,24]. In all cases these group-4 hydrogenases are active under anaerobic, strictly fermentative physiological conditions and so have been termed H2-evolving, hydrogenases are active under anaerobic, strictly fermentative dehydrogenase activity [23,24]. In all cases these group-4 hydrogenases (hydrogenase-4).

From bacterial genome searches, orthologous hyq operons are evident in two additional rhizobial species, R. leguminosarum and B. japonicum both previously classified phenotypically as H2 recyclers [9,55]. In the non-symbiotic but very closely related species Xanthobacter autotrophicus Py2 [26], an orthologous hyq operon is also present, as is the complete nif regulon, implying X. autotrophicus Py2 is also diazotrophic. All such bacteria carrying the hyq operon are obligate oxidative, in which any H2-evolving hydrogenase activities would seem not only superfluous but antithetical.

Metabolic roles for the group-4 hydrogenases are not definitive. All show integral-membrane components with homology to NADH : quinone dehydrogenase (respiratory complex I), which functions as unidirectional NADH oxidant and membrane quinone pool reductant [11,18,19]. Included in this homology are the heterodimeric Ni,Fe-hydrogenase subunits of group-4 hydrogenases (the A. caulinodans HyqGI proteins) which are closely related to the 49 kDa (Nqo4) and 20 kDa (Nqo6) subunits of the Thermus thermophilus (hyperthermophile) respiratory NADH-DH L1 sub-complex, whose crystal structure has been solved by X-ray diffraction at atomic resolution [27]. By structural analogy and genetic homology to the NADH-DH L1 sub-complex, the homologous HyqGI heterodimeric endo-hydrogenase, with its active site facing cell-internally, likely interacts with the integral-membrane, L0-homologous HynBCEF sub-complex and together function as H2 oxidant and membrane quinone reductant. Like both NADH-DH complex and E. coli hydrogenase-4, A. caulinodans Hyq endo-hydrogenase activity is presumably proton-motive, energy-conserving [18] and thus likely drives aerobic respiration.

Given this inferred organization and integral-membrane orientation of the Hyq endo-hydrogenase complex (for which we as yet lack direct experimental evidence), one implication is obvious: the Hyq endo-hydrogenase might physically interact with Mo-dinitrogenase so as to channel evolved H2 as substrate for membrane-driven respiration and oxidative phosphorylation. Coupled respiration would allow quantitative recovery of ATP consumed by Mo-dinitrogenase complex activity in H2 evolution during strictly fermentative metabolism, nevertheless remain capable of H2 oxidation, albeit slowly [11]. Because Mo-dinitrogenase complex activity has exceedingly slow in vivo turnover (<10 sec\textsuperscript–1), any directly coupled Hyq endo-hydrogenase H2 oxidizing activity might operate at correspondingly very slow rates in vivo.
Thus, _exo_-hydrogenase activity is kinetically preferred as agent for exogenous _H_2 oxidation. By contrast, _endo_-hydrogenase activity, via metabolic channeling, might confer an increased efficiency of endogenous _H_2 recycling, thus mitigating energy loss, were such _H_2 to escape to the environment by simple diffusion.

Hydrogen has elicited considerable interest as potential renewable energy source for human civilization. If hydrogen is to be produced at scale as part of a sustainable cycle, external energy source(s) are then required. Solar energy represents an obvious energy coupling source, in principle allowing photoelectrochemical energy source(s) are then required. Solar energy represents an obvious energy coupling source, in principle allowing photoelectrochemical

**Methods**

**Bacterial strains and culture conditions**

_Azorhizobium caulinodans_ ORS571 wild-type (strain 57100), originally isolated from _Sesbania rostrata_ stem-nodes [1], was cultured in both rich (SYPC) and minimal, defined media as previously described [14]. As 57100 wild-type is NAD auxotrophic, defined growth media must be supplemented with nicotinate (or similar) as precursor. However, nicotinate serves strain 57100 as both anabolic (for NAD production) and catabolic (as both utilizable C- and N-sources) supplement. When strain 57100 is cultured in media with limiting primary C- and/or N-sources, nicotinate is rapidly catabolized and exhausted cultures quickly become NAD-limited for growth [28]. Accordingly, to eliminate nicotinate catabolism as a metabolic variable, all experiments reported herein employ _A. caulinodans_ 61305R, a 57100 derivative carrying an _IS50R_ insertion in the (catabolic) nicotinate hydroxylase structural gene, as “virtual” wild-type; 61305R only uses nicotinate as anabolic source and thus requires minimal (1 μM) nicotinate supplementation in all defined media [29].

**Genetic constructions**

_A. caulinodans_ in-frame translational fusion mutants. Precise, in-frame deletion mutagenesis of the _A. caulinodans_ _hupSL_ genes was carried out by “crossover PCR” as previously described [13]. In the first step, separate ~1 kbp genomic fragments immediately proximal to _hupS_ and distal to _hupL_ coding sequences were PCR amplified [13]. These two, ~1 kbp amplified genomic fragments shared an artificial, complementary “crossover” sequence introduced by 21 bp annealed strands. In a second-round PCR, the two amplified DNA fragments were purified, mixed, and used as combination primer-template. A ~2 kbp DNA fragment was then produced when non-homologous template strands annealed via complimentary 21 bp extensions; when an upstream coding-strand annealed to a downstream non-coding strand via the 21 bp crossover extension, 3’-ends on both annealed strands were extended by thermostable DNA polymerase yielding a contiguous ~2 kbp DNA fragment in which the 21 bp crossover sequence fused the ~1 kbp upstream and downstream sequences. In this second-round PCR, terminal “A” and “D” oligodeoxynucleotides (Fig. 1) were also included as primers such that, by standard recursive PCR, this ~2 kbp crossover DNA fragment was further amplified. By design, the 21 bp crossover within the ~1 kbp DNA fragment fuses in-frame an upstream target gene’s translational “start” codon with a downstream target gene’s “stop” codon yielding a translational (e.g., _hupΔSL_) fusion (Fig. 1).

The PCR amplified, crossover DNA fragment carrying the in-frame ~2 kbp _hupΔSL2_ fusion allele was verified by DNA sequencing and introduced into the EcoRI site of plasmid pSUP202 (Table 1) by standard molecular cloning; _E. coli_ strain MH3000 (Table 1) was subject to electroporation with recombinant plasmids, and transformed bacterial colonies were selected for tetracycline (Tc) resistance. In this manner, recombinant plasmid pHupΔ22 was identified (Table 1), purified, and reintroduced by electroporation into _E. coli_ SM10 (Table 1), proficient as donor for bacterial conjugation, again selecting for Tc-resistance. To allow plasmid conjugal transfer, _E. coli_ SM10/ pHupΔ22 as donor was mixed with _A. caulinodans_ 61305R as recipient and plated overnight on SYPC solid medium at 37°C. Conjugal cell mixtures were then selectively plated on solid ORSMM (to counter-select _E. coli_) supplemented with Tc (10 μg/ml) at 37°C. As parental plasmid pSUP202 cannot stably replicate in _A. caulinodans_, transconjugants that are stably Tc-resistant arise after homologous, single recombination events in which the entire plasmid and the target genome are cointegrated [22]. Accordingly, _A. caulinodans_ 61305R _hupSL_ merodiploids were then isolated and confirmed by PCR and DNA sequencing analyses; such strains carried both genomic _hupSL_ and _hupΔSL_ alleles bridged by the integrated SUP202 sequence. To then isolate haploid gene replacement strains, merodiploids were subcultured absent Tc selection in rich GYPC liquid medium and then plated with Tc added at very low (0.125 μg/ml) levels sufficient to 50% inhibit growth of parental wild-type. Pinpoint colonies were identified, retested, and a Tc-sensitive phenotype verified. These putative haploid derivatives arose by a second, single homologous recombination (disintegration) event within the merodiploid, segregating the _hupSL_ alleles. By PCR analysis with Hup-Prox and Hup-Dist as oligodeoxynucleotide primer-pair (Table 1; Fig. 1), resulting haploid strains showed either _hupSL_ or _hupΔSL_ alleles.

Similarly, a haploid 61305R derivative carrying a complete _hyqRBCEFGL_ in-frame deletion allele was isolated using the same crossover PCR technique. Recombinant plasmid pHyqAR17 carried a ~2 kbp _hyqAR17_ allele in the identical 21 bp linker fused in-frame the _hyqR_ “start” codon with the _hyq_ “stop” codon (Table 1; Fig. 2). After gene replacement, haploid strain 66132 carrying the _hyqAR17_ allele was isolated and verified by PCR and DNA sequencing analyses.

_A. caulinodans_ _Hyq_ transcriptional reporter strains. To construct _hyq_ merodiploid transcriptional reporter strains, a 1.8 kbp fragment carrying the _E. coli_ _uidA_ coding sequence was amplified by PCR using synthetic oligodeoxynucleotide primers extended with 6 bp DsI endonuclease recognition sequences (Table 1). As the 21 bp linker sequence used to construct in-frame translational fusions includes a DsI recognition sequence, plasmid pHyqAR17 was partially digested with DsI endonuclease, a 9.8 kbp DNA fragment was isolated, mixed with DsI digested, amplified 1.8 kbp _uidA_ DNA fragment and recombinant plasmids were recovered by standard molecular cloning techniques. After electroporation of _E. coli_ MH3000, and selection for Tc-resistance, _uidA_ recombinant plasmids were identified by plating candidate strains on minimal media supplemented with (0.1 mg ml⁻¹) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGluc), a chromogenic β-glucuronidase substrate, and screening for blue colonies. From PCR and DNA sequencing analysis, recombinant plasmid pHyqRU5 carrying the _hyqR::uidA_ in-frame translational fusion allele was isolated (Table 1). Plasmid pHyqRU5 was introduced to _E. coli_ SM10, and this strain was employed as conjugal donor with _A. caulinodans_ 61305R and 60107R, and Tc-resistant derivatives were selected and isolated. Merodiploid strains 66205 and 66210
(Table 1) carrying both upstream hyqR::uidA+ hyqAB1 and downstream hyqB operon were identified and verified by PCR and DNA sequencing analyses.

**Physiological growth and β-glucuronidase activity measurements.** Starter cultures of *A. caulinoindans* strain 61305R and its derivatives were aerobically cultured in minimal defined NIF liquid medium [14] supplemented with: ammonium (0.5 mM) as sole, limiting N-source and 1 μM nicotinate at 37°C until growth arrested (cell densities ~1×10^6 cells ml⁻¹). For kinetic measurements of diazotrophy, starter cultures were diluted one-thousandfold in NIF medium (with 1 μM added nicotinate) into 30 ml serum vials, sealed with silicone rubber septa, sparged continuously (6 ml min⁻¹) with defined gas mixtures (e.g. 2% O₂, 5% CO₂, bal. N₂), and incubated at 29°C. At least three times per cell-doubling period, culture samples were removed, serially diluted, plated on rich GYP medium [14], incubated 48 hr at 37°C, and colonies were counted, in triplicate. β-glucuronidase activity was measured with as chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) [30]; total protein concentrations were determined in a folin phenol reagent assay [31]. All induction experiments were conducted in triplicate and were repeated until the standard error in β-GUS activities was below 15%.

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**Author Contributions**

Conceived and designed the experiments: RAL. Performed the experiments: GN CT AP LZ RAL. Analyzed the data: GN CT AP LZ RAL. Contributed reagents/materials/analysis tools: RAL. Wrote the paper: RAL.