

A soil actinobacterium scavenges atmospheric H₂ using two membrane-associated, oxygen-dependent [NiFe] hydrogenases

Chris Greening^{a,b}, Michael Berney^{a,c}, Kiel Hards^a, Gregory M. Cook^{a,1}, and Ralf Conrad^{b,1}

^aDepartment of Microbiology and Immunology, University of Otago, Dunedin 9016, New Zealand; ^bMax-Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany; and ^cDepartment of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461

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In the Earth's lower atmosphere, H₂ is maintained at trace concentrations (0.53 ppmv/0.40 nM) and rapidly turned over (lifetime ≤ 2.1 y⁻¹). It is thought that soil microbes, likely actinomycetes, serve as the main global sink for tropospheric H₂. However, no study has ever unambiguously proven that a hydrogenase can oxidize this trace gas. In this work, we demonstrate, by using genetic dissection and sensitive GC measurements, that the soil actinomycete *Mycobacterium smegmatis* mc²155 constitutively oxidizes subatmospheric concentrations of H₂. We show that two membrane-associated, oxygen-dependent [NiFe] hydrogenases mediate this process. Hydrogenase-1 (Hyd1) (MSMEG_2262-2263) is well-adapted to rapidly oxidize H₂ at a range of concentrations [$V_{\max(\text{app})} = 12 \text{ nmol}\cdot\text{g}\cdot\text{dw}^{-1}\cdot\text{min}^{-1}$; $K_{\text{m}(\text{app})} = 180 \text{ nM}$; threshold = 130 pM in the $\Delta\text{hyd}23$ (Hyd1 only) strain], whereas Hyd2 (MSMEG_2719-2720) catalyzes a slower-acting, higher-affinity process [$V_{\max(\text{app})} = 2.5 \text{ nmol}\cdot\text{g}\cdot\text{dw}^{-1}\cdot\text{min}^{-1}$; $K_{\text{m}(\text{app})} = 50 \text{ nM}$; threshold = 50 pM in the $\Delta\text{hyd}13$ (Hyd2 only) strain]. These observations strongly support previous studies that have linked group 5 [NiFe] hydrogenases (e.g., Hyd2) to the oxidation of tropospheric H₂ in soil ecosystems. We further reveal that group 2a [NiFe] hydrogenases (e.g., Hyd1) can contribute to this process. Hydrogenase expression and activity increases in carbon-limited cells, suggesting that scavenging of trace H₂ helps to sustain dormancy. Distinct physiological roles for Hyd1 and Hyd2 during the adaptation to this condition are proposed. Soil organisms harboring high-affinity hydrogenases may be especially competitive, given that they harness a highly dependable fuel source in otherwise unstable environments.

atmospheric chemistry | biogeochemical cycles | enzyme kinetics | mycobacteria

Although hydrogen is the universe's most fundamental and abundant element, H₂ is present at only trace concentrations in the Earth's atmosphere. The biogeochemical cycle of molecular hydrogen results in rapid turnover of tropospheric H₂ (lifetime = 1.4–2.1 y⁻¹) (1) and maintains its levels at low steady-state concentrations (0.53 ppmv/0.40 nM) (2). It is well-established that geochemical and anthropogenic processes are responsible for the majority of net H₂ production (3, 4). The role of biological processes is more complex. Because of intra- and interspecies hydrogen cycling, most microbial H₂-oxidizing and H₂-producing processes are balanced and hence make minor or null net contributions to the global hydrogen budget (5). It has nevertheless been known for several decades that soil ecosystems can oxidize tropospheric H₂ in an apparent enzymatic process (6–8). This ecologically essential process is estimated to be accountable for 80% of the net loss of tropospheric H₂ (3). However, the enzymes responsible remain to be resolved.

GC and tritium exchange measurements in soil samples have demonstrated that H₂ oxidation by soil is a biphasic process, containing both low-affinity ($K_{\text{m}} > 1,000 \text{ nM}$) and high-affinity ($K_{\text{m}} = 10\text{--}70 \text{ nM}$) activities (9, 10). These activities are thought to be catalyzed by [NiFe] hydrogenases, of which there are five defined phylogenetically and functionally distinct groups (11, 12).

The majority of characterized hydrogen-oxidizing soil bacteria (e.g., *Paracoccus denitrificans*, *Ralstonia eutropha*) have a low affinity for H₂ ($K_{\text{m}} \geq 1 \mu\text{M}$, threshold $\geq 1 \text{ nM}$) and hence are probably responsible for the former process; their uptake hydrogenases (principally group 1 [NiFe] hydrogenases) serve to recycle the relatively high levels of H₂ produced by biological processes (8, 13, 14). However, it has recently been shown that certain soil-dwelling actinomycetes are capable of scavenging H₂ at tropospheric concentrations (12, 15). The first isolated high-affinity H₂-oxidizer, *Streptomyces* sp. PCB7, can oxidize H₂ with Michaelis–Menten kinetics with an apparent $K_{\text{m}} \leq 30 \text{ nM}$ and a threshold concentration $\leq 0.1 \text{ nM}$ (15–17). An earlier study demonstrated that *Mycobacterium smegmatis* can oxidize low concentrations of H₂, but did not determine the affinity or threshold of this activity (18).

It is proposed that group 5 [NiFe] hydrogenases are responsible for high-affinity H₂ oxidation in streptomycetes (12, 17). The genes encoding this newly discovered hydrogenase lineage (*hhyLS*) are highly conserved and taxonomically restricted; among sequenced genomes, this hydrogenase is only widespread in environmental actinomycetes, including *Streptomyces*, *Mycobacterium*, *Rhodococcus*, and *Frankia* species (Fig. S1B) (17, 19). However, the evidence that these enzymes are responsible for the observed activity is correlative and somewhat conflicting. High-affinity hydrogenase activity has never been unambiguously determined, e.g., through purifying proteins or making genetic deletions. In addition, many organisms harboring *hhySL* genes

Significance

Molecular hydrogen is present in trace concentrations in the Earth's lower atmosphere and is rapidly turned over through a biogeochemical cycle. Microbial soil processes are responsible for the majority of net uptake of H₂ from the atmosphere, but the enzymes involved have remained elusive. In this communication, we use genetic dissection and GC measurements to show that the soil actinobacterium *Mycobacterium smegmatis* can oxidize trace concentrations of H₂. This process depends on two oxygen-dependent, membrane-associated [NiFe] hydrogenases with picomolar thresholds. Activity is most pronounced during carbon limitation, suggesting that mycobacteria oxidize a dependable trace gas to survive starvation. We propose that these enzymes and their homologs in other actinobacteria and soil phyla constitute the principal sink of global atmospheric H₂.

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¹To whom correspondence may be addressed. E-mail: gregory.cook@otago.ac.nz or conrad@mpi-marburg.mpg.de.

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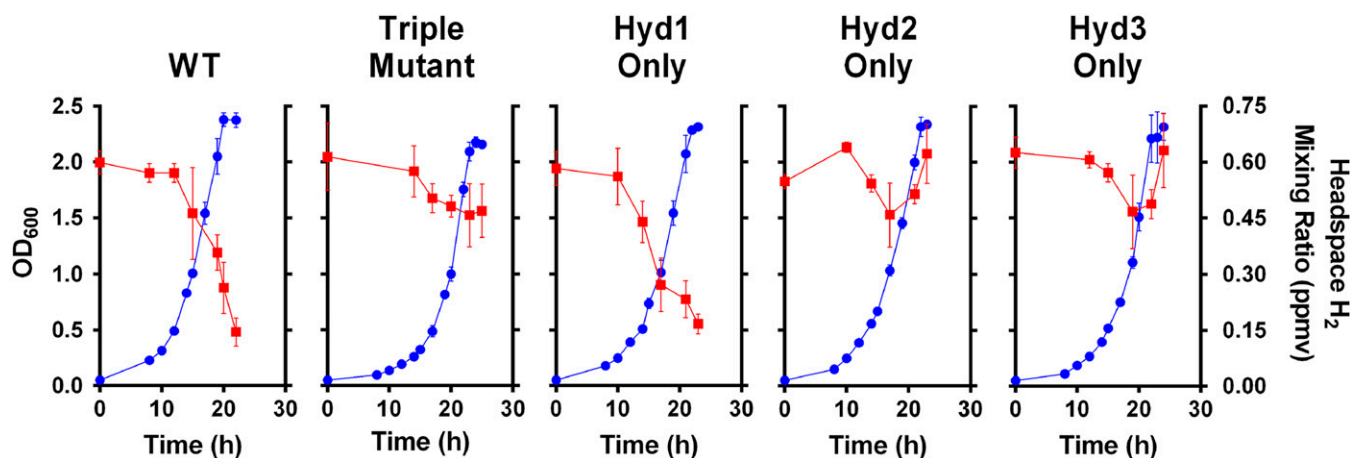


Fig. 1. H₂ oxidation during exponential growth of *M. smegmatis* mc²¹⁵⁵ and derivatives in sealed serum vials. H₂ mixing ratios were measured upon inoculation of culture and throughout growth. All strains entered stationary phase at OD ~ 2.5 (~1.5 × 10⁸ cfu·mL⁻¹) after consuming the majority of the oxygen available in the headspace and hence becoming oxygen-limited. Blue circles represent OD₆₀₀ of the cultures. Red squares represent the H₂ mixing ratio in the headspace. Error bars are SDs from three biological replicates.

only have low to medium affinities for H₂ (e.g., *Streptomyces scabiei*, *Streptomyces griseoflavus*) (17). Furthermore, the recently purified group 5 [NiFe] hydrogenase from the β-proteobacterium *R. eutropha* is a low-affinity, low-activity enzyme (20).

Our laboratory has used *M. smegmatis* as a model organism to study hydrogen metabolism in soil actinomycetes. This saprophytic, nonsporulating bacterium is known for its ability to survive in nonreplicating states when carbon and oxygen sources become limiting (21, 22). The organism expresses three phylogenetically distinct [NiFe] hydrogenases, Hydrogenase-1 (Hyd1; MSMEG_2262-2263), Hydrogenase-2 (Hyd2; MSMEG_2719-2720), and Hydrogenase-3 (Hyd3; MSMEG_3928-3932) (19, 23). The group 2a [NiFe] hydrogenase Hyd1 and the group 5 [NiFe] hydrogenase Hyd2 are known to oxidize H₂ (23). They are expressed at low levels during exponential growth and are significantly up-regulated during carbon or oxygen limitation (19, 23). By contrast, the group 3b enzyme Hyd3 (MSMEG-3928-3932) is predicted to couple NADPH oxidation to H₂ evolution and is induced in oxygen-limitation by the dormancy response regulator DosR (23). Homologs of all three hydrogenases are distributed in other actinomycetes (Fig. S1). In this study, to confirm the determinants of high-affinity H₂ oxidation, we compared the kinetics of H₂ uptake by *M. smegmatis* mc²¹⁵⁵ and strains harboring markerless deletions of its hydrogenases. We definitively confirm that Hyd1 and Hyd2 can oxidize tropospheric H₂ and have distinct but overlapping characteristics.

Results

***M. smegmatis* mc²¹⁵⁵ Constitutively Oxidizes Tropospheric H₂.** H₂ oxidation was measured in exponentially growing and carbon-limited cultures of *M. smegmatis* mc²¹⁵⁵ in sealed serum vials. In both conditions, the wild-type (WT) strain oxidized the majority of the H₂ in the headspace (Fig. 1 and Fig. S2). Activity only occurred when the cultures were aerated and agitated, suggesting that O₂ serves as the terminal electron acceptor. Consistent with [NiFe] hydrogenases being responsible for this activity, oxidation was fourfold faster when exogenous NiSO₄ was introduced in the media (Fig. S2). Oxidation continued when subtropospheric concentrations of H₂ were available. During exponential growth in a headspace of ambient air, the WT strain consumed the majority of the H₂ available (initial mixing ratio ~ 0.60 ppmv; final mixing ratio ~ 0.14 ppmv). This activity was dependent on Hyd1 and did not occur in strains harboring markerless deletions of this enzyme (Δ hyd1, Δ hyd12, Δ hyd13, and Δ hyd123; Fig. 1).

Hyd1 and Hyd2 Oxidize Tropospheric H₂ in Kinetically Distinct Activities.

Hydrogenase activity was subsequently measured in nonreplicating carbon-limited cultures of the *M. smegmatis* strains over an extended time course. Consistent with the increased expression of Hyd1 and Hyd2 during carbon limitation (23), Hyd1 was more active in this condition and Hyd2 activity could be resolved (Fig. 2). The WT strain mc²¹⁵⁵ oxidized the available headspace H₂ (initial mixing ratio ~ 6.3 ppmv) in a first-order kinetic process (Fig. 2 and Fig. S3). This activity was mainly attributable to the group 2a [NiFe] hydrogenase Hyd1; the Δ hyd23 (Hyd1 only) strain rapidly oxidized H₂ to subtropospheric concentrations (final mixing ratio ~ 0.21 ppmv). However, high-affinity activity corresponding to the group 5 [NiFe] hydrogenase Hyd2 was also observed; the Δ hyd13 (Hyd2 only) strain slowly oxidized the headspace H₂ and functioned at even lower concentrations than the Δ hyd23 strain (final mixing ratio ~ 0.07 ppmv). The strain lacking Hyd3 (Δ hyd3) behaved similarly to WT, reinforcing that this enzyme is not expressed in

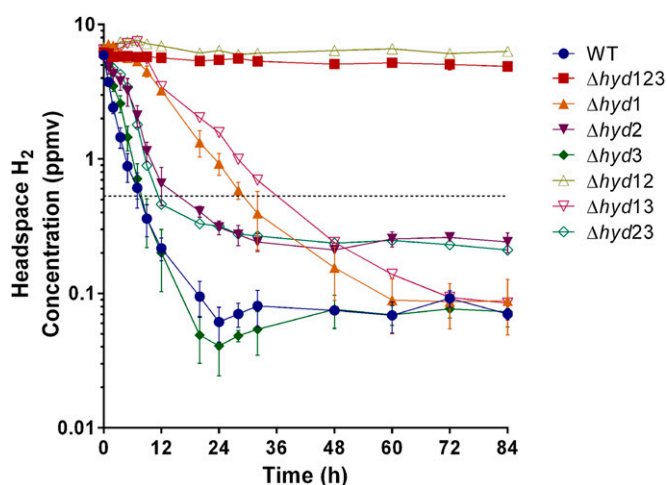


Fig. 2. H₂ oxidation by carbon-limited cultures of *M. smegmatis* mc²¹⁵⁵ and derivatives. All strains were inoculated to a density of 3 × 10⁷ cfu·mL⁻¹ (OD₆₀₀ = 0.4). H₂ mixing ratios were measured upon inoculation of culture and at regular intervals over 84 h. The mixing ratios are displayed on a logarithmic scale. Error bars represent SDs from three biological replicates. The dashed lines represent the average global mixing ratio of tropospheric H₂ (0.53 ppmv).

Table 1. Kinetic parameters of hydrogenase activity measured in whole cells

Parameter	WT	Hyd1 only	Hyd2 only
$V_{\max(\text{app})}$, nmol g dw ⁻¹ min ⁻¹	16.3 ± 1.5	12.3 ± 1.7	2.5 ± 0.8
$k_{(\text{app})}$, nmol g dw ⁻¹ min ⁻¹	0.069 ± 0.005	0.067 ± 0.013	0.019 ± 0.0015
$K_{m(\text{app})}$, nM	113 ± 11	184 ± 40	51 ± 16
Threshold, pM	51 ± 13	133 ± 7	54 ± 3

SDs were calculated from three biologically independent replicates.

aerobic cultures. In the strain lacking any hydrogenases ($\Delta\text{hyd}123$), no significant changes in headspace H₂ concentration could be observed.

To further profile the hydrogenases, the kinetic parameters of H₂ oxidation were determined in carbon-limited cultures of the WT, $\Delta\text{hyd}23$ (Hyd1 only), and $\Delta\text{hyd}13$ (Hyd2 only) strains incubated at low (6.3 ppmv) and high (500, 1,000, and 2,000 ppmv) initial mixing ratios of H₂ (Table 1). In both cases, Hyd1 consumed the majority of the H₂ present and Hyd2 activity was fivefold lower. Whereas Hyd1 is well-adapted to function at a wide range of H₂ concentrations [$K_{m(\text{app})}$ = 184 ± 40 nM; threshold = 133 ± 7 pM], Hyd2 can oxidize lower concentrations of H₂ overall [$K_{m(\text{app})}$ = 51 ± 16; threshold = 54 ± 3 pM]. Although it is clear that Hyd1 is the dominant uptake hydrogenase in *M. smegmatis*, it remains to be fully resolved whether the enzyme is intrinsically faster than Hyd2 or instead more abundant in the cell.

Hydrogen Oxidation Is Membrane-Associated. The localization of the uptake hydrogenases was determined by using zymographic and amperometric assays on cell fractions (Fig. 3). Hyd2 efficiently coupled the oxidation of H₂ to the reduction of the artificial electron acceptor/redox dye nitroblue tetrazolium chloride (24). This activity was enriched in the membrane fraction (Fig. 3B). Attempts to stain with more electronegative acceptors, such as benzyl viologen [E'_0 = -360 mV (24)], were unsuccessful. Although Hyd1 activity could not be visualized zymographically, this highly active enzyme could be detected by using an H₂ microsensor. As previously observed in cyanobacterial group 2a [NiFe] hydrogenases (25), its activity was concentrated in the membrane fractions of the WT and $\Delta\text{hyd}23$ strains (Fig. 3C). Hyd1 and Hyd2 lack predicted transmembrane helices and signal peptides, and are not transcribed with genes encoding probable membrane proteins (PFAM/TMHMM analysis). They are therefore unlikely to be classical membrane-bound proteins and instead may associate with the cytosolic side of the membrane through amphiphilic helices or protein-protein interactions.

Discussion

In summary, this work demonstrates that *M. smegmatis* mc²155 oxidizes tropospheric H₂ by using two membrane-associated, oxygen-dependent [NiFe] hydrogenases. We definitively confirm that group 5 [NiFe] hydrogenases can mediate high-affinity H₂ oxidation and provide strong support for the hypothesis that these enzymes are the principal sinks for atmospheric H₂. The kinetic parameters of whole-cell H₂ oxidation by Hyd2, as observed in isolation in the $\Delta\text{hyd}13$ strain and deleted in the $\Delta\text{hyd}2$ strain, are very similar to the high-affinity processes observed in whole soil samples (9, 10) and cultured streptomycetes (12, 15) (Fig. 4). Genes encoding group 5 [NiFe] hydrogenases are widespread in soil members of the order *Actinomycetales* and are also distributed in undercultured soil phyla such as *Acidobacteria* (Fig. S1B). It is also evident from this study that some group 2a [NiFe] hydrogenases have the capacity to oxidize low concentrations of H₂; however, these enzymes are likely to make a smaller contribution to the global hydrogen budget, given they are found in fewer

sequenced actinomycetes (Fig. S1A) (23) and primarily serve to recycle nitrogenase-derived H₂ in other organisms (e.g., cyanobacteria) (25).

The ability to oxidize tropospheric H₂ may serve as a major selection pressure in soil ecosystems. These environments are

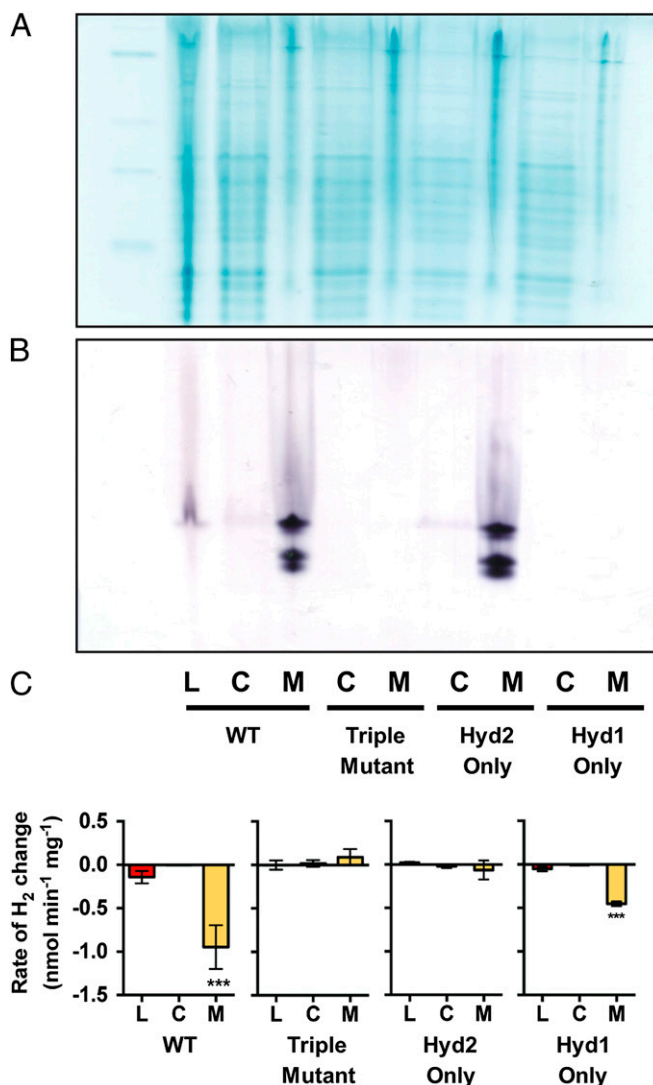


Fig. 3. Determination of uptake hydrogenase localization. Protein samples of each cell fraction (5 μ g) were separated on native polyacrylamide gels. (A) Total protein content was stained by using Coomassie brilliant blue. (B) Uptake hydrogenase activity was detected by using the artificial electron acceptor nitroblue tetrazolium chloride. (C) Cell fractions were added to a reaction chamber saturated with H₂ and O₂. A H₂ microsensor determined the rates of hydrogen consumption (negative values) or production (positive values). Error bars represent SDs from three technical replicates. C, cytosolic fraction; L, crude cell lysate; M, membrane fraction.

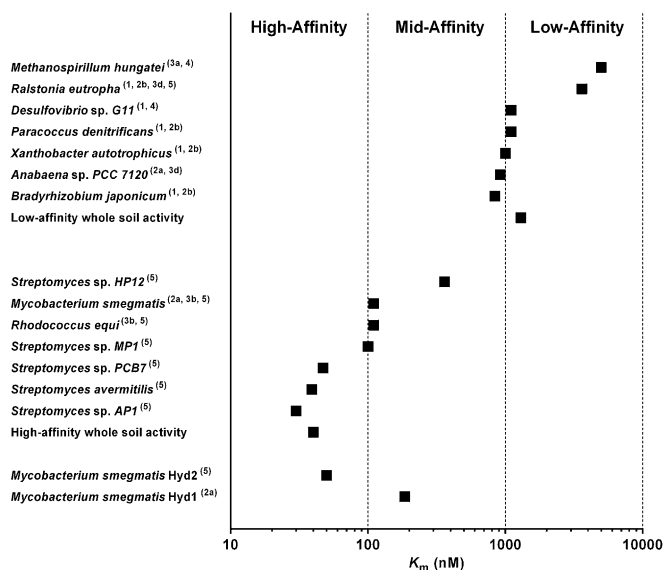


Fig. 4. The wide spectrum of affinities for H_2 observed in hydrogen-oxidizing organisms. The types of [NiFe] hydrogenase encoded by each organism are shown in brackets. Pure cultures of the soil actinomycetes *M. smegmatis* (this work), *R. equi* (26), and certain *Streptomyces* species (12, 15, 17) have high affinities for H_2 ($K_m = 30$ – 130 nM). These activities depend on group 5 [NiFe] hydrogenases and correlate with the high-affinity portion of H_2 oxidation in whole soils (10, 16). By contrast, hydrogen-oxidizing soil *Proteobacteria* such as *Bradyrhizobium japonicum* (28), *P. denitrificans* (29), *Xanthobacter autotrophicus* (14), and *R. eutropha* (20) have low affinities for H_2 ($K_m = 1$ – 5 μ M) similar to that observed for low-affinity portion of H_2 oxidation in whole soils (10). H_2 affinities of the cyanobacterium *Anabaena* sp. PCC 7120 (30), sulfate-reducing bacterium *Desulfovibrio* sp. G11, and methanogen *Methanospirillum hungatei* (31) are also shown for reference. Many organisms harboring group 5 [NiFe] hydrogenases (e.g., *R. eutropha*) and group 2a [NiFe] hydrogenases (e.g., *Anabaena* sp. PCC 7120) still have low affinities for H_2 .

characterized by significant interspecies competition, rapid nutrient fluxes, and changing physical conditions. Although present in trace concentrations, tropospheric H_2 is ubiquitous, unlimited, and energy-rich; it therefore constitutes a dependable fuel source in an otherwise unstable environment. It is now clear that *M. smegmatis* grows mixotrophically on H_2 and organic carbon sources during exponential growth in pure cultures, in accordance with our previous expression and phenotypic studies (23). However, the expression (23) and activities of Hyd1 and Hyd2 are significantly higher during carbon limitation. Group 5 [NiFe] hydrogenases are also more active during energy limitation in other actinomycetes (e.g., *Rhodococcus equi*) and sporulation in streptomycetes (e.g., *Streptomyces* sp. PCB7) (12, 26). In such conditions, H_2 may substitute as the primary electron donor and be used to maintain redox balance and a membrane potential. The finding that group 5 [NiFe] hydrogenases can sustain catalytic activity at a wide range of pH values, temperatures, and O_2 concentrations further suggests that these enzymes confer robustness and flexibility in response to environmental shifts (20).

Hyd1 and Hyd2 share many characteristics, including similar expression profiles (23), high-affinity oxidation activities, and membrane association. However, it is clear that their physiological roles in the cell are not redundant. Despite continued activity of Hyd1, the growth yield of the $\Delta hyd2$ strain was 40% lower than the WT in continuous culture (19). Hyd2 must therefore use tropospheric H_2 for an important and unique purpose in the cell, e.g., to maintain the quinone pool or intracellular cofactors in a reduced state. In contrast, Hyd1 is likely to be a respiratory enzyme that sustains a membrane potential by coupling H_2

oxidation to O_2 reduction through a physical association with the electron transport chain. This enzyme has a high $V_{max(app)}$ and is efficient at a range of H_2 concentrations. It can metabolize the high concentrations that will occur in certain micro-environments [e.g., in waterlogged or rhizobial soils (27)] or as a result of Hyd3 activity during oxygen limitation. The enzyme also enables *M. smegmatis* to oxidize tropospheric H_2 at significantly faster rates than cultured streptomycetes harboring only group 5 [NiFe] hydrogenases (12).

Hyd1 and Hyd2 are the first hydrogenases to have been unambiguously shown to mediate high-affinity H_2 oxidation. Their apparent K_m s are more than 10-fold lower than most purified uptake hydrogenases (Fig. 4). However, the features responsible for this adaptation remain to be established; it remains unclear whether the hydrogenases themselves are intrinsically high-affinity enzymes or whether their affinities are modulated by their cellular context (e.g., through their redox partners and interactions with the respiratory chain). Curiously, a wide spectrum of apparent K_m values have been observed for group 5 [NiFe] hydrogenases in other organisms (Fig. 4) (12, 26). This is surprising given these enzymes are encoded in relatively conserved operons and share very high amino acid sequence identities (>70%). Our multiple sequence alignments reveal no consistent amino acid substitutions between apparent high-affinity (e.g., *M. smegmatis*, *Streptomyces avermitilis*) and low-affinity (e.g., *R. eutropha*, *S. scabiei*) members. Hyd1 is also the first group 2a [NiFe] hydrogenase shown to oxidize tropospheric H_2 . It is notable that this enzyme is cotranscribed with a putative [2Fe-2S] protein (MSMEG_2268), which is likely to serve as the physiological electron acceptor (23). Genes encoding homologous proteins are transcribed with other group 5 [NiFe] hydrogenases (17), including Hyd2 (MSMEG_2718) (23), but are absent from most cyanobacterial genomes. We propose that such proteins may have a relatively high redox potential (>–200 mV), thus ensuring that the oxidation of trace concentrations of H_2 is thermodynamically favorable. It is also conceivable that unusual features of the redox metabolism or respiratory chain of *M. smegmatis* may influence the organism's affinity for H_2 . Biochemical and biophysical studies of purified proteins are clearly required to develop an understanding of the high-affinity activities, oxygen tolerance, and redox partners of Hyd1 and Hyd2.

Materials and Methods

Bacterial Strains and Growth Conditions. All bacterial strains used in this study are listed in Table S1. *M. smegmatis* mc²155 (32) and derived mutants (19, 23) were maintained on lysogeny broth agar plates (33) supplemented with 0.05% (wt/vol) tyloxapol (Sigma-Aldrich). For broth culture, *M. smegmatis* was grown at 37 °C in modified Hartmans-de Bont minimal medium (34) supplemented with 25 mM glycerol, 0.05% tyloxapol, and 10 μ M $NiSO_4$. Cultures were incubated with agitation (200 rpm) at 37 °C toward carbon limitation (30 mL medium in a 125-mL aerated flask) or oxygen limitation (30 mL medium in a 120-mL rubber-stoppered serum vial). For fractionation studies, cultures were grown into carbon limitation in larger volumes (750 mL medium in a 2,500-mL aerated flask). All cultures were inoculated to an initial optical density of 0.05. Optical densities to assess growth were measured at 600 nm in a Jenway 6300 spectrometer. Cultures were diluted in 0.85% saline solution to bring the OD₆₀₀ below 0.5 when measured in cuvettes of 1 cm light path length. To count cfus per milliliter, the cultures were serially diluted in PBS solution (pH 7.0) and spread onto agar plates.

GC Measurements. To measure high-affinity H_2 uptake, carbon-limited cultures of *M. smegmatis* mc²155 and derivatives were injected into serum vials containing 30 mL Hartmans-de Bont medium supplemented with 0.05% tyloxapol to a density of 3×10^7 cfu-mL⁻¹ (OD₆₀₀ = 0.4). Standard H_2 gas (1,000 ppmv \pm 2%; Messer Schweiz) was added to headspaces to obtain initial mixing ratios of 6.3 ± 0.7 ppmv H_2 . The change in mixing ratio was measured as a function of time through GC. Aliquots of headspace air (250 μ L) were sampled by using a gas-tight syringe (VICI Precision Sampling) and loaded on to a Trace Analytical Reduced Gas Analyzer (SRI Instruments). Serum vials were continuously agitated (120 rpm) to enhance transfer of H_2

from the gas phase to the liquid phase. Initial tests showed that H₂ consumption was proportional to the amount of cells and thus not limited by gas transfer. For V_{max} determinations, the rate of H₂ uptake was measured after addition of pure H₂ gas (Messer Schweiz) at initial mixing ratios of 500, 1,000, and 2,000 ppmv. K_m was estimated by dividing the V_{max} by the first-order rate constant *k* as previously described (35). The kinetics of H₂ uptake were also measured in exponentially growing cells (initial mixing ratio = 0.60 ± 0.07 ppmv H₂). All experiments were performed by using biological triplicates. To avoid gas buildup, serum vials were sealed with rubber stoppers only after autoclaving. The reproducibility of the measurements was assessed before and during each time course by analysis of standard H₂ gas (2.0 ppmv ± 5%, 50 ppmv ± 2%, and 1,000 ppmv ± 2% H₂; Messer Schweiz).

Preparation of Cell Fractions. *M. smegmatis* mc²155 and derivatives were fractionated into membranes and cytosols. Cultures (750 mL) were pelleted by centrifugation (15 min, 5,000 × *g*, 4 °C), washed in PBS solution, and resuspended in 25 mL lysis buffer [50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, 1 mM PMSF, 15 mM MgCl₂, 5 mg·mL⁻¹ lysozyme, 1 mg DNase]. The mixture was incubated (60 min, 50 rpm, 37 °C) and passed through a cell disruptor (35,000 psi, four times; Constant Systems). After removal of unbroken cells by centrifugation (10 min, 10,000 × *g*, 4 °C), the crude cell lysate was ultracentrifuged (45 min, 150,000 × *g*, 4 °C) to yield cytosols (supernatant) and membranes (pellets). The protein concentrations of the crude cell lysates, cytosols, and membranes were measured by using the BCA method (Pierce) against BSA standards.

Activity Staining on Native Polyacrylamide Gels. Samples of 5 μg protein from each cell fraction were loaded on to two native 7.5% (wt/vol) polyacrylamide gels prepared as previously described (36). The gels were run simultaneously (25 V, 3 h) in Tris-glycine buffer (pH 8.3). One gel was stained with Coomassie brilliant blue to visualize total protein. The other was stained for hydrogenase activity (H₂-nitroblue tetrazolium oxidoreductase activity) in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.5 mM

nitroblue tetrazolium chloride. The samples were incubated overnight in a hydrogen-rich atmosphere [5% (vol/vol) H₂, 10% (vol/vol) CO₂, 85% (vol/vol) N₂] in an anaerobic chamber (Coy Laboratory Products).

Amperometric Hydrogen Measurements. Hydrogenase activity of the cell fractions was amperometrically measured as previously described (23). Hydrogen-saturated PBS solution (0.1 mL), oxygen-saturated PBS solution (0.1 mL), and diluted cell fractions (0.7 mL; OD₆₀₀ = 1.5) were introduced and stirred in a 0.9-mL assay chamber. The H₂ concentration was measured over time using a Unisense H₂-MR hydrogen microsensor (Unisense) polarized at +800 mV and calibrated according to the manufacturer's instructions. After linear change in hydrogen concentration was observed, the initial rates were calculated for a period of 20 s and normalized to protein concentration.

Taxonomic Analysis. The large subunit protein sequences of all predicted group 2a, group 5, and group 3b [NiFe] hydrogenases in sequenced genomes were retrieved from the National Center for Biotechnology Information database. For retrieval, the large subunits of the three [NiFe]-hydrogenases from *M. smegmatis* were used as reference sequences for the Blast Local Alignment Search Tool (37). The classifications were validated by examining the N-terminal L1 and C-terminal L2 signature motifs of each sequence (38).

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- Ehhalt DH, Rohrer F (2009) The tropospheric cycle of H₂: A critical review. *Tellus B Chem Phys Meteorol* 61(3):500–535.
- Novelli PC, et al. (1999) Molecular hydrogen in the troposphere: Global distribution and budget. *J Geophys Res Atmos* 104(23):30427–30444.
- Rhee TS, Brenninkmeijer CAM, Röckmann T (2006) The overwhelming role of soils in the global atmospheric hydrogen cycle. *Atmos Chem Phys* 6(6):1611–1625.
- Constant P, Poissant L, Villemur R (2009) Tropospheric H₂ budget and the response of its soil uptake under the changing environment. *Sci Total Environ* 407(6):1809–1823.
- Schwartz E, Fritsch J, Friedrich B (2013) H₂-Metabolizing Prokaryotes. *The Prokaryotes: Prokaryotic Physiology and Biochemistry*, eds Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thomson F (Springer, Berlin), 4th Ed, pp 119–199.
- Conrad R, Seiler W (1980) Contribution of hydrogen production by biological nitrogen fixation to the global hydrogen budget. *J Geophys Res* 85(11):5493–5498.
- Conrad R, Aragno M, Seiler W (1983) Production and consumption of hydrogen in a eutrophic lake. *Appl Environ Microbiol* 45(2):502–510.
- Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). *Microbiol Rev* 60(4):609–640.
- Schuler S, Conrad R (1990) Soils contain two different activities for oxidation of hydrogen. *FEMS Microbiol Ecol* 73(1):77–84.
- Häring V, Conrad R (1994) Demonstration of two different H₂-oxidizing activities in soil using an H₂ consumption and a tritium exchange assay. *Biol Fertil Soils* 17(2):125–128.
- Vignais PM, Billoud B, Meyer J (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* 25(4):455–501.
- Constant P, Chowdhury SP, Pratscher J, Conrad R (2010) Streptomycetes contributing to atmospheric molecular hydrogen soil uptake are widespread and encode a putative high-affinity [NiFe]-hydrogenase. *Environ Microbiol* 12(3):821–829.
- Conrad R, Seiler W (1979) The role of hydrogen bacteria during the decomposition of hydrogen by soil. *FEMS Microbiol Lett* 6(3):143–145.
- Conrad R, Aragno M, Seiler W (1983) The inability of hydrogen bacteria to utilize atmospheric hydrogen is due to threshold and affinity for hydrogen. *FEMS Microbiol Lett* 18(3):207–210.
- Constant P, Poissant L, Villemur R (2008) Isolation of *Streptomyces* sp. PCB7, the first microorganism demonstrating high-affinity uptake of tropospheric H₂. *ISME J* 2(10):1066–1076.
- Constant P, Chowdhury SP, Hesse L, Conrad R (2011) Co-localization of atmospheric H₂ oxidation activity and high affinity H₂-oxidizing bacteria in non-axenic soil and sterile soil amended with *Streptomyces* sp. PCB7. *Soil Biol Biochem* 43(9):1888–1893.
- Constant P, Chowdhury SP, Hesse L, Pratscher J, Conrad R (2011) Genome data mining and soil survey for the novel group 5 [NiFe]-hydrogenase to explore the diversity and ecological importance of presumptive high-affinity (H₂)-oxidizing bacteria. *Appl Environ Microbiol* 77(17):6027–6035.
- King GM (2003) Uptake of carbon monoxide and hydrogen at environmentally relevant concentrations by mycobacteria. *Appl Environ Microbiol* 69(12):7266–7272.
- Berney M, Cook GM (2010) Unique flexibility in energy metabolism allows mycobacteria to combat starvation and hypoxia. *PLoS ONE* 5(1):e8614.
- Schäfer C, Friedrich B, Lenz O (2013) Novel, oxygen-insensitive group 5 [NiFe]-hydrogenase in *Ralstonia eutropha*. *Appl Environ Microbiol* 79(17):5137–5145.
- Hartmans S, de Bont JAM, Stackebrandt E (2006) The genus *Mycobacterium*—nonmedical. *The Prokaryotes Third Edition: Archaea, Bacteria: Firmicutes, Actinomycetes*, eds Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (Springer, Berlin), pp 889–918.
- Smeulders MJ, Keer J, Speight RA, Williams HD (1999) Adaptation of *Mycobacterium smegmatis* to stationary phase. *J Bacteriol* 181(1):270–283.
- Berney M, Greening C, Hards K, Collins DM, Cook GM (2014) Three different [NiFe]-hydrogenases confer metabolic plasticity in the obligate aerobic *Mycobacterium smegmatis*. *Environ Microbiol* 16(1):318–330.
- Pinske C, Jaroschinsky M, Sargent F, Sawers G (2012) Zymographic differentiation of [NiFe]-hydrogenases 1, 2 and 3 of *Escherichia coli* K-12. *BMC Microbiol* 12:134.
- Tamagnini P, et al. (2007) Cyanobacterial hydrogenases: Diversity, regulation and applications. *FEMS Microbiol Rev* 31(6):692–720.
- Meredith LK, et al. (2013) Consumption of atmospheric hydrogen during the life cycle of soil-dwelling actinobacteria. *Environ Microbiol Rep*, 10.1111/1758-2229.12116.
- Conrad R, Seiler W (1979) Field measurements of hydrogen evolution by nitrogen-fixing legumes. *Soil Biol Biochem* 11(6):689–690.
- Klüber HD, Conrad R (1993) Ferric iron-reducing *Shewanella putrefaciens* and N₂-fixing *Bradyrhizobium japonicum* with uptake hydrogenase are unable to oxidize atmospheric H₂. *FEMS Microbiol Lett* 111(2-3):337–341.
- Häring V, Conrad R (1991) Kinetics of H₂ oxidation in respiring and denitrifying *Paracoccus denitrificans*. *FEMS Microbiol Lett* 78(2-3):259–264.
- Houchins JP, Burris RH (1981) Occurrence and localization of two distinct hydrogenases in the heterocystous cyanobacterium *Anabaena* sp. strain 7120. *J Bacteriol* 146(1):209–214.
- Robinson JA, Tiedje JM (1984) Competition between sulfate-reducing and methanogenic bacteria for H₂ under resting and growing conditions. *Arch Microbiol* 137(1):26–32.
- Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR, Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 4(11):1911–1919.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
- Berney M, Weimar MR, Heikal A, Cook GM (2012) Regulation of proline metabolism in mycobacteria and its role in carbon metabolism under hypoxia. *Mol Microbiol* 84(4):664–681.
- Conrad R, Goodwin S, Zeikus JG (1987) Hydrogen metabolism in a mildly acidic lake sediment (Knaack Lake). *FEMS Microbiol Ecol* 45(4):243–249.
- Walker JM (2002) Nondenaturing Polyacrylamide Gel Electrophoresis of Proteins. *The Protein Protocols Handbook*, ed Walker JM (Springer, Berlin), 2nd Ed, pp 57–60.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403–410.
- Vignais PM, Billoud B (2007) Occurrence, classification, and biological function of hydrogenases: An overview. *Chem Rev* 107(10):4206–4272.