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Consumption of atmospheric hydrogen during the life cycle of soil-dwelling actinobacteria

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Summary

Microbe-mediated soil uptake is the largest and most uncertain variable in the budget of atmospheric hydrogen (H_2). The diversity and ecophysiological role of soil microorganisms that can consume low atmospheric abundances of H_2 with high-affinity [NiFe]-hydrogenases is unknown. We expanded the library of atmospheric H_2-consuming strains to include four soil Harvard Forest Isolate (HFI) *Streptomyces* spp., *Streptomyces cattleya* and *Rhodococcus equi* by assaying for high-affinity hydrogenase (*hhyL*) genes and quantifying H_2 uptake rates. We find that aerial structures (hyphae and spores) are important for *Streptomyces* H_2 consumption; uptake was not observed in *S. griseoflavus* Tu4000 (deficient in aerial structures) and was reduced by physical disruption of *Streptomyces* sp. HFI8 aerial structures. H_2 consumption depended on the life cycle stage in developmentally distinct actinobacteria: *Streptomyces* sp. HFI8 (sporulating) and *R. equi* (non-sporulating, non-filamentous). Strain HFI8 took up H_2 only after forming aerial hyphae and sporulating, while *R. equi* only consumed H_2 in the late exponential and stationary phase. These observations suggest that conditions favouring H_2 uptake by actinobacteria are associated with energy and nutrient limitation. Thus, H_2 may be an important energy source for soil microorganisms inhabiting systems in which nutrients are frequently limited.

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Introduction

Microbe-mediated soil uptake is a leading driver of variability in atmospheric H_2 and accounts for 60–90% of the total H_2 sink; however, the dependence of this sink on environmental parameters is poorly constrained by field and lab measurements (Xiao *et al.*, 2007; recently reviewed by Ehhalt and Rohrer, 2009). Atmospheric H_2 is an abundant reduced trace gas (global average of 530 ppb) that influences the atmospheric chemistry of the troposphere and the protective stratospheric ozone layer (Novelli *et al.*, 1999). Most notably, the reaction of H_2 with the hydroxyl radical (•OH) attenuates the amount of •OH available to scavenge potent greenhouse gases, like methane (CH_4), from the atmosphere. The H_2 soil sink may play a considerable role in buffering anthropogenic H_2 emissions, which constitute approximately 50% of atmospheric H_2 sources (Ehhalt and Rohrer, 2009). A process-level understanding of the H_2 soil sink is required to understand the natural variability of atmospheric H_2 and its sensitivity to changes in climate and anthropogenic activities.

Early studies established the H_2 soil sink as a biological process because of the enzymatic nature of H_2 consumption (Conrad and Seiler, 1981; Schuler and Conrad, 1990; Häring and Conrad, 1994). Initially, free soil hydrogenases were thought to be the primary drivers of the H_2 soil sink because chemical fumigation of soils had little effect on soil H_2 uptake rates but significantly reduced the active microbial consumption or production of other trace gases, e.g., the active microbial uptake of CO (Conrad and Seiler, 1981; Conrad *et al.*, 1983a; Conrad, 1996). Only indirect evidence existed to support the notion that the H_2 soil sink was an active microbial process (Conrad and Seiler, 1981; Conrad *et al.*, 1983b; King, 2003a) until the isolation of *Streptomyces* sp. PCB7, the first microorganism to exhibit significant consumption of atmospheric H_2 (Constant *et al.*, 2008). This organism demonstrated high-affinity (K_m ~ 10–50 ppm), low-threshold (< 0.1 ppm) H_2 uptake kinetics characteristic of uptake by environmental soil samples (Conrad, 1996). Previously, only low-affinity (K_m ~ 1000 ppm), high-threshold (> 0.5 ppm) H_2-oxidizing

microorganisms were characterized, which were unable to consume H_2 at atmospheric concentrations (Conrad *et al*., 1983a; Conrad, 1996; Guo and Conrad, 2008; summarized by Constant *et al*., 2009).

Streptomyces spp. are ubiquitous soil microorganisms that degrade recalcitrant materials in soils (Kieser *et al*., 2000). Theoretically, the observed rates of atmospheric H_2 soil consumption can sustain the maintenance energy requirements for typical numbers of *Streptomyces* spp. cells in soils (Conrad, 1999; Constant *et al*., 2010; 2011a). However, the importance of atmospheric H_2 as a source of energy to soil microorganisms remains unknown. Atmospheric H_2 uptake was specifically linked to a group 5 [NiFe]-hydrogenase gene cluster containing genes that encode for the small and large hydrogenase subunits, *hhyS* and *hhyL*, respectively (Constant *et al*., 2010). The *hhyL* gene is distributed unevenly among the Actinobacteria, Proteobacteria, Chloroflexi and Acidobacteria phyla (e.g., many, but not all, *Streptomyces* spp. possess the gene) (Constant *et al*., 2010; 2011b). The link between high-affinity H_2 uptake and *hhyL* has been reported in nine *Streptomyces* spp. and in *Mycobacterium smegmatis* (King, 2003a; Constant *et al*., 2011b), but it remains untested in many soil microorganisms. Additional research adding to the library of atmospheric H_2-oxidizing bacteria is needed to identify the key microorganisms involved in H_2 biogeochemical cycling. Information about the genes and ecophysiology of these organisms can improve the process-level understanding of the H_2 soil sink (Conrad, 1996; Madsen, 2005).

The life cycle of *Streptomyces* is complex and controls the timing of many physiological activities, which may include H_2 uptake (Kieser *et al*., 2000; Schrempf, 2008; Flärdh and Buttner, 2009). In soils, *Streptomyces* exist predominantly as inactive spores, which germinate in response to environmental triggers such as moisture and nutrient availability (Kieser *et al*., 2000) and grow vegetatively, producing a network of mycelia that grow into the substrate (Flärdh and Buttner, 2009). Over time and in response to environmental triggers such as nutrient depletion or physiological stresses, the colony differentiates to form hydrophobic aerial hyphae that break the substrate surface tension and grow into the air, forming a millimetre-scale canopy in immediate contact with the atmosphere (Kieser *et al*., 2000; Schrempf, 2008). Finally, aerial hyphae differentiate and septate to form chains of resistant spores (Flärdh and Buttner, 2009). In cultures of *Streptomyces* sp. PCB7 growing on soil particles, H_2 uptake coincided with the presence of aerial hyphae and spores (Constant *et al*., 2008). It is unknown if H_2 uptake occurs at the same life cycle stage in other *Streptomyces* strains and how long uptake persists in the spore stage. Furthermore, the timing of atmospheric H_2 uptake in microbes that possess *hhyL* but do not sporulate has not been measured.

The goal of this paper is to address two questions. First, our study asks whether environmental isolates and culture collection strains with the genetic potential for atmospheric H_2 uptake, i.e., the *hhyL* gene, actually exhibit atmospheric H_2 uptake. To expand the library of atmospheric H_2-oxidizing bacteria, we quantify H_2 uptake rates by novel *Streptomyces* soil isolates that contain the *hhyL* and by three previously isolated and sequenced strains of actinobacteria whose *hhyL* sequences span the known *hhyL* diversity. Second, we investigate how H_2 uptake varies over organismal life cycle in one sporulating and one non-sporulating microorganism, *Streptomyces* sp. HFI8 and *Rhodococcus equi*, respectively. These experiments probe the advantage of atmospheric H_2 consumption to microbes and relationship between environmental conditions, physiology of soil microbes, and H_2.

Results

H_2 *uptake by microbial soil isolates and culture collection strains possessing hhyL*

Candidate *Streptomyces* strains, referred to henceforth as Harvard Forest Isolate (HFI) strains, were isolated from Harvard Forest soils. Polymerase chain reaction (PCR) amplification revealed that *hhyL* encoding the high-affinity [NiFe]-hydrogenase was present in six out of nine tested strains. Four of these strains (HFI6, HFI7, HFI8 and HFI9) were successfully retained in culture and were used to test the link between *hhyL* and H_2 uptake activity. These strains exhibited distinctive *Streptomyces* traits such as pigmentation, a fuzzy appearance indicating the production of aerial hyphae (Figs S1 and S2), and the distinctive earthy scent of geosmin (Schrempf, 2008). The 16S rRNA gene sequences of the new isolates fell within the *Streptomyces* genus and were 100% identical to several different strains of *Streptomyces* spp. (Table S1). Of two clusters that were defined by Constant and colleagues (2011b) based on a deeply rooted split (99% of bootstrap replicates) in the phylogenetic tree of *hhyL* amino acid sequences (Fig. S3), the HFI6–HFI9 *hhyL* sequences group with *hhyL* Cluster 1. In addition to our *Streptomyces* isolates, we examined three culture collection strains to broaden representation across the *hhyL* clusters and genera: *S. griseoflavus* Tu4000 (Cluster 1), *R. equi* (Actinobacterium, Cluster 1) and *S. cattleya* (Cluster 2).

To test whether organisms with *hhyL* gene sequences consume H_2, we measured the uptake of atmospheric H_2 in sporulated *Streptomyces* cultures and in stationary stage of *R. equi*. The presence of *hhyL* predicted atmospheric H_2 uptake activity in HFI strains 6–9, *S. cattleya*,

and *R. equi*, but not in *S. griseoflavus* Tu4000 (Table 1). Atmospheric H_2 uptake was observed in strains with *hhyL* from Cluster 1 (*Streptomyces* strains HFI6–HFI9 and *R. equi*) and Cluster 2 (*S. cattleya*). The biomass-weighted H_2 uptake rates of these isolates spanned nearly two orders of magnitude (from 10 to 780 nmol min^{-1} g^{-1}), and the *Streptomyces* strains that took up H_2 did so at rates more than 10-fold greater than dense stationary phase cultures of *R. equi* (Table 1). *Rhodococcus equi* consumed atmospheric H_2, both when grown on solid Reasoner's 2A (R2A) medium and in liquid triptic soy broth (TSB) medium (data not shown). Uptake rates of *Streptomyces* cultures were measured on solid medium because *Streptomyces* cultures typically do not progress through their full developmental cycle in liquid medium (Flärdh and Buttner, 2009). The Michaelis–Menten substrate affinity was determined from the *x*-intercept of Lineweaver–Burk (LB) plots of the inverse relationship between the first-order H_2 uptake rate and initial headspace H_2 concentrations between 0 and 35 ppm. This method can be more error prone than the non-inverse approach performed over a greater range of initial H_2 mole fractions, but it better restricts H_2 uptake by low-affinity hydrogenases and has enough sensitivity to distinguish high- and low-affinity uptake kinetics. K_m values of HFI strains were typically low (40–80 ppm for HFI strains), which indicated that enzymatic processing of H_2 is tuned to operate efficiently at atmospheric levels of H_2 (high-affinity uptake). *Streptomyces cattleya* and *R. equi* appeared have high- or intermediate-affinity K_m values (< 1000 ppm) but did not pass the quality control measures (Experimental procedures) for inclusion in Table 1. The minimum H_2 concentration, or threshold, consumed by each HFI strain ranged from 0.12 to 0.15 ppm, which is well below typical atmospheric mole fractions of around 0.53 ppm (Table 1). *Streptomyces cattleya* and *R. equi* thresholds were also below atmospheric levels at least below 0.45 and 0.30 ppm respectively (Table 1). This study augments the library of organisms that contain *hhyL* sequences and take up atmospheric H_2 with high-affinity and a low-threshold from 10 to 16 strains.

H_2 *uptake correlates with lifecycle stage in* Streptomyces *sp. HFI8*

We randomly selected *Streptomyces* sp. HFI8 from our HFI strains as a representative organism to determine whether high-affinity H_2 consumption depended on the stage of the life cycle and how long uptake lasted in the sporulation stage. Microscopy revealed the progression of strain HFI8 through developmental stages over 44 days on solid agar (Fig. S4). Following germination, the colonies of strain HFI8 grew as substrate mycelia (Fig. S4A). By day 1.8, the lawn reached its maximal aerial coverage and grew upward as aerial hyphae formed and then sporulated (Fig. S4B). The co-occurrence of partially septated aerial hyphae and spores indicated that the events were not simultaneous throughout the colony (Fig. S4B). Measurements of H_2 uptake revealed that H_2 consumption began only after the formation of aerial hyphae and sporulation around day 2 (Fig. 1). Aerial hyphae formation and sporulation are stages of the life cycle often associated with nutrient limitation in *Streptomyces* spp. H_2 uptake reached a maximum rate (9.4 ± 2.3 nmol h^{-1}) on day 3.8, 2 days after sporulation had begun and then slowly decreased over the next 40 days, dropping below the detection limit of $\pm$ 0.24 nmol h^{-1}. Most cells between days 2.9 and 44 were a lawn of 'dormant' spores that had completed the full life cycle (Fig. S4C–H). H_2 oxidation rates by dormant spores declined slowly over the 44-day experiment to negligible rates (Fig. 1). All three replicates displayed similar timing, but the H_2 uptake rates were systematically lower in the third replicate, although the area coverage of the lawn and biomass was not demonstrably different among the replicates. A cursory set of measurements (data not shown) indicated similar trends in H_2 uptake over the life cycle

Table 1. H_2 oxidation rates weighted by biomass (final protein mass) for HFI strains and strains from culture collections (*S. cattleya*, *S. griseoflavus*, *R. equi*) at typical atmospheric (~0.53 ppm) H_2 mole fractions.

Strain	H_2 oxidation rate (nmol min^{-1} g^{-1})	K_m* (ppm)	V_{max}* (μmol min^{-1} g^{-1})	Threshold (ppm)
Streptomyces sp. HFI6	780	80	180	< 0.15
Streptomyces sp. HFI7	420	60	78	< 0.12
Streptomyces sp. HFI8	240	40	30	< 0.15
Streptomyces sp. HFI9	100	40	14	< 0.12
Streptomyces griseoflavus Tu4000	0	–	–	–
Streptomyces cattleya	130	**	**	< 0.45
Rhodococcus equi	10	**	**	< 0.30

H_2 uptake affinity (K_m), the maximum reaction rate (V_{max}) and the minimum threshold for consumption are listed for each culture.
*Determined by the Lineweaver–Burke method over a 0–35 ppm H_2 range, which is less precise than the non-inverse approach, but avoids interference by low-affinity hydrogenases.
**Kinetic parameters determination did not pass quality check (Experimental procedures).

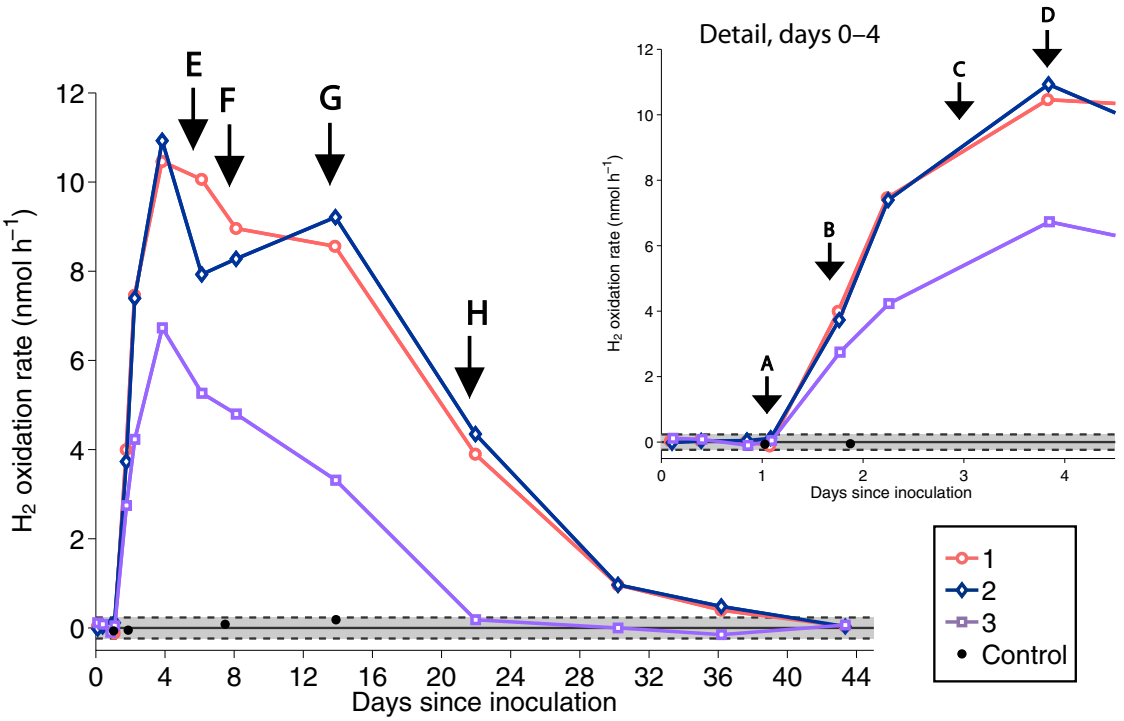

Fig. 1. High-resolution time series of H_2 uptake in three replicate cultures of *Streptomyces* sp. HFI8. The lettered arrows at various time points correspond to the micrographs of the life cycle shown in Fig. S4: (A) the substrate mycelium, (B) the formation of aerial hyphae and onset of sporulation, (C–H) cultures contain mainly spores. Enlarged inset shows the higher resolution measurements taken during the first 4 days. The detection limit (dashed lines) of ±0.24 nmol h^{-1} is reported as the double standard deviation of four values measured in uninoculated control vials (black dots).

of *Streptomyces* sp. HFI6, *Streptomyces* sp. HFI7, *Streptomyces* sp. HFI9, and *S. cattleya*.

Because the formation of aerial biomass (hyphae and spores) occurred at the same time as the onset of H_2 consumption in *Streptomyces*, we asked whether H_2 uptake activity was physically located in the aerial biomass. We isolated the aerial fraction (spores and aerial hyphae) of strain HFI8 cultures by gently rolling glass beads over the entire surface of the colony and transferring the beads and aerial biomass to an empty, sterile glass vial (Fig. S5). H_2 uptake rates were measured in whole cultures before the transfer, in the vials with the transferred aerial fraction, and in the original vial with the substrate fraction that remained after the glass bead procedure (Table 2, Samples 1–6; Fig. S5). The experiment lasted 2–4 h following the aerial biomass transfer. H_2 uptake in the transferred aerial biomass fraction was consistently low, typically near or below the limit of detection of ± 0.24 nmol h^{-1}, and was thus often statistically indistinguishable from zero. Low uptake rates in the aerial fraction were not the result of poor biomass transfer efficiency by the glass bead procedure; glass beads transferred a significant proportion (Tables 2 and 0.7 ± 0.6 mg) of the aerial biomass from the replicate cultures of that could be collected using a metal spatula (1.2 ± 0.5 mg). The drop in uptake also cannot be explained by aging over this period because this occurs over the course of days or weeks and not hours (Fig. 1). No reduction in H_2 uptake stemming from reduced spore viability was expected because the biomass transfer procedure by glass beads is based on established methods for harvesting viable spores (e.g., Hirsch and Ensign, 1976; Hardisson *et al.*, 1978). Furthermore, the number of viable spores in bead-treated cultures was indistinguishable from the number of viable spores obtained by transferring aerial biomass by a metal spatula from replicate vials incubated at the same time. This test was done by harvesting spores by the two methods, plating spore suspension dilutions and counting the number of colony forming units as a function of the initial amount of biomass (protein mass) in the spore suspensions.

We found that the net H_2 uptake diminished after the separation of the aerial biomass from the substrate biomass (Table 2). Even in replicates where glass beads were gently rolled over strain HFI8 lawns and all biomass was left in the original vial, net H_2 uptake was significantly reduced (Table 2, Samples 7–12). The larger the initial H_2 oxidation rate, the larger percentage reduction by the

Table 2. Effect of physical disturbances of the aerial structure on H_2 oxidation rates in sporulated cultures of *Streptomyces* sp. HFI8.

				H_2 oxidation rate (nmol h^{-1})			
ID	Day	Beads (g)	Aerial biomass (mg)	Whole	Aerial + glass beads	Substrate	Change in net uptake (%)
1	2	10	–	4.1	0.6	1.5	– 1.9 (– 48%)
2	8	10	0.3	6.8	0.3	2.9	– 3.6 (– 52%)
3	15	10	0.1	6.9	0.3	1.7	– 4.9 (– 71%)
4	9	2.5	1.5	3.6	0.1	2.1	– 1.4 (– 40%)
5	9	5	0.6	3.8	0.1	2.2	– 1.4 (– 37%)
6	9	10	1.1	3.8	0.1	2.1	– 1.6 (– 43%)
				Whole	Whole + glass beads		
7	9	10	–	2.2	1.3		– 0.9 (– 41%)
8	9	10	–	2.8	2.1		– 0.7 (– 25%)
9	9	10	–	2.4	1.5		– 0.9 (– 38%)
10	9	10	–	1.5	2.0		– 0.3 (– 20%)
11	9	10	–	2.4	2.0		– 0.4 (– 18%)
12	9	10	–	2.2	1.9		– 0.3 (– 13%)

Gently rolling 4 mm diameter glass beads over culture lawns (Fig. S5) reduced the observed H_2 uptake. The H_2 oxidation rates (the 5th column) in 12 whole cultures of strain HFI8 growing in serum vials on solid R2A medium were measured between 2 and 15 days after inoculation (the 2nd column). In samples 1–6, the aerial biomass was isolated from substrate biomass using glass beads to transfer aerial biomass to an empty, sterile vial. The amount of transferred biomass was measured by protein assay (the 4th column); no protein data available (dash) for untransferred samples and one unmeasured replicate. We tested using different amounts (2.5 and 10 g) of 4 mm diameter glass beads (the 3rd column). H_2 uptake is reported for the fraction of aerial biomass transferred to glass beads (the 6th column) and for the fraction of the lawn remaining in the original vial in the medium (the 7th column) measured within 2–4 h. In samples 7–12, all biomass was left in the original vial, and H_2 oxidation rates were measured before and after treatment with glass beads. The difference in uptake because of the procedure (the sum of the uptake rates reported in the 6th and the 7th column minus the uptake before transfer in the 5nd column) is reported in the 8th column.

glass beads (Fig. S6, linear fit, $R^2 = 0.93$), regardless of culture age or the amount of glass beads used for transfer (Samples 1–12). These experiments suggested that the colony structure and the presence of intact aerial hyphae were important for H_2 uptake.

H_2 *uptake correlates with the growth stage of* R. equi

Only some microbes containing *hhyL* are sporulating *Streptomyces* (Fig. S3). To test whether H_2 uptake by non-sporulating Actinobacterium *R. equi* is related to its lifecycle, we measured the uptake of H_2 by this organism at various stages of growth in liquid cultures (Fig. 2). The growth phases were determined from optical density (OD) measurements of the cultures. *Rhodococcus equi* did not consume measurable quantities of H_2 during the exponential growth phase (days 1–4) but started taking up H_2 in the late exponential growth phase (days 4–7) and in the stationary phase (days 7–17) until the end of the experiment (Fig. 2). The late exponential phase and stationary phase growth stages are associated with nutrient limitation.

The low H_2 uptake rates by *R. equi* were much closer to the experimental detection limit than *Streptomyces* sp. HFI8. This suggested that the lack of uptake could be related to low *R. equi* cell densities in late exponential and early stationary phase rather the altered cell physiology. To test this, we concentrated cells from a culture in exponential growth phase (day 1.9) into either fresh medium or sterile water to match the cell densities (Fig. 2B) of H_2-oxidizing cultures in the late exponential and early stationary phases (comparable with those on days 4–6). In spite of the comparable cell densities, cells concentrated in this manner did not consume H_2 (-0.075 ± 0.15 nmol h^{-1}, Fig. 2A). In addition, we diluted cells in stationary phase (day 7.8) into fresh medium or water to obtain suspensions whose cell densities matched those during days 2–3 of the exponential phase (Fig. 2). Although H_2 oxidation rates of the exponentially growing cultures on days 2 and 3 were below the limit of detection (± 0.12 nmol h^{-1}), comparably dense cells derived from the diluted stationary phase cultures took up H_2 (0.43 ± 0.047 nmol h^{-1}). All cultures were shaken vigorously to ensure the delivery of H_2 into the medium. Some extracellular factors of relevance to H_2 uptake, such as extracellular hydrogenases, may have been carried over into the diluted suspensions. The decrease in the uptake of H_2 by stationary phase cells (74% of undiluted uptake) did not scale with the dilution (22% of the undiluted cell biomass), which corresponds to a relative mismatch factor of 3.5 in H_2 uptake vs dilution. The reason is unclear and could result from H_2 substrate diffusion limitation in very dense cultures, which was partially alleviated upon dilution. If the cultures were diffusion-limited for H_2 substrate, the observed H_2 oxidation rate (Table 1) and H_2 uptake rates during late exponential and stationary phases (Fig. 2) may underestimate the potential H_2 uptake by cultures of *R. equi*. The uptake of H_2 only by stationary

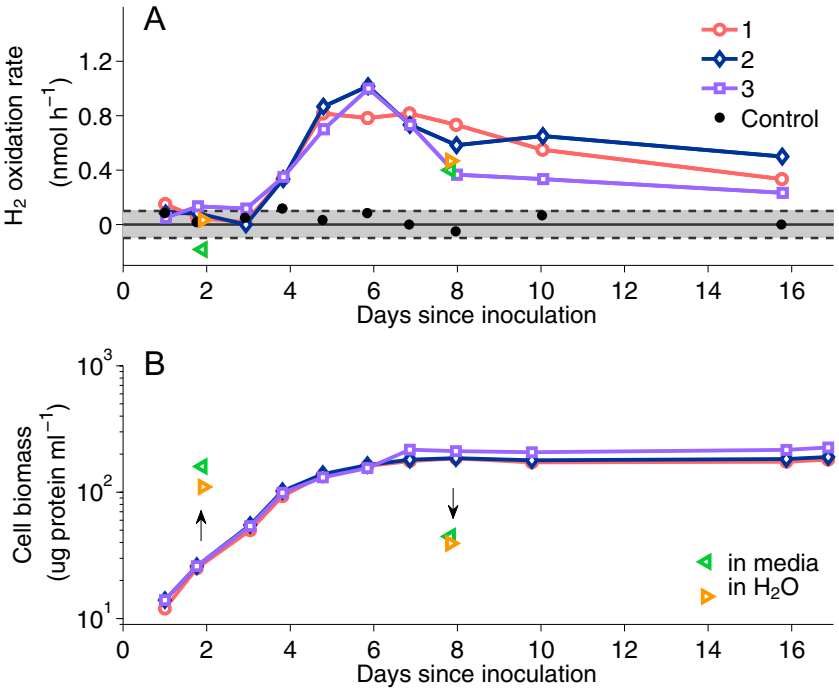

Fig. 2. Consumption of H_2 by *Rhodococcus equi* in liquid culture. (A) H_2 oxidation rate, (B) cell biomass (protein concentration). All data are shown for three liquid culture replicates. The detection limit (dashed lines) is of $\pm$ 0.12 nmol h^{-1} and calculated as the double standard deviation of 10 values measured in uninoculated control vials (black dots). Coloured triangles show the results of concentration and dilution experiments. Cells in exponential phase were concentrated in either fresh TSB medium (green) or water (orange) to match protein concentrations in late exponential and stationary phase. Alternatively, cells from stationary phase were diluted in either fresh TSB medium (green) or water (orange) to protein concentrations similar to those in exponential phase cultures.

phase cells, either in the old culture medium or when resuspended in fresh medium or water, related the uptake of H_2 to the late exponential and stationary phases. Overall, these tests linked *R. equi* H_2 consumption with growth phase.

Discussion

Link between hhyL and H_2 *uptake*

Our results confirm links between *hhyL* and H_2 uptake in *R. equi*, four *Streptomyces* HFI soil isolates from Cluster 1, and *S. cattleya* from Cluster 2, thereby providing additional support for the use of the high-affinity hydrogenase gene *hhyL* as a predictor for the capability to consume atmospheric hydrogen. H_2 uptake by *hhyL* by strains from Clusters 1 and 2 indicate that the phylogenetic divergence between the two groups does not compromise atmospheric H_2 uptake activity by *hhyL* or its prediction. Strains HFI6–HFI9 exhibit high H_2 uptake affinities and low uptake thresholds. Culture collection strains exhibit more variable H_2 uptake kinetics in keeping with a recent suggestion that H_2-consuming microorganisms exhibit a continuum of affinities rather than a discrete grouping of high and low affinities (Constant *et al.*, 2010). Current observations of high-affinity H_2 uptake are limited to the *Actinobacteria*, and future studies are required to determine whether H_2 uptake occurs in the other phyla containing the *hhyL* gene, such as Chloroflexi, Planctomycetes, Verrucomicrobia and Proteobacteria (Fig. S3). A genome data-mining investigation revealed the ubiquity of *hhyL* in DNA extracted from forest, desert, agricultural and peat soils samples, and although some evidence suggests a correlation between soil H_2 uptake rates and the number of H_2-oxidizing bacteria, no correlation was found between *hhyL* DNA copies and soil H_2 uptake rates (Constant *et al.*, 2011a,b). Future work should be aimed both at understanding the diversity and ecophysiology of these *hhyL*-containing microorganisms and at developing methods to predict H_2 uptake activity across ecosystems.

H_2 *uptake and the developmental cycle of actinobacteria*

Our results support a correlation between the developmental stage of *Streptomyces* spp. and high-affinity H_2 uptake in two ways. First, we did not observe any H_2 uptake in the substrate mycelium developmental phase of *Streptomyces* sp. HFI8. H_2 uptake began only after the formation of aerial hyphae and sporulation. Second, we found that *S. griseoflavus* Tu4000, which grew predominantly as substrate mycelium, did not take up H_2. We propose that the impaired development (i.e. lack of aerial hyphae and/or spores) of *S. griseoflavus* Tu4000 may impair the production or activity of its high-affinity hydrogenase. In culture, *S. griseoflavus* Tu4000 is smooth and waxy, and does not produce the aerial hyphae typical of *Streptomyces* grown on solid culture (Figs S1 and S2). *Streptomyces griseoflavus* Tu4000 may belong to a class of *bld* (bald) mutants that are often deficient in aerial hyphae production (Kieser *et al.*, 2000). Sporulation efficiency is also often reduced in *bld* mutants

(Szabó and Vitalis, 1992), and *S. griseoflavus* Tu4000 does not form spores on various types of media (J. Blodgett, pers. comm.), including our cultures. To our knowledge, *S. griseoflavus* Tu4000 is the first *hhyL*-containing *Streptomyces* sp. found to be unable to oxidize atmospheric H_2 under the same experimental conditions that lead to H_2 oxidation by other *Streptomyces* spp. High-affinity H_2 uptake is also absent from Cluster 1 *hhyL* containing cultures of a gram-negative beta-proteobacterium *Ralstonia eutropha* H16 (formerly known as *Alcaligenes eutropha* 16) grown on solid medium and tested for uptake in suspensions (Conrad *et al*., 1983a). Future experiments could compare sporulating *Streptomyces* with their *bld* mutants or stimulate the formation of aerial hyphae and/or sporulation in *bld Streptomyces* spp. mutants by application of exogenous δ-butyrolactone factor (Ueda *et al*., 2000; Straight and Kolter, 2009) and determine the effect of this stimulation on H_2 oxidation or *hhyL* expression. In summary, the combined lack of aerial hyphae, spores and H_2 uptake in *S. griseoflavus* Tu4000 and the co-occurrence of these phenotypes in strain HFI8 underscored a strong developmental control of atmospheric H_2 uptake in *Streptomyces*. These observations motivate the use of *Streptomyces* mutants arrested at different points in the developmental cycle to investigate the regulation and physiological role of *hhyL* in sporulating actinobacteria.

Our measurements of H_2 uptake in HFI8 colonies disturbed by glass beads indicate that H_2 uptake depends on the physical structure of *Streptomyces* aggregates. Cultures treated by glass beads take up less H_2, suggesting that the activity of the hydrogenase is impaired by the disturbance of the aerial structures. H_2 uptake by the disrupted colony could decrease because of loss in structural support, loss in signaling and nutrient transport within the bacterial lawn (Miguélez *et al*., 1999), or reduction in the aerial hyphae surface area in contact with the air. Therefore, we attribute the observed decrease in H_2 uptake to physical destruction of the lawn and colony structure of *Streptomyces*.

The H_2 uptake by non-sporulating batch cultures of *R. equi* occurs only during late exponential and stationary phase, suggesting that its H_2 consumption may support metabolism under nutrient-limiting conditions. Similarly, H_2 uptake by strain HFI8 is present only during those stages of its life cycle associated with nutrient-limiting conditions, suggesting that H_2 may be an important energy source for *Streptomyces* under stress. This is consistent with previous reports of H_2 oxidation by *M. smegmatis*, a non-sporulating Actinobacterium with a Cluster 1 high-affinity [NiFe]-hydrogenase that can persist for many years in host tissue in a nutrient-deprived stationary phase (Smeulders *et al*., 1999; King, 2003a). *Mycobacterium smegmatis* expresses the hydrogenase gene under starvation conditions and mutants lacking this hydrogenase have a reduced growth yield under these conditions (Berney and Cook, 2010). Therefore, the ability to scavenge low concentrations of H_2 may be an important adaptation of various sporulating and non-sporulating actinobacteria (Prescott, 1991; Smeulders *et al*., 1999; Scherr and Nguyen, 2009). This could be particularly true in terrestrial environments where nutrient concentrations are often low for extended periods and atmospheric H_2 is available.

Implications for soil H_2 *uptake in the environment*

Uptake of atmospheric H_2 by spores, which are often considered to be metabolically dormant, may have consequences for both the sporulating microbes and the cycling of H_2 in the environment. H_2 oxidation rates in cultures of strain HFI8 continue to increase for 2 days after the onset of sporulation. This could reflect heterogeneity in the sample because not all cells sporulate simultaneously, or maximum H_2 uptake by already formed spores. In any case, measurable H_2 oxidation in sporulated cultures persists for over a month, such that the time-integrated H_2 oxidation in any culture is much larger in spore state than at any other stage in the life cycle. Net H_2 consumption by HFI8 is at least 10-fold larger in the spore state (days 4–44) than during the growth of substrate mycelium (through day 1.1) and formation of aerial hyphae (after day 1.8) combined. One should also keep in mind that the H_2 uptake rates measured in culture studies depend on the specific medium and may not be directly translated to different media or soil types, where the nutritional characteristics, moisture levels, and cell abundances likely differ. The persistence of H_2 oxidation by *Streptomyces* spp. may have consequences for environmental H_2 cycling and environmental conditions that promote the removal of atmospheric H_2. Conditions that favour germination and growth, including soil moisture and nutrient availability (Kieser *et al*., 2000), may increase the population of *Streptomyces* spp. in the substrate mycelium phase and actually limit the amount of H_2 oxidized by soils. During moisture- or nutrient-limiting conditions, a greater fraction of the population of *Streptomyces* spp. will be in life cycle stages linked with H_2 uptake (aerial hyphae and spores). Counterintuitively, H_2 uptake by *Streptomyces* spp. may be most significant when the environmental conditions are the harshest. H_2 uptake in spores under our experimental conditions is reduced to negligible levels after about a month (Fig. 1), indicating that H_2 uptake may be very low in environments where conditions are harsh for long periods, such as deserts.

Ultimately, the goal of studying microbial influences on trace gas fluxes is to understand and predict emergent

biogeochemical cycling in the environment. This study describes H_2 consumption by two developmentally distinct actinobacteria under nutrient-limiting conditions. Field measurements along a chronosequence of recent volcanic deposits support this notion by suggesting that relative uptake of H_2 by the soil microbial community (normalized to soil respiration rates) is higher in soils limited in organic carbon (King, 2003b). However, insignificant or even opposing trends also exist (Conrad and Seiler, 1985; Rahn *et al*., 2002), which may be driven by other factors. Future studies are also needed to determine the impact of nutrient- and moisture-limiting conditions on H_2 uptake by soils and to consider the significance and implications of the energetic supply from H_2 for the microorganisms in the competitive soil environment. A better understanding of the process-level controls on microbe-mediated H_2 soil uptake is critical for evaluating the impact of a changing climate on the soil H_2 uptake and the impact of continued anthropogenic H_2 emissions on atmospheric chemistry and climate.

Experimental procedures

Microbial strains

Streptomyces spp. were isolated from soils within the footprint of the Environmental Measurement Site atmospheric trace gas flux tower at the Harvard Forest Long Term Ecological Research site in Petersham, MA, USA (42°32'N, 72°11'W). Atmospheric H_2 fluxes were concurrently measured at the same site (Meredith, 2012). Harvard Forest is a mixed deciduous forest with acidic soils originating from sandy loam glacial till (Allen, 1995). Most H_2 consumption occurs within the first few centimetres of soil beneath the litter layer (Yonemura *et al*., 2000; Smith-Downey *et al*., 2008); therefore, samples were collected from the uppermost six inches of soil after removal of the leaf litter. Sporulating soil organisms such as *Streptomyces* spp. were enriched for using desiccation and chemical destruction (El-Nakeeb and Lechevalier, 1963; Schrempf, 2008). Soils were dried for 3–4 h at 55°C. Dry soil samples (1 g) were ground with a mortar and pestle, and were combined with $CaCO_3$ (1 g). The soil mixtures were incubated for 2 days at 28°C in 100 × 15 mm polystyrene Petri dishes (sterile, polystyrene, 100 × 15 mm, VWR International, Radnor, PA, USA), with moistened filter paper (11.0 cm diameter, Grade 1, Whatman® International, Kent, ME, USA) fitted in the lids to maintain a humid environment. After this period, incubated soil mixtures were suspended in 100 ml sterile water and thoroughly vortexed. After settling for 30 min, soil suspensions were serially diluted, and the 10^0, 10^{-2} and 10^{-4} dilutions were spread onto R2A plates (Difco™ R2A, BD, Franklin Lakes, NJ, USA) that had been treated with 88 mg cycoheximide/L medium (Porter *et al*., 1960). After incubation at 30°C for 3–5 days, microbial colonies were screened for the presence of any of the following four distinctive *Streptomyces* traits: (i) antibiotic inhibition of neighbouring growth (i.e., zone of clearing), (ii) a fuzzy appearance indicating the production of aerial hyphae (Figs S1 and S2), (iii) pigmentation, or (iv) the distinctive earthy scent of geosmin (Schrempf, 2008). Those exhibiting any of the traits were serially transferred onto fresh R2A plates until pure isolates were obtained. The resulting set of isolates, henceforth referred to as HFI, was maintained in culture on R2A agar at room temperature. Strains HFI6, HFI7, HFI8 and HFI9 were deposited to the U.S. Department of Agriculture NRRL Culture Collection for preservation as NRRL B-24941, NRRL B-24943, NRRL B-24942 and NRRL B-24940 respectively.

Strains from culture collections that were used in this study have published genomes accessible in the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). *Streptomyces griseoflavus* Tu4000 (accession NZ GG657758) was kindly provided by the genome authors and collaborators (Michael Fischbach, John Clardy, Joshua Blodgett). The following strains were obtained from culture collections: *R. equi* ATCC 33707™ (accession CM001149) and *S. cattleya* NRRL 8057 (accession NC 016111).

DNA extraction and PCR amplification

DNA was extracted using the PowerSoil® DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, USA) from colonies. PCR amplification of 16S rRNA and *hhyL* genes, respectively, was performed using a Mastercycler® pro (Eppindorf, Hamburg, Germany) in 25 μl reaction volumes with the following reaction mixture: 12.125 μl ddH_2O, 1.25 μl bovine serum albumin (BSA) (Roche, Indianapolis, IN, USA), 2.5 μl 10x Ex Taq Buffer (TaKaRa Bio, Japan), 0.125 (5 units μl^{-1}) Ex Taq (TaKaRa Bio), 2 μl dNTP (2.5 mM TaKaRa Bio) and 2.5 μl of each primer suspended at 3 μM (IDT, Coralville, IA, USA). The 16S rRNA gene was amplified using universal primers 27F:5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R:5'-ACGGCTACCTTGTTACGACTT-3' (Lane, 1991), and *hhyL* gene was amplified using NiFe244F:5'-GGGAT CTGCGGGGACAACCA-3' and NiFe-1640R:5'-TGCACGG CGTCCTCGTACGG-3' (Constant *et al*., 2010). The following program was used: 5 min initial denaturation at 95°C, followed by 30 cycles consisting of 30 s template denaturation at 95°C, 30 s hold at the primer annealing temperature, 1.5 min extension at 72°C and a final extension at 72°C for 5 min. Annealing temperatures of 50 and 60.7°C were used for the amplification of the 16S rRNA and *hhyL* genes respectively. The *hhyL* annealing temperature was optimized over a temperature gradient spanning eight temperatures between 50 and 62.2°C using *S. griseoflavus* Tu4000 DNA as template.

Each HFI strain was evaluated for the presence of a putative group 5 [NiFe]-hydrogenase by gel electrophoresis of the *hhyL* gene PCR reaction product. Gels were cast (1% agarose, 5 μl GelRed nucleic acid stain [Biotum, Hayward, CA, USA]), loaded (5 μl PCR product and 2 μl DNA loading dye [Fermentas, Glen Burnie, MD, USA]), run (100 V for 1 h) and visualized (UVP MultiDoc-It™ Digital Imaging System [UVP, Upland, CA, USA]) to verify successful PCR amplification. Migration of HFI strain PCR product was compared with the *S. griseoflavus* Tu4000 *hhyL* gene as a positive control and to the DNA Molecular Weight Marker X (Roche) ladder for reference.

Gene sequencing and sequence analysis

PCR products were sequenced at Genewiz (Cambridge, MA, USA) following the manufacturer's sample preparation guidelines. Both 16S rRNA and *hhyL* gene sequences (trimmed for >Q30) were identified by BLASTN (Altschul *et al*., 1990) and listed in Table S1. Hydrogenase *hhyL* amino acid sequences were aligned using ClustalW (Larkin *et al*., 2007), and phylogenetic analyses were carried out in Mega 5.2 (Tamura *et al*., 2011). Relationships were determined using a maximum likelihood method based on the Whelan and Goldman model (Whelan and Goldman, 2001) and checked for consistency using parsimony. The *hhyL* gene from archaeon *Sulfolobus islandicus* HVE10/4 was used as an outgroup. A 100 bootstrap maximum likelihood tree was constructed using Mega 5.2.

The gene sequences obtained for strains HFI6, HFI7, HFI8 and HFI9 were deposited in GenBank under accession numbers KC661265, KC661266, KF444073 and KF444074 for the 16S rRNA genes and under accession numbers KC661267, KC661268, KC661269 and KC661270 for the *hhyL* genes. 16S rRNA gene sequences were compared with published sequences in the NCBI gene databases (BLASTN, http://blast.ncbi.nlm.nih.gov) for phylogenetic identification (Table S1).

H_2 *uptake assays*

H_2 oxidation rates were determined routinely by measuring the decrease in H_2 mole fractions in the microbial culture headspace over time. Microbial strains were cultivated aerobically on solid (R2A) or liquid (TSB) medium inside 160 ml glass serum vials. H_2 uptake rate measurements were initiated by isolating the serum vial headspace from the atmosphere with a crimped stopper and vials were slightly pressurized after closure by adding 15 ml of sterile lab air. Liquid cultures were continuously agitated at 200 r.p.m. during the H_2 uptake assay to facilitate gas exchange across the air-liquid interface. The change in headspace H_2 was measured three times at approximately 40-min intervals. H_2 uptake followed apparent first-order kinetics over the small range (0.1–4 ppm) of laboratory atmospheric H_2 mole fractions: $H_2(t) = H_2(0)e^{-bt}$. First-order rate constants were determined from the slope (–b) of the logarithmic decrease in the headspace H_2 mole fraction. H_2 oxidation rates are reported at a H_2 mole fraction of 530 ppb, the estimated global mean (Novelli *et al*., 1999).

H_2 mole fractions were measured using a gas chromatograph (GC, Model 2014, Shimadzu Co., Kyoto, Japan) retrofitted with a helium ionization pulsed discharge detector (HePDD, D-4-I-SH17-R Model, Valco Instruments Co. Inc., Houston, TX, USA). The instrument is similar to a recently described system for measuring H_2 at atmospheric levels (approximately 530 ppb) and has an improved precision, linearity and stability compared with methods that use a mercuric oxide detector (Novelli *et al*., 2009). Details of the instrument design and performance are publically accessible in Meredith (2012). Stainless steel flasks containing compressed air were used as working standards with ambient H_2 mole fractions. These were calibrated using the GC-HePDD system against a tertiary standard (514.3 ppb H_2 in air, aluminum 150A tank, Airgas, Radnor) tied to the H_2 scale of the National Oceanic & Atmospheric Administration Earth System Research Laboratory Global Monitoring Division. Precisions, assessed by repeated standard measurements, were typically < 1% (1 sigma) on the Shimadzu GC-HePDD.

The precision for H_2 oxidation rate measurements is taken as two times the standard deviation of measurements of the H_2 uptake in sterile control vials containing the same (liquid or solid) medium as the culture vials. This precision serves as the effective detection limit, that is, the minimum H_2 oxidation rate that is distinguishable from zero by the measurement. Detection limits were determined separately for the time series of H_2 uptake rates measured in control vials for strain HFI8 and *R. equi* because of the difference in medium and were between (±0.12 and ±0.24 nmol h^{-1}).

The Michaelis–Menten substrate affinity (K_m) describes the affinity of H_2 uptake, relevant to the broad range of H_2 concentrations that occur in soils (H_2 mole fractions ranging from 0.01 to 1000 ppm) (Schink, 1997; Constant *et al*., 2008). Kinetic parameters of H_2 uptake were determined in sporulated *Streptomyces* cultures and in the stationary phase cultures of *R. equi*. The dependency of H_2 uptake rates on initial H_2 mole fractions were determined over a range of initial headspace H_2 mole fractions (set at about eight levels between 0.5 and 35 ppm H_2 by injecting a 1% H_2 in N_2 mix into the sealed headspace). Headspace H_2 was measured twice, 15 min apart in each culture containing different initial H_2 concentrations, and H_2 uptake was calculated from the linear uptake rate. The K_m and the maximum reaction rate (V_{max}) for each strain was determined from LB plots of the inverse of the uptake rate (1/V) vs the inverse of the substrate concentration (1/S) the initial H_2 mole fraction. K_m was determined as the $K_m = -1/x$-intercept and Vmax as $V_{max} = 1/y$-intercept (Constant *et al*., 2008). As a cross-check for the quality of the reported kinetic parameters, Eadie–Hofstee (EH) plots of V vs V/S were used to determine K_m from $K_m = -$slope. K_m and V_{max} values were reported for a given strain only if the LB and EH K_m values methods agreed within 50%. A typical LB and EH plot is shown in Fig. S7. H_2 uptake thresholds were determined after allowing the cultures to take up H_2 mole fractions for at least 90 min until headspace H_2 mole fractions reached stable values.

Lifecycle analysis of Streptomyces *spp.*

The life cycle of *Streptomyces* spp. cultures was tracked in parallel with the H_2 uptake to test the influence of developmental stage on atmospheric H_2 uptake. Serum vials (160 ml) containing 10 ml of R2A medium were inoculated with 100 µl of the spore suspension onto the agar surface. Control vials were supplemented with 100 µl sterile H_2O. The developmental stages were assessed by microscopy, using a Zeiss Axio Imager.M1 microscope and Axio Cam MRm camera using Axio Vision (4.8) software (Zeiss, Peabody, MA, USA).

Growth rates of filamentous organisms grown on solid media are difficult to measure; instead, photographs of the fractional area covered by *Streptomyces* colonies in the serum vial were used as an indication of growth rate. Final aerial biomass was quantified by a protein assay. Aerial biomass was aseptically harvested using a metal spatula and

transferred to 1.5 ml tubes containing 0.3 g of glass beads (0.2 mm diameter) and 0.7 ml water. Cells were vortexed for 5 min at 2000 r.p.m. followed by cooling on ice and then sonicated with three 30 s bursts and 1 min intermittent cooling on ice. Residues of membranes and nucleic acids were removed by transferring 0.5 ml of the protein extract to Costar® Spin-X® microcentrifuge filter tubes (Corning, Inc., Corning, NY, USA) and centrifuging at 10 000 r.p.m. for 15 min. Protein concentrations were determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA) and a Synergy 2 Microplate Reader (BioTek, Winooski, VT, USA) controlled by Gen5 (1.04.5) software.

To determine whether H_2 uptake in the aerial fraction (containing hyphae and spores) of a *Streptomyces* culture would continue to take up H_2 when separated from the substrate mycelium and medium, H_2 uptake rates were measured before and after by gently rolling between 2.5 and 10 g of 4-mm glass beads (Table 2) over *Streptomyces* sp. HFI8 lawns of various ages (2–15 days). The lawns grew on R2A solid medium in a serum vial, and the aerial biomass was transferred to a sterile glass serum vial containing no medium (Fig. S5). H_2 uptake rates were measured in the original culture vial, the lawn was treated with the glass beads, and aerial biomass was transferred immediately by moving the glass beads to a sterile vial. H_2 uptake rates were measured over the next 2–4 h in the sterile vial containing the isolated aerial biomass on glass beads and in the original vial containing medium and the remaining substrate mycelium. The amount of biomass that was transferred was quantified using the protein assay described earlier. This procedure was performed for six replicates at different time points after sporulation and with different amounts of glass beads (Table 2, Samples 1–6). In addition, the effect of the glass beads on H_2 uptake in the absence of transfer was tested in six control samples. These samples were treated with the glass beads, but the beads remained in the original vials (Table 2, Samples 7–12) and H_2 uptake rates were measured in the same vials before and after disruption by glass beads.

Growth phase analysis of R. equi

The relationship between the growth phase and H_2 uptake of *R. equi* was assessed in liquid cultures. *Rhodococcus equi* was inoculated by adding 100 µl of a cell suspension into 20 ml sterile TSB (Bacto™ Triptic soy broth, BD) liquid medium in 160 ml glass serum vials. All cultures were incubated at 30°C and shaken at 200 rpm. Growth was monitored by measuring the OD of *R. equi* cultures as the absorbance at 600 nm at 25°C in the Synergy 2 Microplate Reader. The relationship between OD and protein concentration was established by constructing a calibration curve between OD measurements of serial dilutions with known protein concentrations. *Rhodococcus equi* protein concentrations were determined using the same general procedure as described for the *Streptomyces* spp. The growth phase in *R. equi* cultures were established using the semilogarithmic plot of the growth curve (Fig. 2), where the exponential growth phase is taken as the period with the maximum, sustained positive slope. Late exponential phase was defined as the time when the growth rate slowed down, as identified by a decreasing slope of the growth curve. Finally, stationary phase occurred where the growth curve slope was zero.

H_2 uptake by *R. equi* was low. A concentration/dilution experiment was performed to test whether the negligible H_2 oxidation rates at low cell densities in early exponential growth phase were the result of a lack of H_2 oxidation activity or the low signal-to-noise ratio because of the small number of active cells. *Rhodococcus equi* cultures were inoculated at the beginning of the experiment, concentrated in exponential phase on day 1.9 by centrifugation at 8000 r.p.m. for 10 min, and re-suspended into either fresh TSB or in sterile H_2O to final densities of 160 and 110-µg protein ml^{-1} in TSB and H_2O respectively. This was within the range of densities observed in late exponential and stationary phases (100–230 µg protein ml^{-1}). Additionally, a sample was taken on day 7.8 in stationary phase (at a density of 190 µg protein ml^{-1}) and was diluted in TSB or sterile H_2O to cellular densities of 45 and 38 µg protein ml^{-1}, respectively, to match the density in the early exponential phase (10–100 µg protein ml^{-1}). For both the concentration and dilution experiments, the cell pellets resulting from centrifugation were not washed during the procedure so that some extracellular material and original culture medium (< 1 ml) was diluted into fresh TSB or H_2O to a maximum final concentration of 1/5th. H_2 uptake rates in the headspace of the concentrated or diluted samples were measured as described earlier.

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Supporting information

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Fig. S1. Photographs of *Streptomyces griseoflavus* Tu4000 and *Streptomyces* sp. HFI6–HFI9 soil isolate colonies on R2A medium plates. *S. griseoflavus* Tu4000 had the smooth and waxy appearance of a *bld* (bald) *Streptomyces* mutant, while strains HFI6–HFI9 formed fuzzy colonies consistent with the presence of aerial hyphae. The pigmentation of strains HFI6–HFI8 was light pink, and strain HFI9 was darker with a brown exudate secreted into the surrounding medium. HFI6–HFI9 strains had the strong scent of geosmin, while *S. griseoflavus* Tu4000 did not.

Fig. S2. Photomicrographs of *Streptomyces griseoflavus* Tu4000 and *Streptomyces* sp. HFI6–HFI9 soil isolate cultures on R2A medium plates. The same samples are photographed in Fig. S1. Only substrate mycelia are visible in the *S. griseoflavus* Tu4000 colony, while HFI6–HFI9 strains had plentiful aerial hyphae.

Fig. S3. Molecular phylogenetic analysis of *hhyL* sequences by the maximum likelihood method. The diversity of the high-affinity group 5 [NiFe]-hydrogenase (*hhyL*) sequences of the strains (bold) we tested for H_2 uptake (Table 1) are compared with *hhyL* sequences from the NCBI microbial genome database in this amino acid tree. The *hhyL*-containing *Streptomyces* sequences form two distinct clusters at a deep 99% bootstrap branch: Cluster 1 and Cluster 2. Isolates that have been tested for H_2 uptake are marked to indicate whether (*) or not (†) high-affinity H_2 uptake was observed. Culture collection strains investigated in this study were selected to broaden representation across the clusters and genera: *Streptomyces griseoflavus* Tu4000 (Cluster 1), *Rhodococcus equi* (*Actinobacterium*, Cluster 1) and *Streptomyces cattleya* (Cluster 2). Strains HFI6, HFI7, HFI8 and *S. griseoflavus* Tu4000 *hhyL* are closely related to Cluster 1 *Streptomyces* spp. soil isolates that take up H_2 (summarized in Constant *et al.*, 2011b), while strain HFI9 *hhyL* is more closely related to the *R. equi hhyL*. Cluster 2 *S. cattleya hhyL* is closely related to *Streptomyces* sp. AP1 *hhyL*, which also consumes H_2 (Constant *et al.*, 2011b). Other culture collection strains that have been tested for H_2 uptake include *Ralstonia eutropha* H16 (Conrad *et al.*, 1983a) and *Mycobacterium smegmatis* (King, 2003b). The evolutionary history was inferred by using the maximum likelihood method based on the Whelan and Goldman (2001) model. The *hhyL* gene from an archaeon *Sulfolobus islandicus* HVE10/4 was used as the outgroup. The bootstrap values are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using a WAG model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.2590]). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 amino acid sequences. There were a total of 427 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Fig. S4. Microscopic observations of the development of *Streptomyces* sp. HFI8 show that strain HFI8 underwent the full lifecycle from spore to spore in less than 1.8 days, after which nearly all the viable cells existed as spores. Each panel shows a representative micrograph of the culture taken on a different day after the inoculation. Image A shows the substrate mycelium that grows after the germination of inoculated spores. By day 1.8 (B), septated aerial hyphae (punctuated tubular branches) and fully formed spores (round cells) are present. Mainly spores are present from day 2.9 to day 22 (C–H), and the same persists until day 44 (not shown).

Fig. S5. Photograph of serum vials during the aerial biomass removal experiments (Table 2, Samples 1–6) illustrates the separation of the aerial hyphae and spores from the substrate mycelium: (A) vial containing an *whole* intact strain HFI8 culture (Table 2, column 5); (B) vial from which the aerial biomass had been isolated using glass beads leaving behind the remaining *substrate* mycelium (Table 2, column 7); and (C) vial containing the isolated *aerial* biomass on the surface of the glass beads (Table 2, column 6). In some samples, glass beads were rolled on the whole colony surface (A) and

were left in the same vial with no biomass transfer (Table 2, Samples 7–12).

Fig. S6. Scatter plot of the initial H_2 oxidation rate vs the reduction in H_2 uptake by *Streptomyces* sp. HFI8 during the glass bead procedure (Tables 2, 5th and 8th columns). The larger the initial H_2 oxidation rate, the larger percentage reduction by the glass beads ($R^2 = 0.93$), regardless of culture age or the amount of glass beads used for transfer.

Fig. S7. Determination of H_2 uptake kinetic parameters by the Lineweaver–Burk (LB) and Eadie–Hofstee (EH) methods for *Streptomyces* sp. HFI8. The H_2 uptake rate (V) nmol h^{-1} and initial H_2 concentration (S) in ppm are used to generate the LB plot as 1/V vs 1/S and EH as V vs V/S. K_m was determined from the LB plot as the $K_m = -1/x$-intercept (38 ppm) and V_{max} as $V_{max} = 1/y$-intercept (30 μmol min^{-1} g^{-1}). K_m was determined from the EH plot as K_m = -slope (22 ppm). K_m and V_{max} values were reported for a given strain only if the LB and EH K_m values methods agreed within 50%.

Table S1. The top database matches for strains HFI6–HFI9 16S rRNA gene, and *hhyL* nucleotide sequences indicate that the strains are *Streptomyces* spp. containing *hhyL* sequences. The GenBank accession number is listed for each deposited sequence. The results of NCBI Megablast BLAST search are listed for each sequence, where queries were made for the 16S rRNA sequences against the 16S rRNA gene sequence database and for the *hhyL* sequences against the entire nucleotide sequence database. The top match for each BLAST search is listed along with the total score, E value and maximum identity of the match. Strains HFI6–HFI9 16S rRNA gene sequences were 100% identical to several different strains of *Streptomyces* spp. Strains HFI6–HFI9 *hhyL* sequences are highly similar to published cultured and uncultured *hhyL* sequences, of which some were submitted to public databases as *hydB*-like genes, although the *hhyL* terminology has been more recently adopted (Constant *et al*., 2011b).