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Respiratory Membrane *endo*-Hydrogenase Activity in the Microaerophile *Azorhizobium caulinodans* Is Bidirectional

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Abstract

Background: The microaerophilic bacterium *Azorhizobium caulinodans,* when fixing N_2 both in pure cultures held at 20 μ*M* dissolved O_2 tension and as endosymbiont of *Sesbania rostrata* legume nodules, employs a novel, respiratory-membrane *endo*-hydrogenase to oxidize and recycle endogenous H_2 produced by soluble Mo-dinitrogenase activity at the expense of O_2.

Methods and Findings: From a bioinformatic analysis, this *endo*-hydrogenase is a core (6 subunit) version of (14 subunit) NADH:ubiquinone oxidoreductase (respiratory complex I). In pure *A. caulinodans* liquid cultures, when O_2 levels are lowered to <1 μ*M* dissolved O_2 tension (true microaerobic physiology), *in vivo endo*-hydrogenase activity reverses and continuously evolves H_2 at high rates. In essence, H^+ ions then supplement scarce O_2 as respiratory-membrane electron acceptor. Paradoxically, from thermodynamic considerations, such hydrogenic respiratory-membrane electron transfer need largely uncouple oxidative phosphorylation, required for growth of non-phototrophic aerobic bacteria, *A. caulinodans* included.

Conclusions: *A. caulinodans in vivo endo*-hydrogenase catalytic activity is bidirectional. To our knowledge, this study is the first demonstration of hydrogenic respiratory-membrane electron transfer among aerobic (non-fermentative) bacteria. When compared with O_2 tolerant hydrogenases in other organisms, *A. caulinodans in vivo endo*-hydrogenase mediated H_2 production rates (50,000 pmol $10^9 \cdot cells^{-1}\ min^{-1}$) are at least one-thousandfold higher. Conceivably, *A. caulinodans* respiratory-membrane hydrogenesis might initiate H_2 crossfeeding among spatially organized bacterial populations whose individual cells adopt distinct metabolic states in response to variant O_2 availability. Such organized, physiologically heterogeneous cell populations might benefit from augmented energy transduction and growth rates of the populations, considered as a whole.

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Introduction

Given the relatively low (-414 m*V*) potential in aqueous solution for the standard hydrogen bio-electrochemical half-cell, hydrogen gas (H_2) is a strong electron (e^-) donor, whereas in the back-reaction, combining H^+ ions are weak e^- acceptors. Hydrogenases, which catalyze this reaction, are widely distributed among bacteria [1]. Diverse aerobic bacteria employ O_2-tolerant (group-1) hydrogenases as e^- donor, oxidizing substrate H_2 at the expense of substrate O_2 as preferred e^- acceptor, driving oxidative phosphorylation. These group-1 hydrogenases, both soluble and membrane-associated, typically include globular, heterodimeric catalytic proteins. In the stably membrane-associated group-1 hydrogenases, each catalytic heterodimer is first exported and then stably complexes with a membrane-integral diheme *b*-type cytochrome [2]. Resulting heterotrimeric complexes are typically denoted 'uptake hydrogenases' as they operate *in vivo* as unidirectional catalysts of H_2 oxidation. Product H^+ ions, released on the exterior (*exo*) face of cell membranes, directly contribute to trans-membrane proton-motive force absolving these activities of any, obvious chemiosmotic (ion-pumping) workload. These stably membrane-associated, group-1 heterotrimeric complexes may be termed *exo*-hydrogenases.

The reversible or H_2 evolving (group-4) hydrogenases, also membrane-associated, are encoded by completely divergent gene-sets. The group-4 hydrogenases are typically employed by anaerobic bacteria to produce H_2 as fermentative end-product and in so doing, facilitate overall cellular oxidation-reduction balance [1]. In anaerobes, the hydrogenesis (H_2 production from H^+ ions) reaction involves direct coupling of group-4 hydrogenases as e^- acceptor with various e^- donors such as formate and carbon monoxide dehydrogenases as membrane-integral complexes [3,4]. In contrast to group-1 membrane-associated uptake hydrogenases, the catalytic heterodimers of group-4 hydrogenases are oriented to the cytosolic (*endo*) face of cell membranes [5,6] and so may be termed *endo*-hydrogenases.

However, *endo*-hydrogenases are not exclusive to fermentative anaerobes. We recently reported on a novel *endo*-hydrogenase in the

aerobic microaerophile *Azorhizobium caulinodans* which requires oxidative phosphorylation for growth. Indeed, *A. caulinodans* employs both membrane-associated *exo*- and *endo*-hydrogenases when respiring with H_2 as e^- donor. In chemolithotropic cultures with exogenous H_2 as sole energy source, *A. caulinodans* primarily relies on *exo*-hydrogenase activity [7]. Archetype *A. caulinodans* strain ORS571 was originally isolated as N_2-fixing endosymbiont of stem- and root-nodules in *Sesbania rostrata*, an annual legume indigenous to the Atlantic coastal Sahel [8]. *A. caulinodans* ORS571 may be cultured diazotrophically (N_2 as sole N-source) and organotrophically (oxidizable organic acids as C- and energy source) under a reduced (2%) atmosphere [9]. Its sole N_2 fixing activity, Mo-dinitrogenase, also produces stoichiometric H_2 in an ATP-dependent process [10,11]. In such diazotrophic liquid cultures, respiratory-membrane uptake hydrogenase activity allows input of endogenous H_2 as fuel for oxidative phosphorylation, recovering invested ATP. In contrast to use of exogenous H_2, in endogenous H_2 uptake, *endo*-hydrogenase activity predominates [7]. In *Sesbania rostrata* (legume) nodules actively fixing N_2, *A. caulinodans* endosymbionts employ both *exo*- and *endo*-hydrogenases to recycle endogenous H_2 produced by Mo-dinitrogenase activity [12]. In these cases, both *exo*- and *endo*-hydrogenases function as uptake hydrogenases.

However, as we demonstrate here, *A. caulinodans endo*-hydrogenase *in vivo* activity is bidirectional, reversing in response to physiological O_2 availability. Given sufficient O_2, *endo*-hydrogenase operates in H_2 uptake mode. Under strict O_2 limitation, *endo*-hydrogenase reverses and operates in hydrogenesis mode at extraordinarily high *in vivo* rates. Hitherto, *endo*-hydrogenase mediated hydrogenesis has been masked as it occurs in N_2 fixing pure cultures in which Mo-dinitrogenase activity itself also produces H_2 [10,11]. Because *exo*-hydrogenase invariably operates in H_2 uptake mode, *exo*- and *endo*-hydrogenases then function at cross-purposes, yielding a novel and seemingly paradoxical physiology.

Results

Hyq *endo*-hydrogenase is a Core Homolog of L-type Respiratory Complex I

The *Azorhizobium caulinodans hyq*$^+$ operon (Entrez Gene identifier: AZC4360–AZC4355) encodes an *endo*-hydrogenase including six discrete structural proteins as well as a transcriptional activator [7]. From SUPERFAMILY analysis, a hidden Markov model library of protein structures [13], the six catalytic *A. caulinodans* Hyq proteins all have close homologs among the Nuo proteins of NADH:quinone oxidoreductase, commonly referred to as 'L-type' respiratory complex I (Table 1). Bacterial respiratory complex I typically includes 14 subunits equally divided into membrane-integral (L_O) and cytosol-interfacing, membrane-peripheral (L_1) subcomplexes [14,15]. In pairwise primary amino acid sequence alignments, four Hyq proteins (HyqBCEF) and four L_O subcomplex NuoHJLM proteins are ~60% conserved (Table 1; Figs. S1, S2, S3, S4, S5, S6). For the L_1 subcomplexes, two (HyqGI) proteins are homologs of three (NuoCDB) proteins. From SUPERFAMILY analysis, HyqG corresponds to a fused NuoC::D (SSF56762). The HyqG (504 residues) N-terminal domain (residues 1–156) is homologous to NuoC, and its C-terminal domain (residues 157–504) is homologous to NuoD. Because its (SSF56762) superfamily also includes the group-1 hydrogenase catalytic (large) subunit, HyqG together with HyqI presumably catalyze hydrogenase activity. HyqI, a small FeS protein, is a NuoB (SSF56770) homolog; all HyqI orthologs show conserved cys-55, cys-58 (Cys-X-X-Cys), cys-112 and cys-152 residues likely coordinating a N2-type, high-potential 4Fe4S center, which in respiratory complex I serves as immediate e^- donor to membrane quinone [16,17]. The binding site for complex I membrane quinone, its e^- acceptor, is a cavity formed between a four-helix bundle of NuoD, the H1 helix of NuoB, and transmembrane helix 1 of NuoH [14,15,18], all of which elements are conserved in Hyq *endo*-hydrogenases (Table 1; Figs. S1, S2, S3, S4, S5, S6). By inference, the Hyq *endo*-hydrogenase of microaerophiles constitutes a core L-type H_2:ubiquinone oxidoreductase (Fig. 1).

In Growth-optimized *A. caulinodans* Diazotrophic Liquid Cultures held at 20 μ*M* DOT, *endo*-hydrogenase Activity Serves *in vivo* as Respiratory Membrane e^- donor for Uptake of Endogenous H_2

To recapitulate, *A. caulinodans* operates distinct, respiratory-membrane *exo*- and *endo*-hydrogenases; unlinked Δ*hyqRI*7 (*endo*-hydrogenase) and Δ*hupSL*2 (*exo*-hydrogenase) complete deletion alleles of relevant structural genes were previously isolated. In growth-optimized liquid diazotrophic cultures open to the environment, *exo*-hydrogenase mutants grow normally, whereas *endo*-hydrogenase mutants grow slowly [7]. To more accurately measure relative contributions of both *exo*- and *endo*-hydrogenase activities to *in vivo* recycling of H_2 produced by Mo-dinitrogenase activity, H_2 evolution rates of diazotrophic liquid batch cultures under continuous sparge have now been measured. *A. caulinodans* strains were batch cultured at 29°C in defined liquid media lacking utilizable-N; N_2 as sole N-source was provided by continuous sparge with (2% O_2, 5% CO_2, bal. N_2) gas mixture optimized for *A. caulinodans* N_2-dependent growth (Materials). Dissolved O_2 tension (DOT) in these sparged cultures held steady in the range of 18–20 μ*M* O_2 as measured potentiometrically with a Clark-type polarographic electrode (Thermo-Orion 97–08). Culture exit gas streams were periodically sampled and analyzed for evolved H_2 by gas chromatography (Materials). In these diazotrophic cultures, both *A. caulinodans* Δ*hyqRI* (*endo*-hydrogenase) mutant 66132 and Δ*hyqRI*, Δ*hupSL* (*exo*-, *endo*-hydrogenase) double-mutant 66204 showed tenfold elevated H_2 evolution rates relative to both *hyq*$^+$, *hup*$^+$ parent 61305R and Δ*hupSL exo*-hydrogenase mutant 66081 (Table 2B).

Sparge rates for all liquid cultures were standardized to allow culture atmosphere exhaust rates of 0.5 min^{-1}. In principle, for these growth-optimized diazotrophic cultures, relative abilities of *exo*- and *endo*-hydrogenases to recycle endogenous H_2 might vary with sparge rates. Increased sparge rates proportionally decreased exit gas H_2 levels of all cultures; relative H_2 evolution rates among cultures were not affected. Culture sparges were slowed to the minimum rate still maintaining stable 20 μ*M* DOT. Nonetheless, Δ*hyqRI* single mutant 66132 still evolved tenfold more H_2 than did Δ*hupSL exo*-hydrogenase mutant 66081. Because Δ*hyqRI* mutants invariably evolved more H_2 than did Δ*hupSL* mutants at 20 μ*M* culture DOT, *endo*-hydrogenase activity is disproportionately responsible for recycling endogenous H_2 produced by Mo-dinitrogenase activity in growth-optimized liquid cultures.

Similarly, when defined media were supplemented with 5 m*M* L-glutamine, measurable H_2 evolution by all strains was negligible. In *A. caulinodans* cultures, L-glutamine sufficiency yields complete repression of the N_2 fixation regulon, including *nifD*$^+$*K*$^+$ genes encoding Mo-dinitrogenase [19] as well as *hyq*$^+$ genes encoding *endo*-hydrogenase [7]. *A. caulinodans* ORS571 wild-type also grows aerobically with either nitrate or nitrite as sole utilizable N-source; both nitrate (AZC0679) and nitrite (AZC0680–AZC0682) reductases are soluble and assimilatory; neither nitrate nor nitric oxide serves as respiratory e^- acceptor nor are these activities suggested by analysis of the complete

Table 1. *A. caulinodans* Nuo (NADH:quinone oxidoreductase) and Hyq (*endo*-hydrogenase) structural homologs.

A. caulinodans complex I	EntrezGene identifier	*T. thermophilus* complex I	*A. caulinodans* hydrogenase	EntrezGene identifier	Identity[††]%	Conserved[††]%
L_1 subcomplex (membrane-peripheral)						
NuoB	AZC_1668	Nqo6	HyqI	AZC_4355	31	66
NuoC	AZC_1669	Nqo5	HyqG(N-term.)[†]	AZC_4356	23	57
NuoD	AZC_1670	Nqo4	HyqG(C-term.)[‡]		26	62
NuoE	AZC_1671	Nqo2				
NuoF	AZC_1672	Nqo1				
NuoG	AZC_1674	Nqo3				
NuoI	AZC_1676	Nqo9				
L_O subcomplex (membrane-integral)						
NuoA	AZC_1667	Nqo7				
NuoH	AZC_1675	Nqo8	HyqC	AZC_4359	23	58
NuoJ	AZC_1677	Nqo10	HyqE	AZC_4358	18	54
NuoK	AZC_1678	Nqo11				
NuoL	AZC_1679	Nqo12	HyqB	AZC_4360	24	55
NuoM	AZC_1680	Nqo13	HyqF	AZC_4357	22	61
NuoN	AZC_1667	Nqo14				

[†]5′-end of *hyqG* encodes residues 1–156;
[‡]3′-end of *hyqG* encodes residues 157–504;
[††]CLUSTAL 2.1 pairwise alignments.
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genome sequence [20]. Similarly, *A. caulinodans* test strains were aerobically cultured in defined medium supplemented with 5 m*M* nitrate as utilizable N-source. Upon reaching a cell density of $\sim 1\times10^8$ ml^{-1}, exponentially growing liquid cultures were shifted to 2% O_2 sparge and H_2 levels of exit gases were monitored as before. In this protocol, H_2 was evolved by *nifK*⁻ Δ*hupSL* double- mutant 66216R at baseline levels (Table 2A). By comparison, *exo*-, *endo*-hydrogenase double-mutant 66204 evolved H_2 at levels corresponding to those of optimized diazotrophic cultures (Table 2A,2B). Accordingly, physiological H_2 evolution at 20 μ*M* DOT was thus entirely owed to and benchmarked optimal Mo-dinitrogenase activity.

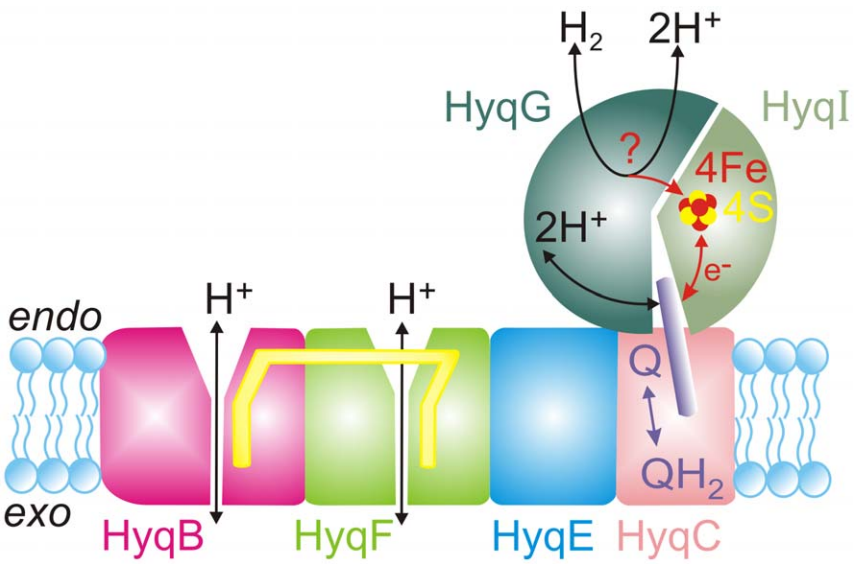

Figure 1. Structure-function rendering of L-type Hyq *endo*-hydrogenase by analogy and homology to respiratory complex I. Inferred membrane ubiquinone (Q) or ubiquinol (QH_2) binding at the interface of HyqC, HyqG and Hyq I requires partial (14Å) extraction from the respiratory membrane hydrophobic phase; yellow rods represent linked transmembrane and transverse α-helices [14]. Any HyqG catalytic site remains speculative; *in vivo* activity is in principle fully reversible (see Discussion).
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Table 2. H_2 evolution by *A. caulinodans* diazotrophic cultures.

A. caulinodans	Genotype	H_2 evolved[†]	relative H_2 evolved[‡]
(A) N_2 and NO_3^- as N-sources (20 μM DOT)			
66204	*ΔhyqRI ΔhupSL*	460	46.±5.0
66216R	*nifK ΔhupSL*	10	1.0±0.2
(B) N_2 as sole N-source (20 μM DOT; growth optimized)			
61305R	*nif*$^+$ *hyq*$^+$ *hup*$^+$	12	1.0±0.2
66081	*ΔhupSL*	16	1.3±0.3
66132	*ΔhyqRI*	175	15.±1.6
66204	*ΔhyqRI ΔhupSL*	540	45.±5.0
(C) N_2 as sole N-source (<1 μM DOT; microaerobic)			
61305R	*nif*$^+$ *hyq*$^+$ *hup*$^+$	7,100	1.0±0.2
66081	*ΔhupSL*	61,000	9.0±1.0
66132	*ΔhyqRI*	2,600	0.4±0.04
66204	*ΔhyqRI ΔhupSL*	14,000	2.2±0.4
(D) N_2 and NO_3^- as N-source (<1 μM DOT; microaerobic)			
60107R	*nifA*	1,100	1.0±0.2
66216R	*nifK ΔhupSL*	56,600	51.±3.0

[†]pmol 10^9·cells^{-1} min^{-1} (typical, single experiment);
[‡]multiple experiments.
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In Microaerobic (<1 μ*M* DOT) *A. caulinodans* Cultures, *in vivo endo*-hydrogenase Activity Reverses, Driving Hydrogenic Respiratory Membrane e^- transfer at Extraordinarily High Rates

For *A. caulinodans* chemostat cultures sparged with 0.2% or more O_2, elevated H_2 production is not observed [21]. This critical O_2 level corresponds to ≥0.9 μ*M* DOT, allowing *A. caulinodans* 57100 to be continuously cultured with succinate as C-source and N_2 as N-source with O_2 rate-limiting for growth [22]. In similar continuous cultures at <1 μ*M* DOT, *A. caulinodans* 57100 dinitrogenase activity levels decrease twofold [23] when compared to optimum (10–20 μ*M* DOT) diazotrophic culture conditions [21,22,23]. Accordingly, similar diazotrophic liquid batch cultures were initially sparged with 2% O_2, 5% CO_2, bal. N_2 for 24 hr allowing cell densities to reach $\sim 1\times10^8$ ml^{-1}, at which point sparge gas O_2 levels were decreased to 0.11%. In response, culture DOT levels declined precipitously, breaching 1 μ*M* DOT, true microaerobic physiology, defined as DOT insufficient to sustain conventional cytochrome *aa*$_3$ oxidase activity [2]. *A. caulinodans* microaerobic cultures employ two ultra-high O_2 affinity terminal oxidases, cyt*cbb*$_3$ and cyt*bd*, to maintain active oxidative phosphorylation [25]. When microaerobic cultures were supplemented with 5 m*M* L-glutamine and sampled periodically for viable cell counts by plating (Materials), all strains maintained exponential growth for 72+ hr; for all strains, microaerobic cell doubling-times were 8.1±1.5 hr at 29°C. Indeed, when strains were inoculated at low cell densities ($\sim 1\times10^6$ ml^{-1}) and cultured microaerobically (0.11% O_2 sparge) in minimal defined medium supplemented with 2.5 m*M* L-glutamine, all strains and cultures grew completely, and measurable H_2 evolution in sparged culture exit gases was insignificant. Whereas, when microaerobic cultures were supplied with sparged (95%) N_2 gas as sole N-source, no diazotrophy (cell doubling-times >20 hr) for any strain was measured. Neither was microaerobic growth observed when cultures were supplemented with 5 m*M* nitrate or nitrite. When 5 m*M* ammonium was supplied, microaerobic growth of test strains was variable. Strain 60107R *nifA* and 66132 Δ*hyq* cultures both yielded cell-doubling times of 9.5±0.5 hr; parental strain 61305R cultures yielded cell-doubling times of 14±0.8 hr; for all other strains tested, cell-doubling times exceeded 20 hr. In all cases, microaerobic growth with ammonium as N-source inversely correlated with H_2 evolution rates (Discussion).

Methylene blue (3,7-*bis*[dimethylamino]-phenothiazin-5-ium) serves as alternative e^- acceptor for respiratory complex I and, when reduced, as e^- donor to cyt*c*-dependent cytochrome oxidases, bypassing cytochrome *bc*$_1$ (respiratory complex III) activity and uncoupling oxidative phosphorylation [26]. Accordingly, methylene blue was deployed in microaerobic culture samples as *in vivo* respiratory e^- transfer probe. At experimentally sampled time points, culture samples were withdrawn into a gas tight syringe containing anoxic (colorless) methylene blue solution (2 μ*M* final); all culture samples initially turned visibly blue. However, when enclosed syringes were then held at 29°C, within 60 min all culture samples turned completely colorless (anoxic). When thus sampled, all microaerobic *A. caulinodans* cultures supplied excess succinate as C- and energy source retained respiratory-membrane e^- transfer activity for the duration of experiments (days).

Exit gas streams of sparged microaerobic cultures were sampled and H_2 evolution was again measured by gas chromatography. Relative to optimized diazotrophic cultures (20 μ*M* DOT), H_2 evolution of microaerobic (<1 μ*M* DOT), diazotrophic cultures dramatically increased. In parental 61305R cultures, microaerobic H_2 evolution rates increased more than fiftyfold. For *ΔhupSL* (*exo*-hydrogenase) mutant 66081, H_2 evolution rates increased almost four-thousandfold, which output persisted for 72+ hr. Yet, in *endo*-hydrogenase mutant 66132 cultures, H_2 evolution rates increased only fifteen-fold (Table 2C). H_2 evolution rates of all microaerobic batch cultures were sustained 72+ hr given sufficient oxidizable organic-C (succinate) as energy substrate. In conclusion, the extraordinarily high H_2 evolution rates of strain-specific microaerobic cultures required both *endo*-hydrogenase present and *exo*-hydrogenase absent.

Recall that Mo-dinitrogenase operates in concert with *endo*-hydrogenase activity given optimum (20 μ*M* DOT) diazotrophic physiology. To what extent does Mo-dinitrogenase activity contribute to H_2 output by microaerobic (<1 μ*M* DOT) cultures? To test this hypothesis, similar microaerobic shift experiments were conducted with cultures grown with and maintained on 5 m*M* nitrate as N-source, which allows full transcriptional derepression of the N_2 fixation regulon [19]. Upon microaerobic shift, nitrate-grown *nifK*$^-$ Δ*hupSL* double-mutant 66216R likewise showed exceedingly high H_2 output (Table 2D). Note that for all strains, microaerobic cultures with nitrate fail, implying nitrate is not then a competing e^- acceptor. Moreover, nitrate itself has no inducible effect on H_2 evolution; *nifA* null mutant 60107R, which entirely lacks ability to derepress the N_2 fixation regulon [19], shows negligible H_2 output in microaerobic nitrate-supplemented culture (Table 2D). Thus, Mo-dinitrogenase activity itself contributes little (<10%) of the exceedingly high H_2 output by Δ*hupSL* mutant microaerobic cultures. Moreover, because Δ*hyqRI* Δ*hupSL* double-mutant 66204 retains 20% microaerobic H_2 evolution rates when compared to Δ*hupSL* single-mutant 66081 (Table 2C), an additional, uncharacterized H_2 source is then operative. As it is absent in *nifA* mutant 60107R (Table 2D), this additional

microaerobic H_2 source also seems associated with the N_2 fixation regulation. In summary, *endo*-hydrogenase activity is itself responsible for ~80% of the H_2 evolved, *i.e.*, net hydrogenic respiratory membrane e^- transfer rates, by microaerobic cultures in which the N_2 fixation regulon is derepressed.

Discussion

In summary, *A. caulinodans endo*-hydrogenase is a bidirectional catalyst whose *in vivo* activity reverses in response to physiological O_2 availability. In optimized diazotrophic (20 μ*M* DOT) cultures, *endo*-hydrogenase operates in H_2 uptake mode, consuming endogenous H_2 as respiratory e^- donor. In microaerobic (<1 μ*M* DOT) cultures, membrane-integral *endo*-hydrogenase switches to hydrogenic respiratory-membrane e^- transfer mode, employing H^+ ions as terminal e^- acceptor. Accordingly, *endo*-hydrogenase shares a microaerobic terminal oxidase role with cytochrome *cbb*$_3$ and cytochrome *bd* [25], whereas respiratory complexes I (NADH:quinone oxidoreductase) and II (succinate dehydrogenase) serve as net e^- donors. Measured *in vivo* respiratory-membrane *endo*-hydrogenase H_2 production rates (45,000 pmol 10^9·cells^{-1} min^{-1}) are orders of magnitude higher than previously observed for O_2 tolerant hydrogenases in non-fermentative microorganisms. In *A. caulinodans exo*-hydrogenase mutants lacking microaerobic H_2 uptake activity, respiratory membrane *endo*-hydrogenase mediated H_2 production in liquid batch cultures persists at high rates for 72+ hr. Sum totals of evolved H_2 ($2e^-$ reduction) at 72 hr are 230±30 μmol per 10^9 cells, representing net oxidation of some 25% of total (340 μmol) succinate supplied these cultures as sole organotrophic energy source and quantitatively converted to *poly*-β-hydroxbutyrate as organic end-product ($5e^-$ oxidation per succinate; [24,27]).

Similarly, hydrogenic Mo-dinitrogenase activity, at most 10% of hydrogenic *endo*-hydrogenase activity, consumes almost tenfold more NADH on a mole:mole basis (4 NADH for reductant; 5+ NADH as substrate for oxidative phosphorylation to make the required 16 ATP) [10,11]. Then, Mo-dinitrogenase activity itself consumes similar amounts of succinate. Unsurprisingly, for all strains tested, diazotrophic (N_2 as sole N-source) microaerobic liquid batch cultures open to the environment fail (cell doubling-times >20 hr). Whereas, all strains may be successfully cultured microaerobically with L-glutamine provided as N-source absent all hydrogenesis. When provided ammonium and N_2 as N-sources, some strains grow microaerobically, albeit slowly as significant N_2 fixation persists; any growth inversely correlates with hydrogenesis by diazotrophic microaerobic cultures (Table 2C). When atmospheric N_2 is entirely replaced by argon, ammonium-supplemented microaerobic cultures indeed grow [27], hence Mo-dinitrogenase activity is explicitly responsible for failed microaerobic growth. Earlier, we reported *A. caulinodans* microaerobic diazotrophic liquid suspension cultures showed increased spectrophotometric absorbance at 600 nm [25]. In more recent experiments, growth in microaerobic diazotrophic liquid suspension cultures was measured by removing samples and aerobically plating for viable cell counts on rich media (Materials) as, in these cultures, viable cell counts do not correlate with increased spectrophotometric absorbance at 600 nm. Likewise, colony growth tests on solid media reflect multiple cell physiology states, and colony growth is facilitated by more efficient H_2 recycling at increased cell densities [12].

Among bacteria with known genome sequences, eight genera (A. caulinodans, Azospirillum brasilense, Beijerinckia indica, Bradyrhizobium japonicum; Rhizobium leguminosarum bv. viciae, Rhodopseudomonas palustris, Rhodocista centenaria, Xanthobacter autotrophicus), all microaerophiles capable of N_2 fixation, possess orthologous hyq^+ operons encoding endo-hydrogenase [12]. Of the six, inferred endo-hydrogenase subunits, five have close homologs in L-type respiratory complex I (Table 1). The sixth (HyqG) protein is homologous to a fused NuoC/D protein. The presumed HyqG H_2 catalytic site shared among conserved group-4 hydrogenases is yet undetermined. The group-4 HyqG superfamily (SSF56762) also includes the group-1 exo-hydrogenase catalytic subunit, which possesses a heteronuclear Ni,Fe catalytic center coordinated by four, completely conserved Cys residues, of which two bridge the catalytic Ni and Fe = C = O binuclear center [28]. To the contrary, inferred HyqG proteins from eight microaerophilic genera all lack both N-terminal and C-terminal Cys-X-X-Cys motifs. Rather, three Cys residues (A. caulinodans Cys-258, Cys-491, and Cys-497) are completely conserved by the HyqG family (Fig. S7). The NuoD (Nqo4) proteins of bacterial respiratory complex I, also members of this same Superfamily, neither possess the Cys-X-X-Cys pairs nor do they exhibit a Ni,Fe binuclear center. Therefore, that HyqG actually carries a binuclear Ni,Fe catalytic site seems uncertain, if not unlikely. The binding site for respiratory complex I membrane ubiquinone, its ultimate e^- acceptor, is a cavity formed between a four-helix bundle of NuoD (Nqo4), the H1 helix of NuoB (Nqo6), and transmembrane helix 1 of NuoH (Nqo8) [14,18], all of which motifs are conserved in the Hyq endo-hydrogenase (HyqG, HyqI, and HyqI, respectively).

By inference, the Hyq *endo*-hydrogenase is a membrane-integral H_2:quinone oxidoreductase. Given the strong reducing potential of the biochemical standard hydrogen electrode ($E_o' = -0.414V$) relative to that of ubiquinone ($E_o' = +0.070V$) respiratory-membrane hydrogenesis is a highly endergonic process under standard conditions. In *Rps. palustris*, membrane physiology has been modeled under a variety of dynamic steady-state conditions including microaerobic respiration, whose membrane ubiquinone pools are necessarily highly (>90%) reduced [29]. If membrane ubiquinone pools were poised some 90% reduced, a ubiquinon/ubiquinol half-cell potential (E') of $+0.040V$ (at 25°C) would obtain. By inference, the balanced reaction for *endo*-hydrogenase mediated H_2 uptake, including trans-membrane H^+ pumping, may be written:

$$H_2 + Q + (2)\,H^+_{(N)} \;\Leftrightarrow\; QH_2 + (2)\,H^+_{(P)} \qquad (1)$$

where *N* connotes *endo* and *P* connotes *exo* membrane faces. In purified, reconstituted vesicles, respiratory complex I activity, including H^+ translocation, is fully reversible [30]. If the same holds true for *endo*-hydrogenase *in vivo*, its hydrogenesis mode activity may be written:

$$QH_2 + (2)\,H^+_{(P)} \;\Leftrightarrow\; H_2 + Q + (2)\,H^+_{(N)} \qquad (2)$$

This activity would tap steady-state membrane proton-motive force (Δp), modeled in *Rps. palustris* microaerobic respiratory membranes as $\Delta p = 0.195V$ [29]. Were $2H^+(p)$ counter-transported during steady-state hydrogenesis, E' values at 25°C would be effectively lowered from $+0.040V$ to $-0.350V$, at which steady-state potential the operative hydrogen half-cell H_2 partial pressure (pH_2) would approach 0.7 kPa at 25°C. Indeed, when exit gases of sparged *A. caulinodans* hydrogenic cultures maintained at 30°C were analyzed, pH_2 levels reproducibly approached 0.7 kPa as sustained hydrogenesis rates. In all likelihood, high-level *endo*-hydrogenase dependent H_2 production requires both highly

reduced membrane ubiquinone pools and high Δp values. In *A. caulinodans* microaerobic liquid batch cultures, elevated H_2 production requires supplementation with excess, primary C-source (succinate, L-malate, or L-lactate), which presumably drive reduction of respiratory membrane ubiquinone pools at relatively high rates. Moreover, if indeed consumptive of membrane Δp, respiratory membrane hydrogenesis only operates when sufficient O_2 is also available as respiratory e^- acceptor to regenerate high Δp (respiratory complex I, III, and IV activities). Regardless, any respiratory-membrane hydrogenesis would necessarily largely uncouple oxidative phosphorylation.

For *A. caulinodans* microaerobic respiration, both available (limiting) O_2 and H^+ ions simultaneously serve as e^- acceptors. *A. caulinodans* then employs multiple cyt*c*- and ubiquinol-oxidases [25], resulting in varied respiratory membrane proton-translocation yields. So, no fixed stoichiometries of H_2 relative to H_2O production may be deduced. Moreover, parceling out relative *in vivo* contributions as respiratory membrane e^- acceptors is problematic, given restricted choice. When one or more e^- acceptor activities are absent due to mutation, compensatory flux to soluble e^- acceptors, such as N_2 (Mo-dinitrogenase), NO_3^- (nitrate reductase), and CO_2 (both rubisco and CO dehydrogenase) then obtains. As one example, H_2 evolution rates for *exo- endo*-hydrogenase double-mutant 66204 increased twenty-five-fold when shifted from growth optimal (20 μM DOT) to microaerobic (<1 μM DOT) conditions, presumably owed to restricted choice of available e^- acceptors (*i.e.*, relative absence of O_2). Moreover, restricted choice also extends to e^- donors. Because it successfully reoxidizes >80% of H_2 then produced by combined (>90%) *endo*-hydrogenase and (<10%) Mo-dinitrogenase activities, *exo*-hydrogenase operates as a relatively more competitive microaerobic respiratory membrane e^- donor. Conceivably, these changes in cellular microaerobic respiratory membrane physiology might simply reflect more-reduced ubiquinone pools. Alternatively, respiratory membranes might build *exo*- and/or *endo*-hydrogenases into macromolecular complexes which would preclude simple diffusion control of respiratory e^- transfer by membrane ubiquinone pools.

In *A. caulinodans*, the two, respiratory membrane hydrogenases possess distinct physiological roles; group-4 *endo*-hydrogenase activity is bidirectional and strictly correlates with diazotrophy and endogenous H_2 uptake, whereas group-1 *exo*-hydrogenase activity is unidirectional and also allows chemoautotrophy with exogenous H_2 as energy source [7]. Among capable anaerobes, fermentative membrane hydrogenesis is well described [1,2,5]. Whereas, among obligate aerobes, respiratory membrane hydrogenesis as a sustained physiological process seems counterproductive, as it significantly uncouples oxidative phosphorylation. Indeed, in diazotrophic microaerobic *A. caulinodans* cultures, *exo*-hydrogenase and *endo*-hydrogenase are both highly active and thus seemingly operative at cross-purposes.

However, liquid culture experiments, which strive to allow all bacterial cells a similar physiological milieu, are contrived. In reality, bacterial cell populations experience a dimensional world. We suggest, as one possibility, concomitant H_2 evolution and H_2 uptake might prove useful if partitioned among aerobic and microaerobic bacterial cells in dimensional populations. Varying O_2 microenvironments within organotrophic bacterial colonies or biofilms might *de facto* segregate metabolic physiology, allowing internal O_2-restricted cells to evolve H_2 and external O_2-sufficient cells to take up and use that H_2, driving oxidative phosphorylation. Superficial H_2 oxidizing, O_2 rich cells might then redirect environmental organic-C sources away from catabolism (oxidative phosphorylation substrate) towards anabolism (C-assimilation), augmenting growth rates and proliferation of dimensionally organized and specialized cell populations considered as a whole.

Materials and Methods

Bacterial Strains and Media

Azorhizobium caulinodans ORS571 wild-type (strain 57100; ATCC No. 43989), was originally isolated from *Sesbania rostrata* stem-nodules [8]. Strain 61305R [32], a 57100 derivative carrying an IS*50*R insertion in the (catabolic) nicotinate dehydrogenase structural gene served as 'virtual' wild-type for reported experiments; 61305R uses supplied (3 μM) nicotinate only as anabolic substrate for synthesis of pyridine nucleotides, for which 57100 is auxotrophic [33]. Precise, in-frame deletion mutagenesis of *A. caulinodans* target genes was conducted out by "crossover PCR" as previously described [34]. Strain 66216R was constructed as for strain 66081 with strain 60057R as parent (Table 3). Defined media for all cultures was basal NIF medium (7.5 mM potassium phosphate pH 6.3, 1 mM $MgSO_4$, 0.5 mM $CaCL_2$, 2 μM ferric citrate, 3 μM nicotinate, 1 μM sodium molybdate, 1 μM pantothenate, 0.1 μM D-biotin, and Hutner's "44′ trace elements [35]) supplemented with 20 mM potassium succinate as sole C- and energy source, and 2.5 mM ammonium bicarbonate as N-source. Strains (whose lineage does not include 61305R) and which actively catabolize nicotinate were supplemented with 0.1 mM nicotinate in aerobic cultures; in microaerobic cultures, *A. caulinodans* wild-type 57100 does not measurably catabolize nicotinate.

Physiological Growth Measurements and Evolved H_2 Analyses

Starter cultures of *A. caulinodans* strain 61305R and its derivatives were aerobically cultured in minimal defined NIF liquid medium [8] supplemented with: 0.3 mM ammonium as sole, limiting N-source and 3 μM nicotinate at 37°C until growth arrest (cell densities $\sim 1\times10^8$ cells ml^{-1}). For kinetic measurements of diazotrophy, arrested starter cultures were each diluted one-hundredfold in 20 ml NIF medium; serum vials (30 ml capacity) were sealed with silicone rubber septa, sparged continuously (10 ml min^{-1}) with defined gas mixtures (*e.g.* 2% O_2, 5% CO_2, bal. N_2), and incubated at 29°C. At least three times per cell-doubling period, culture samples were removed, serially diluted, plated on rich GYPC medium [9], and incubated aerobically 48 hr at 37°C; colonies were counted in triplicate. *In vivo* H_2 uptake activities were inferred, coupled to Mo-dinitrogenase activity as H_2 donor, by comparing rates of H_2 evolution from *A. caulinodans hup*$^+$ *hyq*$^+$ (wild-type), Δ*hupSL* (*exo*-hydrogenase) mutant, Δ*hyqRI* (*endo*-hydrogenase) mutant, and Δ*hupSL* Δ*hyqRI* double-mutant cultures (Table 3). Collectively, both hydrogenases account for all *in vivo* H_2 uptake

Table 3. *Azorhizobium caulinodans* strains.

Strain	Genotype	Ref.
57100	ORS571 wild-type	[8]
60035	57100 *nifD35::Vi*	[31]
60035R	60035 *nifD35*::IS*50*R	
60057R	60057 *nifK57*::IS*50*R	
60107R	57100 *nifA107R*	
61305R	57100 Nic^-, 6-OH-Nic^+	[32]
66081	61305R *hupΔSL2*	[7]
66132	61305R *hyqΔRI7*	[7]
66204	61305R *hupΔSL2 hyqΔRI7*	[7]
66216R	60057R *hupΔSL2*	

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activity [12]. (Amperometric cell-free assay of isolated *endo*-hydrogenase H_2 uptake activity with exogenous H_2 as substrate is not at hand.) To measure evolved H_2, sparged culture exit gas streams were sampled and analyzed by gas chromatography (RPC1; Peak Laboratories LLC) fitted with an HgO (reducing compound) photometer as detector [36] and a fixed volume (25 µl) sampling loop. Molar H_2 evolution rates were inferred from measured dilution rates of culture atmospheric volumes. Total cellular protein was measured by the bicinchoninic acid procedure (Sigma-Aldrich Co.); for *A. caulinodans* and related microaerophiles employing oxidative metabolic gearing, mean total cell proteins levels (135±15 fg) are largely independent of cell physiology [24].

Supporting Information

Figure S1 *A. caulinodans* HyqB and NuoL (CLUSTAL W2) alignment.
(EPS)

Figure S2 *A. caulinodans* HyqC and NuoH (CLUSTAL W2) alignment.
(EPS)

Figure S3 *A. caulinodans* HyqE and NuoJ (CLUSTAL W2) alignment.
(EPS)

Figure S4 *A. caulinodans* HyqF and NuoM (CLUSTAL W2) alignment.
(EPS)

Figure S5 *A. caulinodans* HyqG and NuoC/D (CLUSTAL W2) alignment.
(EPS)

Figure S6 *A. caulinodans* HyqI and NuoB (CLUSTAL W2) alignment.
(EPS)

Figure S7 HyqG orthologs (CLUSTAL W2) multiple alignment. From the top, species are: *Rhizobium leguminosarum* (Rhleg), *Azorhizobium caulinodans* (Azoca), *Xanthobacter autotrophicus* (Xanpy2), *Beijerinckia indica* (Beind), *Bradyrhizobium japonicum* (Braja), *Rhodospirillum centenum*, (Rhoce), *Rhodopseudomonas palustris* (BisA53), *Azospirillum brasilense* (Azobr), *Rhodopseudomonas palustris* (HaA2), *Rhodopseudomonas palustris* (BisB18), *Rhodopseudomonas palustris* (BisB5).
(EPS)

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Author Contributions

Conceived and designed the experiments: RL. Performed the experiments: BS MG RL. Analyzed the data: BS MG RL. Contributed reagents/materials/analysis tools: BS MG RL. Wrote the paper: RL.

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