

**Exploring the microbe-mediated soil H₂ sink:
A lab-based study of the physiology and related
H₂ consumption of isolates from the Harvard
Forest LTER**

by

Deepa Rao

Submitted to the Department of Earth, Atmospheric, and Planetary
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Abstract

Atmospheric hydrogen (H₂) is a secondary greenhouse gas that attenuates the removal of methane (CH₄) from the atmosphere. The largest and least understood term in the H₂ biogeochemical cycle, microbe-mediated soil uptake, is responsible for about 80% of Earth's tropospheric H₂ sink. A recent discovery of the first H₂-oxidizing soil microorganism (*Streptomyces* sp. PCB7) containing a low-threshold, high-affinity NiFe-hydrogenase functional at ambient H₂ levels (approx. 530 ppb) made it possible to identify a model organism to characterize microbial H₂-uptake behavior. In the present research, several strains of *Streptomyces* containing the high-affinity NiFe-hydrogenase were isolated from the Harvard Forest LTER and used to characterize H₂ uptake alongside analysis of their life cycles. It was found that containing the gene encoding for the specific hydrogenase predicted H₂ uptake behavior in the wild *Streptomyces* strains and also in more distantly related organisms that contained the gene. The H₂ uptake rates were correlated with the microorganisms' life cycles, reaching a maximal uptake corresponding with spore formation. Understanding how environmental conditions, organismal life cycle, and H₂ uptake are connected can help reduce the uncertainty in atmospheric models. With the rise of H₂-based energy sources and a potential change in the tropospheric concentration of H₂, understanding the sources and sinks of this trace gas is important for the future.

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Chapter 1

Introduction

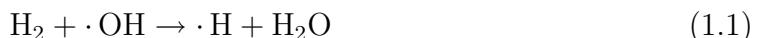
1.1 Motivations for Research

The uncertainties about the microorganisms responsible for global soil H₂ uptake as well as the environmental factors that influence their H₂ uptake activity limit our ability to model present and future atmospheric H₂ and its associated GHG trends (Constant et al., 2009). Our research proposes to help fill the gap in knowledge concerning the nature of H₂ oxidizing organisms by characterizing their H₂ uptake behavior alongside analysis of their life cycles. In particular, a genus of soil microorganism—*Streptomyces*—will be studied because some *Streptomyces* have been found to contain a high-affinity hydrogenase enzyme that can utilize H₂ at ambient tropospheric levels. Furthermore, *Streptomyces* are prevalent in soils, potentially contributing a significant amount to the overall H₂ soil sink. Other high-affinity hydrogenase-containing soil microbes exist outside of the genus *Streptomyces* and this research will begin to explore their H₂ uptake potential as well, with the goal of improving the larger ecological understanding of microbial-mediated soil H₂ uptake. This research focuses on testing high-affinity hydrogenase-containing microbes in a laboratory setting because in environmental soil, many variables affect H₂ flux in the low atmosphere.

1.2 Atmospheric Hydrogen

Present at about 530 ppb, molecular hydrogen (H_2) is the 9th most abundant element in Earth's atmosphere (Novelli, 1999). The rising use of hydrogen-based energy technologies, particularly hydrogen fuel cells, is proposed to increase H_2 input into the atmosphere (Constant et al., 2009). The potential regional and global environmental effects of a hypothetical rise in tropospheric H_2 are very difficult to model due to a large uncertainty in the H_2 biogeochemical cycle.

On Earth, H_2 is an indirect greenhouse gas (GHG) that is integral to maintaining the atmosphere's oxidative state. H_2 attenuates the removal of potent GHGs like methane (CH_4) by reacting with the atmosphere's cleansing hydroxyl radical ($\cdot OH$).



In Reaction (1.1), H_2 reduces $\cdot OH$ to H_2O , thereby eliminating an $\cdot OH$ that could have potentially removed CH_4 from the troposphere via Reaction (1.2). Understanding the sources and sinks of molecular hydrogen is necessary to understand the current impact of anthropogenic H_2 emissions and to predict the future environmental effects of a hydrogen-fuel economy.

Sources of Atmospheric H_2

Annually, 107 ± 15 Tg of H_2 are emitted into the troposphere by approximately equal parts natural and anthropogenic processes (Rhee and Brenninkmeijer, 2006). The photochemical oxidation of hydrocarbons like methane (CH_4) and non-methane hydrocarbons (NMHC) result in the addition of H_2 into the atmosphere. Indirect combustion is a direct emission source of H_2 to the atmosphere, making both biomass burning and fossil fuel combustion emission sources. CH_4 is mainly released from microbial methanogenesis in wetlands but is also derived from the combustion of fossil fuels, domestic ruminant fermentation, rice paddies, legume emissions, and biomass

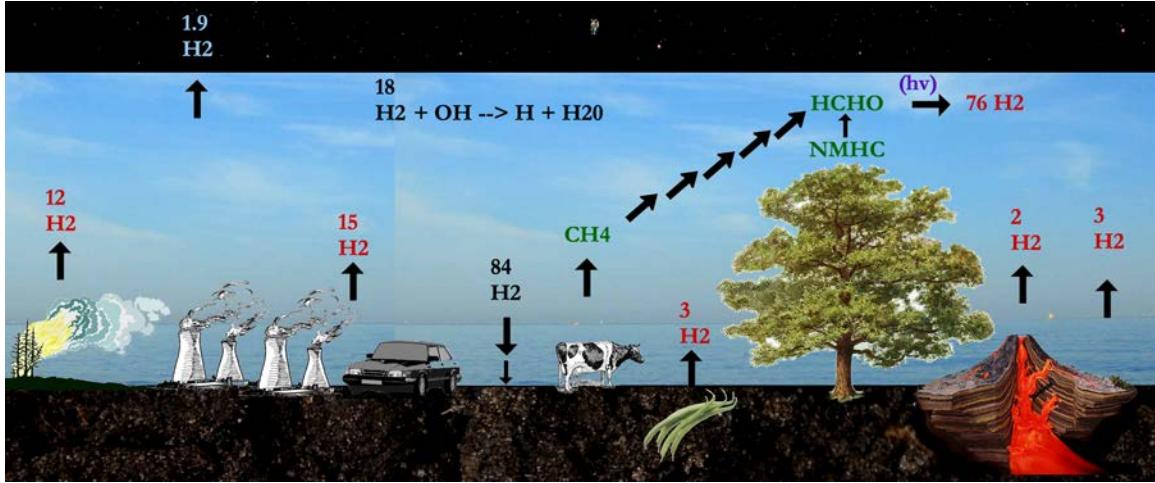


Figure 1-1: The major components of the biogeochemical hydrogen (H_2) cycle on Earth. All units are in Tg/year. Anthropogenic and natural sources are roughly equal H_2 emitters into the atmosphere. The largest H_2 sink is due to microbial-mediated uptake into the soil, accounting for about 80% of all tropospheric H_2 uptake. The remaining 20% of tropospheric H_2 is removed from the atmosphere during its interaction with $\cdot OH$ (Xiao et al., 2007; Novelli, 1999).

burning (Constant et al., 2009).

Sinks for Atmospheric H_2

Atmospheric H_2 has a lifetime of 1-2 years. Approximately 20% of tropospheric H_2 is lost via oxidation by $\cdot OH$ (Reaction (1.1)) (Constant et al., 2008). Microbe-mediated soil uptake compromises the largest tropospheric H_2 sink, accounting for approximately 80% of H_2 losses (Constant et al., 2009; Novelli, 1999; Rhee and Brenninkmeijer, 2006). It is estimated that soil uptake removes 88 ± 11 Tg of H_2 each year (Rhee and Brenninkmeijer, 2006). Although the microbe-mediated soil sink is the largest term in the H_2 budget, it is also the least understood. Much is unknown about the environmental and microbiological controls of H_2 soil uptake.

Microbe-Mediated H_2 Soil Sink: A Brief History

In Germany in the 1980s, the first measurements of H_2 soil deposition velocities (v_d) confirmed soil uptake (dry deposition) to be the largest sink for tropospheric H_2 (Conrad et al., 1983). Deposition velocity is an uptake rate independent of the initial

concentration, measured in units [distance/time]. The relationship between H₂ flux and v_d is

$$\text{Flux}_{H_2} \propto -v_d [\text{H}_2], \quad (1.3)$$

where [H₂] is the concentration.

Several soil environmental factors influence H₂ dry deposition, most notably soil water content, temperature, pH, and organic content (Constant et al., 2008; Conrad et al., 1983). Naturally, as these factors vary, H₂ uptake varies as well. Initially it was believed that abiotic, free soil-hydrogenases were primarily responsible for the tropospheric H₂ sink because sterilizing soils by different methods did not appear to affect uptake rates (Conrad et al., 1983).

Later, in the early 2000s, to further test the nature of the soil sink, soil samples were sterilized, treated with antimicrobial compounds, and depleted in O₂. After these treatments, soil H₂ uptake was reduced by 60-80%, leading to the presumption that microbial-mediated uptake likely had an active and important role in the H₂ soil sink. The remaining 20-40% of H₂ uptake was attributed to free soil hydrogenases that continued to uptake H₂ despite treatments to eliminate biological sources (King, 2003; Conrad, 1981; Conrad et al., 1983; Constant et al., 2009). In order to gain a global understanding of the H₂ soil sink, several measurements of H₂ deposition velocity were taken in various locations and extrapolated to a global scale (Conrad, 1981; Novelli, 1999); however, a lack of both data and understanding of the microbe-mediated soil sink inhibits progress in designing more precise global models of H₂.

Hydrogenases within microorganisms were recognized as minimally contributing factors to the soil sink because only low-affinity ($K_m \sim 1000$ ppmv) soil hydrogenases had been characterized at the time. Low-affinity soil hydrogenases were found in organisms living in extremophilic environments, methanogenic sediments, or around the nitrogen-fixing root nodules on legumes that diffuse H₂ at locally high concentrations. In order to be considered a potential tropospheric H₂ consumer, a microorganism should contain a high-affinity ($K_m < 100$ ppmv) hydrogenase that is active at and below an H₂ concentration of 530 ppbv (ambient H₂ concentration)(Conrad,

1996). Until recently, no such microbe had been found or characterized; only aerobic microorganisms containing a low-affinity hydrogenase enzyme that worked at much higher concentrations of H₂ than atmospheric levels had been studied.

Recent Advances in Understanding of the Microbial H₂ Soil Sink

In 2008, Constant et al. (2008) characterized the first organism found to utilize H₂ at ambient tropospheric levels due to its having a high-affinity, low-threshold NiFe-hydrogenase, which is active at and below ambient tropospheric H₂ levels. The specimen, named *Streptomyces* spp. PCB7 was found to belong a common genus of soil microorganisms, *Streptomyces*. *Streptomyces* sp. PCB7's life cycle and corresponding H₂ uptake of a H₂-utilizing organism were studied in the soil, revealing that H₂ uptake occurred at a maximum rate during a specific point in the organism's life cycle. Their research hypothesized that H₂ uptake was used for the spores' metabolism or that the hydrogenase enzyme was secreted into the environment during the formation of physical structures, called aerial hyphae, by the colony (Constant et al., 2008).

Other, less-prevalent soil microbial species such as *Rhodococcus*, *Saccharopolyspora*, and *Frankia*, also contain the high-affinity NiFe-hydrogenase that may be responsible for a significant amount of the measured H₂ soil sink. Little or no work has currently been done to characterize the H₂ uptake of these strains. Understanding the environmental and biological influences on H₂-oxidation through the high-affinity NiFe-hydrogenase could potentially help explain environmental fluxes in the H₂ soil sink.

1.3 High-affinity NiFe-hydrogenase

NiFe-hydrogenases are reversible metalloenzymes, containing a Ni and Fe ions in the active site. Most often, NiFe-hydrogenases oxidize H₂ by the forward direction of the following Reaction (Constant et al., 2008).



However, it is also possible for the enzyme to catalyze the production of H₂. NiFe-hydrogenases are less sensitive to the ambient concentration of O₂ unlike the FeFe-hydrogenase; for this reason, most microorganisms which oxidize H₂ in aerobic environments contain a NiFe-hydrogenase (Constant et al., 2011). Organisms with NiFe-hydrogenase typically use H₂ as an energy source via uptake and oxidation and sometimes use the enzyme to produce H₂ from protons via Reaction (1.4) (Constant et al., 2011):

A tree (Figure 1-2), based on published genomes from the NCBI database as well as sequences of our strains, revealed that the high-affinity, low-threshold NiFe-hydrogenase gene is present in three main clusters (1, 2, and distantly related hydrogenases). Clusters 1 and 2 contain many common soil microorganisms. The distant high-affinity hydrogenases are typically found in extremophilic bacteria, Several other species contain the high-affinity NiFe-hydrogenase gene and may also have H₂ uptake potential.

1.4 *Streptomyces*

Streptomyces are widespread aerobic soil and sediment-dwelling microorganisms. One gram of soil alone can contain 10⁴ to 10⁷ colony forming units of streptomycetes, accounting for 1-20% or more of all viable soil microorganism counts (Schrempf, 2008). Comprising the largest genus of the phylum Actinobacteria, *Streptomyces* are Gram-positive bacteria that have genomes rich (> 50%) in guanine and cytosine DNA base pairs (GC rich). *Streptomyces* produce many metabolites, most importantly antibiotic compounds used by the *Streptomyces* to inhibit competition in their environment and used by humans for pharmaceuticals.

Life Cycle and Morphology

Streptomyces have a complex life cycle similar to many fungi, with active cells and semi-dormant spores, as depicted in Figure 1-3. Under the proper environmental conditions *Streptomyces* spores enter into the vegetative growth stage of their life cycle,

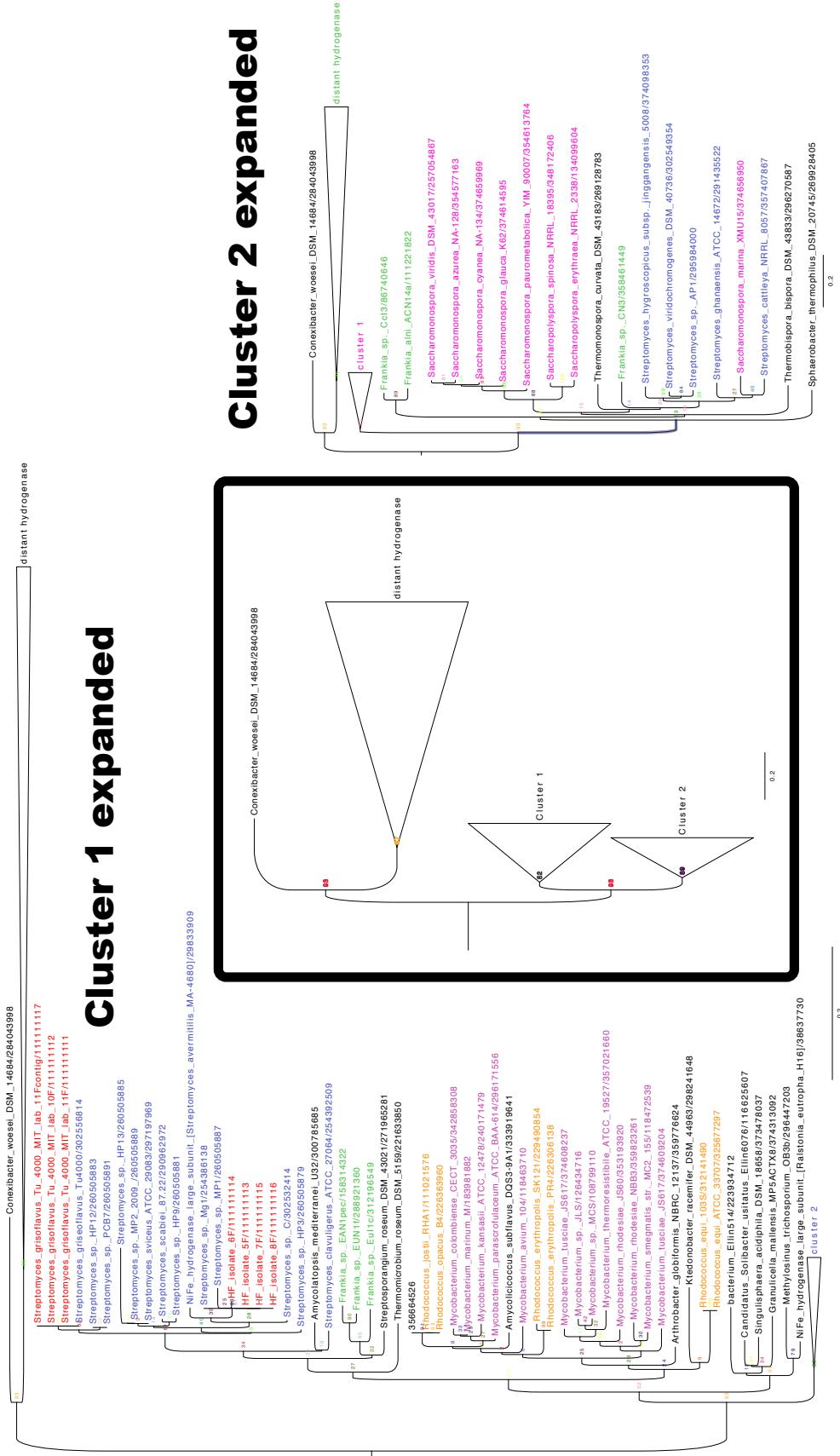


Figure 1-2: A tree of high-affinity hydrogenase amino acid sequences constructed using reference strains from NCBI database of previously published genomes of high-affinity NiFe-hydrogenase containing microorganisms and sequences derived from HFI strains and *S. griseoflavus*. High-affinity NiFe-hydrogenases group into three main clusters, cluster 1, cluster 2, and distant. The strains sequenced in this study are shown in red. (Image by Laura Meredith)

sometimes after decades of dormancy (Schrempf, 2008). This stage is characterized by the formation of branching vegetative hyphae, one or more filamentous cells that are surrounded by a tubular cell wall. The hyphae spread out among particles of soil, increasing the colony's surface area (Schrempf, 2008). A large network of vegetative hyphae forms, at which point it is called a group of mycelia. Mycelia initially grow and embed into the substrate (either agar in the lab or sediment and soil particles) and solubilize organic debris (Flärdh and Buttner, 2009). After some time, the substrate mycelia are lysed and the molecular components are used to form aerial hyphae. Aerial hyphae are encased with hydrophobic proteins that allow them to break the surface tension of the substrate and extend above the colony surface (Schrempf, 2008). Aerial hyphae begin to differentiate into chains of spores, which are separate semi-dormant cells containing genomic DNA, leading to exponential growth. When the aerial hyphae encasing layer is broken, individual spores are released into their surroundings.

At this point of aerial hyphae production and spore differentiation, metabolites, pigments, and antibiotics are produced as chemical protection against potential microbes that could consume the transitioning cells. Volatile compounds—most notably geosmin, an earthy-water scent reminiscent of soil—are also produced (Schrempf, 2008). Similarly, significant H₂ consumption has been found to coincide with maturation of the spores. In the case of Constant et al. (2008)'s isolate *Streptomyces* sp. PCB7, H₂ uptake activity was only detected during the terminal stage of sporulation. The exact reasons for H₂ uptake by *Streptomyces* remains unknown. Characterizing H₂ uptake while analyzing the life cycle can help elucidate the connection between life cycle and H₂ metabolism. This knowledge can later be extrapolated to potentially explain environmental factors correlated to the behavior of the H₂ soil sink.

Ecology

Streptomyces spores are resistant to low nutrient conditions and drought; however, the vegetative state is sensitive to water availability (Schrempf, 2008). Spores are the most common life cycle state of *Streptomyces* in soil. Mycelia are non-motile; how-

Streptomyces Life Cycle

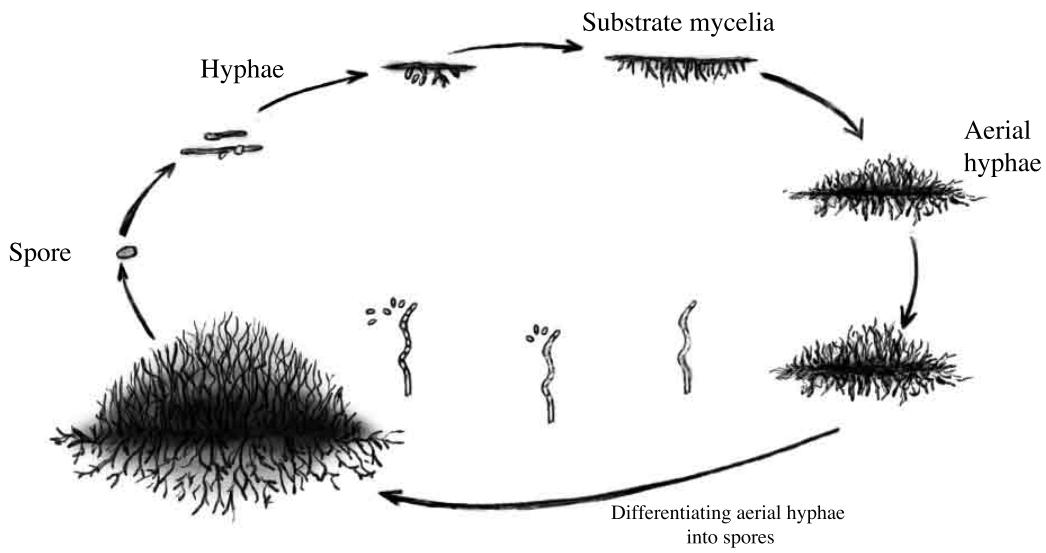


Figure 1-3: The life cycle of *Streptomyces*, a genus of filamentous soil microorganisms with a complex life cycle similar to many fungi. Semi-dormant spores can regenerate under favorable environmental conditions and transition into a vegetative cell state, starting with the formation of substrate mycelia and later the production of aerial hyphae, which ultimately differentiate into spores.

ever, as they branch through a substrate, they differentiate into spores that are easily transported (via wind, water, etc.) and may then colonize another area containing necessary nutrients. Sporulation is a means of dispersal for *Streptomyces* and is induced by nutrient depletion (Schrempf, 2008). The vegetative cycle of *Streptomyces* relies on soil moisture. Thus, it is easy to imagine a constant cycle of growth and dormancy depending on environmental factors in a dynamic ecosystem. Environmental factors trigger transitions in the *Streptomyces* life cycle. If H₂ uptake is related to their life cycle state, then understanding the connections between environmental variables, *Streptomyces* life cycle, and H₂ uptake would lead to a better understanding of the overall soil sink.

1.5 Experiments

Three experiments were designed with the overarching goals of quantifying the H₂-uptake rates and unique life cycles of the isolated strains and *S. griseoflavus*, the reference organism. A basic experimental setup was standard for all three experiments and is first detailed here in the Introduction. An explanation of any variations among the three experiments in the Results section.

1.5.1 Experimental Setup

The acquisition of *S. griseoflavus* from another lab and the isolation of four *Streptomyces* Harvard Forest isolates (HFIs) containing the high-affinity NiFe-hydrogenase resulted in five testable strains, allowing further inquiry into the nature of *Streptomyces*' H₂ oxidation. Significant questions include:

1. Does having the gene for the high-affinity NiFe-hydrogenase correlate with active H₂ oxidation?
2. Do the various HFI strains and *S. griseoflavus* have unique patterns of H₂ uptake?
3. Do observed changes in the *Streptomyces* life cycle correlate to changes in measured H₂ uptake rates?
4. Does measurable H₂ uptake continue into the spore state of the *Streptomyces* life cycle?
5. Is H₂ uptake perhaps maximal at the spore state as suggested by current data?

Although all five strains were found to contain the gene encoding for the high-affinity, low-threshold NiFe-hydrogenase, their atmospheric H₂-oxidation behavior had to be verified. In addition, the H₂-uptake rates of the individual strains were characterized and compared to each other. To supplement the measurements of H₂ uptake activity, the strains' life cycles were monitored to gain a deeper understanding of H₂ uptake in relation to life-cycle state.

The three separate experiments can be conceptually organized according to purpose and frequency of their measurements. Experiment 1 was a High (H) frequency set of measurements that were designed to capture rapid changes in uptake that accompany the early stages of the lifecycle. Experiment 2 was a Medium (M) frequency set of measurements designed to capture the rough shape of H₂ uptake over time in relation to the life cycle. Lastly, Experiment 3 was a set of Low (L) frequency measurements designed to only determine whether or not H₂ uptake occurred, without much detail on the time and life cycle dependence of uptake.

Table 1.1: An organized view of the main experiments

Exp. #	Exp. Frequency	Strain ID	H ₂ Uptake Reps	Media Control
1	High	HFI 8	3 (A-C)	R2A
1	[glass beads]	HFI 8	3 (F-H)	R2A
2	Medium	HFI 6	3 (A-C)	R2A
2	Medium	HFI 7	3 (A-C)	R2A
2	Medium	HFI 8	3 (A-C)	R2A
2	Medium	HFI 9	3 (A-C)	R2A
2	Medium	<i>S. griseoflavus</i>	3 (A-C)	R2A
3	Low	<i>S. cattleya</i>	3 (A-C)	R2A
3	Low	<i>Rhodococcus equi</i>	3 (A-C)	R2A
3	Low	<i>Rhodococcus equi</i>	3 (A-C)	TSB

1.5.2 Experiment 1: High Frequency Measurements of H₂ Uptake During Exponential Growth Stage and Spore State

Based on previous microscopy observations, it was assumed that HFI strains 6, 7, 8, and 9 had similar life cycles, although their speed of development varied. Given that the pattern of growth was similar, one strain, HFI 8, was chosen for Experiment 1. In order to capture the rapid changes in the early stage of the *Streptomyces*' life cycle, high-frequency gas chromatography H₂ uptake measurements and microscopy photos were taken to chronicle changes in H₂ uptake with the colonies' exponential growth phase. Measurements were taken approximately every 8-10 hours for the first

three days of the exponential growth phase of the freshly inoculated HFI 8 strains. Subsequent GC measurements were taken every two days for the completion of the life cycle, and were continued once a week for longer-term life cycle and H₂-uptake observations.

Glass beads

A series of three more replicates of each isolated strain underwent a procedure to test if pure-spore H₂ uptake occurs. Spores were isolated at three separate times by rolling sterile glass beads atop the colonies to collect aerial biomass. The aerial biomass was only harvested using this method after spores were observed (using microscopy) to be plentiful in the serum vial.

1.5.3 Experiment 2: Comparisons of H₂ Uptake Rates and Morphology/Life Cycle Between Similar HFI Strains

Experiment 2 was designed for morphology and H₂ uptake rate comparison among the five *Streptomyces* strains (HFI 6-9 and *S. griseoflavus*). In particular, GC measurements were taken to measure the magnitudes of H₂ uptake and quantify which strains had stronger H₂ oxidation. The strains' life cycles were also observed along with their changing patterns of H₂ uptake over time. This created a basis for correlating life cycle with amount of H₂ oxidation. The strains were measured with the GC for H₂ uptake every other day for 14 days. Accompanying the measurements, photographs and microscopy images were taken of the HFI and *S. griseoflavus* cultures.

1.5.4 Experiment 3: Comparisons of H₂ Uptake Measurements of Dissimilar High-Affinity NiFe-hydrogenase Reference Strains

Experiment 3 was a Low (L) frequency measurement designed to only determine whether or not H₂ uptake occurred by novel strains, without much detail on the time

and life cycle dependence of uptake. *Rhodococcus equi* (*R. equi*) and *Streptomyces cattleya* (*S. cattleya*) were chosen for Experiment 3 strains because *R. equi* contains a Cluster 1 high-affinity hydrogenase but does not belong to the genus *Streptomyces* and *S. cattleya* contains a Cluster 2 high-affinity hydrogenase but is a *Streptomyces*. Furthermore, *R. equi* is a non-spore forming soil microbe. If *R. equi* uptakes H₂, then its biological function for the high-affinity hydrogenase may be quite different than for *Streptomyces*.

Chapter 2

Methods

2.1 Isolation of *Streptomyces*

A single genus of organisms, *Streptomyces*, was isolated from fresh Harvard Forest soil samples after a series of morphological and genetic screening procedures.

Fresh soil samples are teeming with microbes, with as many as one billion organisms per gram of soil, which complicates the isolation of a single genus. The *Streptomyces*' life cycle includes the formation of a specialized cell type called a spore. *Streptomyces* spores are desiccation resistant and slightly heat resistant. They may germinate after periods of dormancy under favorable environmental conditions (You et al., 2005).

In order to isolate *Streptomyces* from the Harvard Forest Long Term Ecological Research (LTER) site, samples from the first 6 inches of soil (where *Streptomyces* are most in contact with atmospheric gases) were collected in sterile falcon tubes and dried in a 55°C oven for 3-4 hours, as per the Soil Spore Enrichment Protocol (Appendix A.4). Heat desiccation increased the relative density of *Streptomyces* spores to other organisms in the soil samples by eliminating non-spore forming, heat-sensitive organisms. Thereafter, the soil samples were potentially rich with *Streptomyces* spores that could later be cultivated.

Following the Soil Spore Enrichment Protocol, 1 g samples of the dried soils were

ground with a mortar and pestle and combined with 1 g of CaCO₃, which destroys the vegetative cells of many bacteria, thereby selecting for the enrichment of spores (Schrempf, 2008) The 2 g mixtures were spread across the bottom of petri plates, whose lids had been fitted with Whatman sheets moistened with sterile water. This humid environment was maintained for 2-10 days at 28°C. After this period, the soil and CaCO₃ mixture was combined with 100 mL of H₂O and agitated in a vortex. The mixture was left to settle for 30 minutes and soil suspensions were made at varying dilutions (10⁰, 10⁻², and 10⁻⁴). 100 µL aliquots of the soil suspension dilutions were plated on R2A agar plates. In order to prevent mold and fungal growth, the R2A plates were treated with 1.75 mg of cycloheximide, an antifungal agent, per plate. A 10⁻² dilution resulted in the most even coverage of plate surface area by microbes and was used for the rest of the isolation procedures.

Microbial growth on the plates was assessed for the four traditional morphological criteria of *Streptomyces*:

1. a zone of clearing, produced by the colony when it secretes antibiotics and thereby inhibits neighboring growth,
2. a fuzzy top coating of the colony that is distinctive of aerial hyphae stage of the *Streptomyces* lifecycle,
3. a pigmentation produced by the colony,
4. the distinctive scent of geosmin that is clearly evident when single strains were individually plated.

Based on these characteristics, colonies that were suspected to be *Streptomyces* were picked with a sterilized loop and plated onto fresh R2A agar plates. These cultures were grown at room temperature and transferred to new plates every few weeks to continue growth and avoid contamination.

Glycerol suspensions were made to preserve the HFI *Streptomyces* strains and *S. griseoflavus*. The microbes were inoculated in 4 mL of R2A broth and grown overnight or until the late exponential growth phase. At this point, 1 mL of a 50% glycerol

solution was added to the broth and culture vials. The contents were transferred to a 10 mL sterile falcon tube and frozen at -80°C until future use.

At this stage, the several strains suspected to be *Streptomyces* are referred to as the Harvard Forest Isolates (HFIs).

2.1.1 Testing PCR Protocols Using *S. griseoflavus* and Gel Electrophoresis

PCR protocols required optimization in order to accurately identify the two gene sequences of interest: 1) the 16S rDNA sequence to confirm which HFI strains actually belonged to the genus *Streptomyces* and 2) the high-affinity NiFe-hydrogenase sequence that was hypothesized to predict H₂ uptake activity.

The DNA of the HFIs was extracted using the PowerSoil DNA Extraction Kit (MoBio Laboratories, Inc.). Specific regions of the HFI DNA extracts containing the 16S rRNA and high-affinity Ni-Fe hydrogenase sequences were then amplified in separate reactions through polymerase chain reaction (PCR). The HFI DNA extracts were prepared for PCR by creating a reaction solution, called the master mix. Prior to making the reaction solution, 16S and high-affinity NiFe-hydrogenase oligo-primers were resuspended in TE to a 100 µM concentration, per the manufacturer's instructions.

In order to identify which of the suspected *Streptomyces* strains contained the gene encoding for high-affinity NiFe-hydrogenase, the reference genome *Streptomyces griseoflavus* Tu4000 (*S. griseoflavus*), which contains a high-affinity NiFe-hydrogenase highly similar to a published genome of PCB7 (Constant et al., 2008), was used as a positive control. *S. griseoflavus* was initially used to test the efficacy of the DNA extraction and PCR protocols, for both 16S and high-affinity NiFe-hydrogenase. In particular, *S. griseoflavus* was used to optimize PCR run cycles to determine both the optimal dilution of DNA template to use in PCR and the most effective annealing

temperature for the high-affinity NiFe-hydrogenase. Later on, the strains containing the high-affinity NiFe-hydrogenase were sequenced to establish whether or not they belonged to the genus *Streptomyces*.

The same protocol for gel electrophoresis was consistently used to confirm successful DNA amplification of the regions of interest. The gels were composed of 1% agarose and 5 μ L of red dye (GelRed Nucleic Acid Stain 10,000x in H₂O, BIOTUM). The DNA samples loaded into the gel were a mixture of 2 μ L loading dye (Fermentas 6x solution #R061) and 5 μ L of PCR product. A 1 kb DNA ladder was used to verify that the PCR product was the correct sequence length. A negative control (no DNA, only 25 μ L of master mix) and the positive control (*Streptomyces griseoflavus* DNA extract) were loaded into the gel for comparison. The gels were run at 100 V for 1 hour and then imaged under UV light using a UVP MultiDoc-It Digital Imaging System (Software Doc-ItLS).

PCR: High-Affinity NiFe-Hydrogenase Annealing Temperature

The efficacy of annealing high-affinity NiFe-hydrogenase primers to *S. griseoflavus* DNA extract was tested over an eight-step temperature gradient in the PCR reaction between 50°C and 62.2°C.

PCR: Dilution Series

DNA template dilutions were tested using gel electrophoresis to find the optimum concentration of template to use in future PCR runs. Dilutions of *S. griseoflavus* DNA extract (ranging from 1 μ L to 5 μ L, increments of 1 μ L) underwent PCR amplification with the high-affinity NiFe-Hydrogenase oligo-primers.

2.1.2 Testing Harvard Forest Isolates for the High-Affinity Hydrogenase

Ten Harvard Forest isolates (HFIs 1-10) were picked—based on the four defining *Streptomyces* characteristics described previously—and tested for the presence of NiFe-

hydrogenase. Their DNA was extracted using the PowerSoil DNA Extraction Kit. The DNA extracted from the HFI strains underwent high-affinity NiFe-hydrogenase and 16S rDNA amplification via PCR protocols that were previously optimized using *S. griseoflavus* as a standard, resulting in a 2 μ L DNA template dilution and 60.7°C as the high-affinity NiFe-hydrogenase annealing temperature. Gel electrophoresis was used to verify successful amplification of the high-affinity NiFe-hydrogenase gene and to determine whether or not it was present in the HFI organisms and also used to preliminarily see if the isolates contained the *Streptomyces* 16S rDNA sequence. Both of the HFI's amplified high-affinity NiFe-hydrogenase and *Streptomyces* 16S rDNA PCR products were run on an electrophoresis gel (1% agarose, 100 V, 1 hr) with a 1 kb DNA ladder, negative control , and a positive control (*S. griseoflavus*). The HFIs that showed a signal on the gel for successful amplification of 16s rDNA were later sequenced to determine their species.

Before sequencing, the extracted and PCR amplified 16S rDNA and high-affinity NiFe-hydrogenase DNA of strains *S. griseoflavus* and HFI 1-9 were purified using the Wizard® SV Gel & PCR Clean-Up System by Promega. The concentrations of purified DNA sequences were measured using a Nanodrop. Based on the measured concentrations, the cleaned PCR products were diluted to approximately 12 μ L for sequencing and submitted to MIT's Biopolymers Laboratory (76-181 Cambridge, MA 01239). Since *Streptomyces*' DNA is GC rich, the DNA was designated as "difficult" for its sequencing profile. Using the sequence data, a tree of the high-affinity hydrogenase amino acids was constructed using SeaView and Figtree software to determine the relationship and lineage of the HFI hydrogenases relative to those of the organisms listed in the NCBI database.

2.2 Characterizing *Streptomyces* Isolates' H₂ Uptake, Life Cycle, and Morphology

In order to observe whether the strains were oxidizing atmospheric H₂, a gas chromatograph (GC) in the Ono Lab at MIT was retrofit with HePDD for measuring H₂ concentration at atmospheric levels (~530 ppb) to high precision (~2 ppb), as described in Appendix A.13. A custom GC method was designed to record chromatograms of H₂ peaks, which can be used to obtain a H₂ mixing ratio, to determine the H₂ uptake rate.

Preparation of HFI Cultures for H₂ Uptake Measurements

In preparation for GC H₂ uptake measurements, several petri plates and 250 mL serum vials were autoclaved, sterilized, and filled with a layer of R2A agar. HFI 6, 7, 8, and 9 *Streptomyces* and *S. griseoflavus* cell suspensions were created by picking mature colonies with spores using sterile loops, suspending the cells in 1 mL of sterile H₂O, and vortexing to create a homogeneous mixture called a cell suspension. For every strain, four R2A plates were treated with 100 µL each of the cell suspensions: three for photography and one for colony counting. 200 µL of cell suspension of each strain was pipetted into each of the five respective R2A agar serum vials. The vials were swirled to cover the R2A agar evenly with the cell suspension. Of the five inoculated vials, three were replicates for GC H₂ uptake measurements, one was for taking microscopy samples, and one was for protein extraction at the conclusion of the experiment. A sixth control serum vial was created for each strain that contained 200 µL of sterile water atop the R2A agar without inoculum. The vials were capped with a sterile cotton bud so that air could be exchanged freely between the headspace and atmosphere, while preventing contamination.

Method for Measuring H₂ Uptake via Gas Chromatography

The individual H₂ uptake rates of each strain over the course of the HFIs' life cycles were quantified at a given frequency by measuring the rate of decrease of H₂ mixing

ratios (χ_{H_2}) via gas chromatography after capping the headspace of serum vials. Three serum vials for each strain were fitted with stoppers, crimped tight, and slightly pressurized with 20 mL of sterile, ambient, lab air. Using a 10 mL air-tight syringe, 4 mL of headspace air were removed from each serum vial and injected into the GC sample loop, to analyze the deposition velocity of H_2 by the organisms. Usually three serum vials were measured in rotation with a GC runtime that was set to a 12-minute cycle. Each sample vial, including a control vial, was measured three times. Upon completion of GC measurements, the vials' stoppers were removed and they were capped with sterile cotton and allowed to re-equilibrate to ambient H_2 concentrations. An example chromatogram is shown in Figure 2-1, where Ne and H_2 peaks occur at 3.3 and 3.7 minutes, respectively. The lines under the peak are the interpolated baseline, above which are the peak area and height, which are proportional to the mixing ratio. The peak areas are compared to peak areas of a calibrated standard to calculate mixing ratios.

H_2 mixing ratios were observed to decrease exponentially in time. Therefore, an exponential curve was fit to determine the decay constant b via

$$\chi_{\text{H}_2}(t) = \chi_{\text{H}_2}(0) e^{-bt}. \quad (2.1)$$

Following (Smith-Downey et al., 2006) and Eq. (2.2),

$$v_d = b \left(\frac{V_{\text{vial}}}{A_{\text{vial}}} \right), \quad (2.2)$$

the deposition velocity v_d was calculated from the decay constant, independently of the initial H_2 mixing ratio in the headspace (where volume = 150 cm³; area of base = 21 cm²) of the serum vial. Using the deposition velocity is important because H_2 mixing ratios in the lab varied from atmospheric levels (530 ppb), sometimes up to eight times higher (4 ppm), which influences the hydrogen flux but not the deposition velocity.

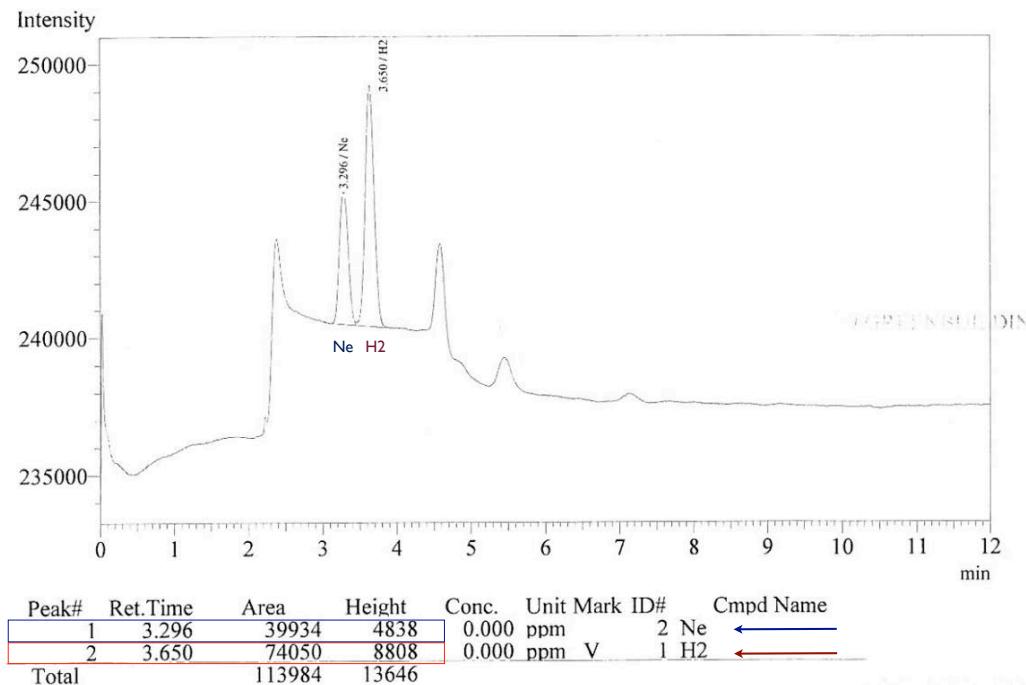


Figure 2-1: An example chromatogram of a serum vial headspace during the quantification of H₂ uptake. The Ne and H₂ peaks occur at 3.3 and 3.7 minutes, respectively. The lines under the peak are the interpolated baseline, above which peak area and height (which are proportional to a mixing ratio) are calculated. The peak areas are compared to peak areas of a calibrated standard to calculate mixing ratios.

Method for Imaging: Microscopy and Photography

Every round of GC measurements was accompanied by imaging, to determine the life cycle state and to track biomass growth in the vials. Photographs were taken of the petri plate surfaces to show colony morphology and growth; and of the underside of the serum vials to gain a rough idea of the percent area covered by microbial growth. Microscope slides of each strain were prepared from colonies in the designated microscopy serum vial. Microscopy (using the Zeiss Axio Imager.M1, AxioCam MRm, AxioVision software version 4.8, Bosak Lab at MIT) was used to determine the life cycle stage by searching for aerial hyphae and spores.

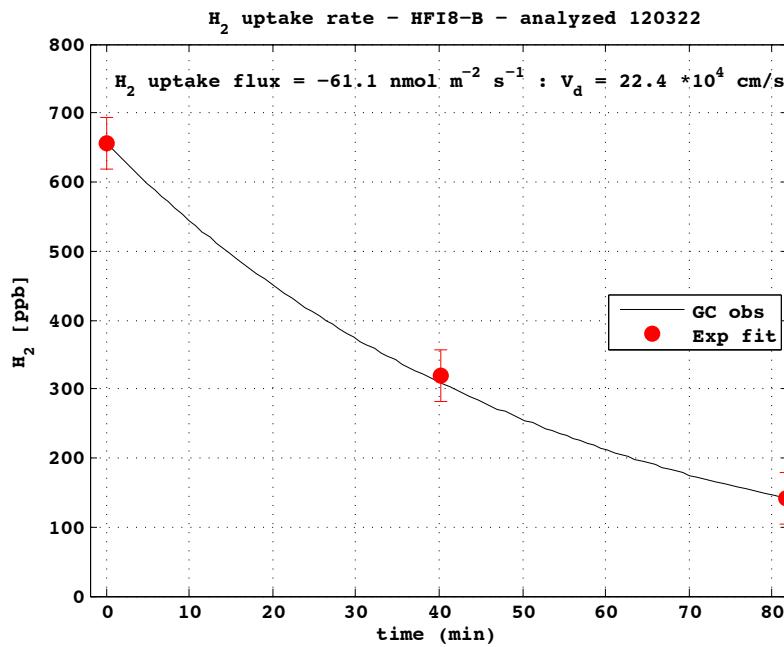


Figure 2-2: An example calculation of χ_{H_2} , v_d based on the original H_2 mixing ratios measured in the capped vial headspace as measured with the GC. For this example of HFI 8 (replicate B) analyzed on 03/22/2012, the exponential decay constant $b = 3.1362 \times 10^{-4} \text{ s}^{-1}$ and thus $v_d = 22.4 \times 10^{-4} \text{ cm/s}$.

Quantifying Biomass via Protein Extraction and CFUs

At the conclusion of the three experiments, a final round of GC measurements, microscopy, and photos were taken. As a preliminary measure, the colony forming units (CFUs) were measured on the plates to estimate the number of viable organisms in the cell suspensions and a rough idea of percent area coverage was assessed using photography.

In order to quantify the amount of uptake relative to biomass at the conclusion of Experiment 2, a protein bioassay was conducted to estimate cell density in the serum vials. Cellular material was removed from above the agar using a metal spatula, and protein was extracted following the Protein Extraction and Assay Protocol in Appendix A. The protein concentration was then determined using the Thermo Scientific Pierce BCA Protein Assay Kit and a BioTek Synergy 2 Microplate Reader (Gen5 1.04.5). A low-protein-concentration standard curve was created for the HFI

samples. The same protein extraction and biomass assay methods will be applied to Experiment 1 at its conclusion, but will not be complete by publication of this thesis.

Glass Beads

In Experiment 1, once spores were observed (using microscopy) to be plentiful in the serum vial, the colonies' aerial biomass was removed by rolling 10 g of 4 mm glass beads on the agar surface and transferring the beads with the collected biomass to a fresh, sterile serum vial. The serum vials were tested using the same GC method above to measure H₂ uptake of the separated biomass. Both sets of H₂ uptake measurements (aerial biomass on beads vs. remaining biomass in the serum vial) were compared. Prior to measuring the uptake of the vials containing glass beads, the original vials containing the undisturbed colonies were measured. Aerial biomass was separated at different times from the three replicates (F day 2; G day 9; H day 16). The aerial biomass was expected to consist primarily of spores.

Chapter 3

Results

3.1 Enrichment Protocol for *Streptomyces* Selection

After conducting the enrichment protocol on fresh Harvard Forest soils to isolate spores and plating several dilutions of the resulting soil suspensions, the plates were analyzed for potential *Streptomyces* candidates. Nine microbial colonies, referred to as Harvard Forest Isolates (HFIs), were selected and maintained in culture after assessing them for the characteristics mentioned in the Methods section.

At first, many of the plates containing potential *Streptomyces* colonies also contained fungi. An antifungal agent, cycloheximide, was used to eliminate fungal growth. With only microbial growth on the plates, it was easier to identify the distinguishing traits of *Streptomyces* described above. Figure 3-1 is a synthesis of the isolation procedure based on physiology of the microbes.

3.2 PCR Amplification of the High-Affinity NiFe-Hydrogenase Gene

To test if the HFIs contained the specific hydrogenase of interest, Polymerase Chain Reaction (PCR) was used to amplify the high-affinity NiFe-

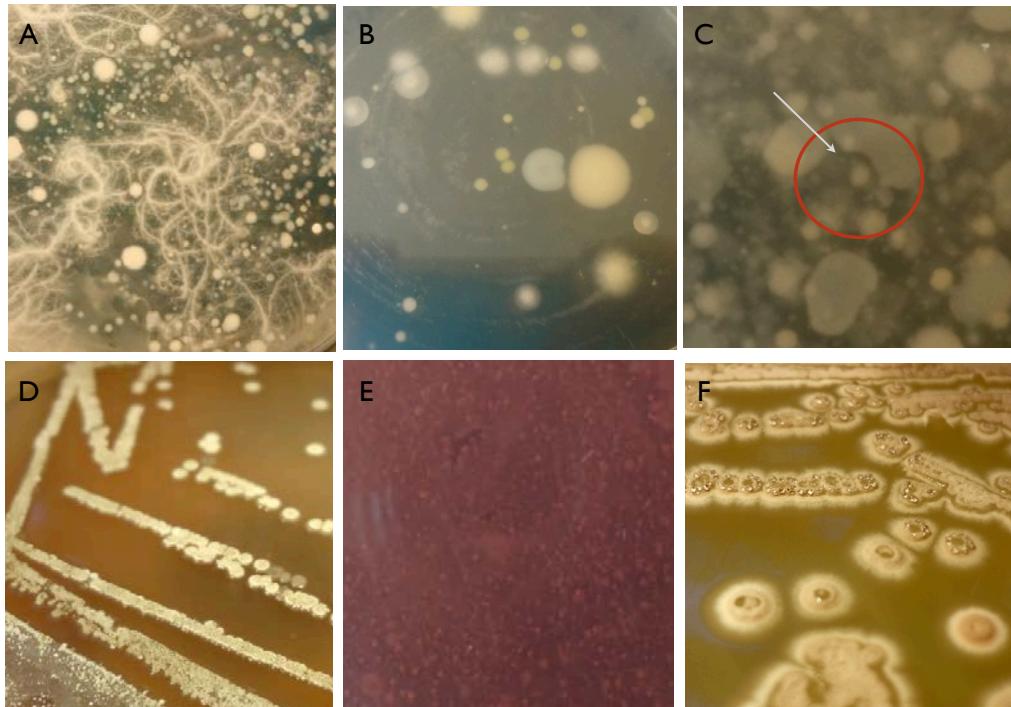


Figure 3-1: A) Serial dilutions of soil from the Enrichment protocol were plated on R2A agar. Note the fungal growth covering much of the surface area of the plate. B) In order to grow a plate of purely microbes and no fungi, cycloheximide, an antifungal agent, was applied. Potential *Streptomyces* were selected based on the following characteristics: C) zone of clearing due to antibiotic production, D) fuzzy appearance of colonies due to aerial hyphae growth. The suspected *Streptomyces* were plated on separate plates to observe growth. At this point, E) the colonies produced pigment (in this case a purple-brown color pigment was secretion by the colonies) and F) either had water droplets condensed on surface of the colony or some excretion. Lastly, when a single colony was grown on the plate the distinctive scent of geosmin was strongly present.

hydrogenase gene using published primers. Gel electrophoresis was used to visualize if PCR amplification was successful and if any of the HFI strains contained the high-affinity NiFe-hydrogenase gene.

Identifying the optimal high-affinity NiFe-hydrogenase primer annealing temperature for *S. griseoflavus* DNA extract was necessary to ensure that PCR effectively amplified the proper genomic sequence of interest. In this case, forward(f) and reverse(r) primers were used for high-affinity NiFe-hydrogenase. The f and r high-affinity NiFe-hydrogenase primers have different sequence lengths and therefore slightly different annealing temperatures. A PCR run cycle program was created to have an eight-step

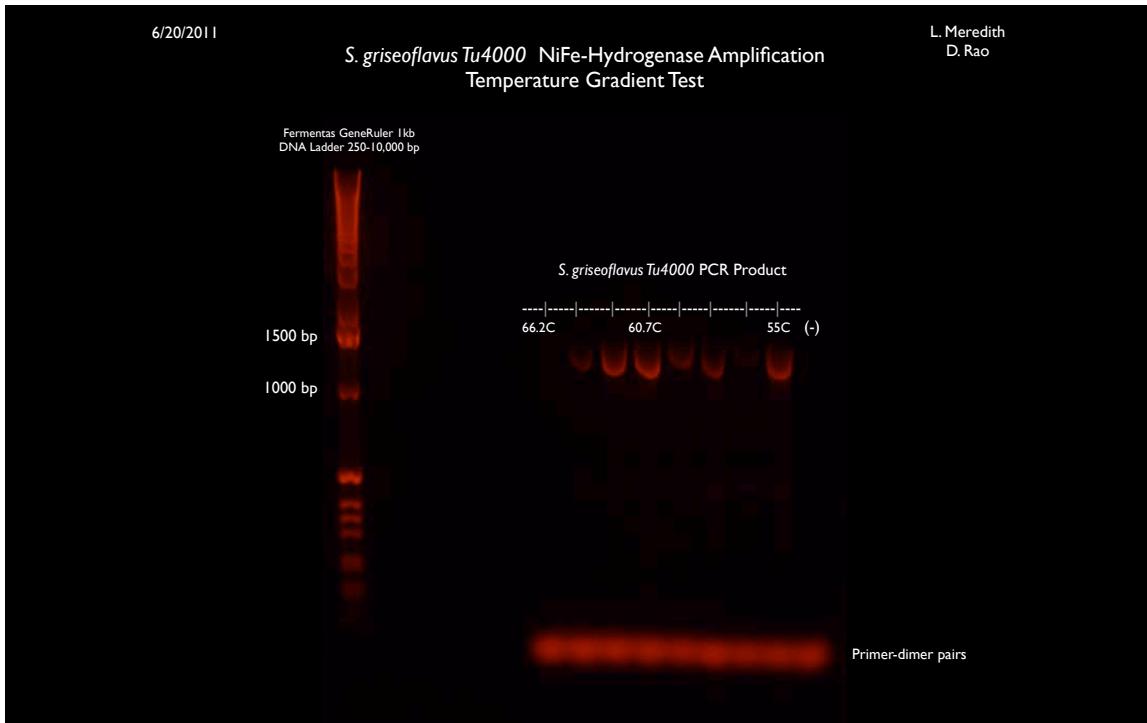


Figure 3-2: An electrophoresis gel (1% agarose, 100 V, 1 hr) was run to determine the ideal PCR annealing temperature for both forward and reverse high-affinity NiFe-hydrogenase to *S. griseoflavus Tu4000* DNA extract based on the temperature-gradient PCR run (55°C to 66.2°C). The optimal annealing temperature for both f and r high-affinity NiFe-hydrogenase primers was chosen to be 60.7°C

temperature gradient from 55°C to 66.2°C to test the efficiency of DNA amplification at specific temperatures. 55°C was on the low end of the annealing temperature spectrum and 66.2°C was on the high end and likely to denature the DNA. An intermediary temperature of 60.7°C efficiently annealed the primers and was not too hot as to denature the DNA. Figure 3-2 shows the efficacy of the range of tested annealing temperatures.

Over the course of a PCR run cycle, HFI DNA was amplified across several orders of magnitude, creating thousands to millions of copies of the DNA sequences of interest. The final concentration of DNA at the end of the PCR run was not measured and therefore unknown prior to being loaded into the electrophoresis gels. Initially, the DNA primer concentration used in PCR was too high. The resulting amplified DNA (PCR product) concentration was correspondingly too high for obtaining a clear gel electrophoresis signal. If an excess amount of DNA is loaded, the mass will not move

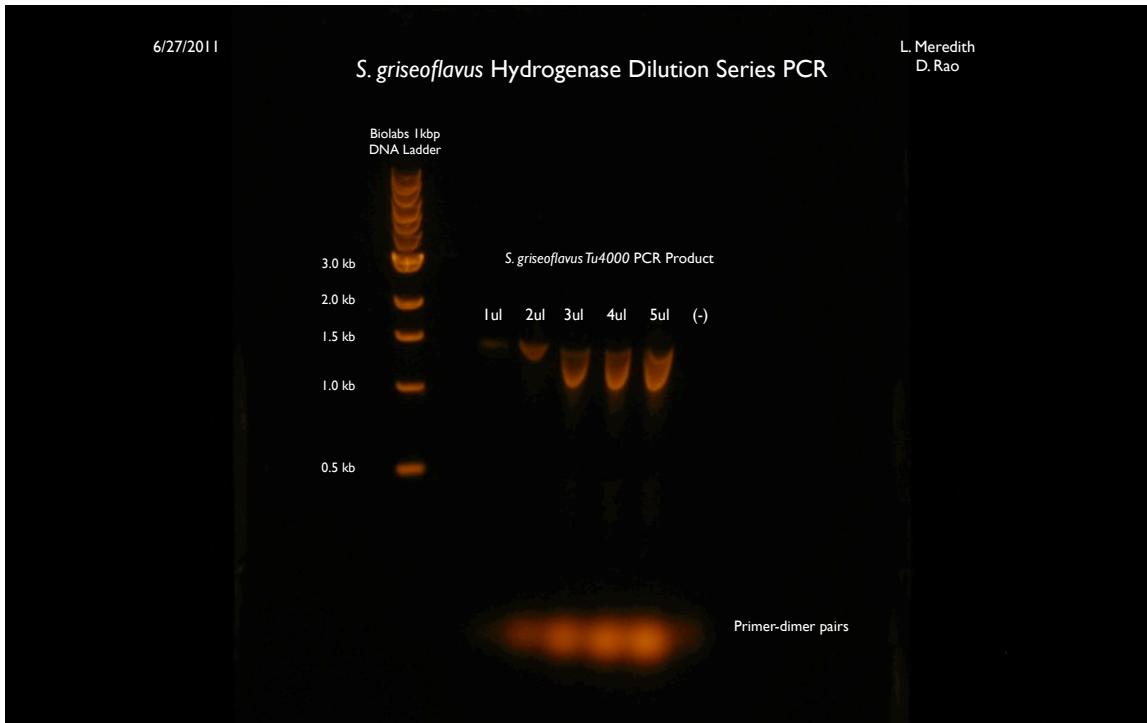


Figure 3-3: A gel (1% agarose, 100 V, 1 hr) was run to determine the optimal concentration of DNA template to use in PCR reactions. The dilution series of *S. griseoflavus* DNA extract revealed that a concentration of 2 μ L was optimal amount for future PCR runs.

efficiently through the gel.

Gel electrophoresis was used to determine the optimum concentration of DNA template to use for future PCR runs. Dilutions of *S. griseoflavus* DNA template (ranging from 1 μ L to 5 μ L, in increments of 1 μ L) underwent PCR amplification with the forward and reverse high-affinity NiFe-hydrogenase oligo-primer. Using the wrong concentration of DNA in the initial PCR reaction resulted in small, faint bands (1 μ L) or smeared and uneven bands (3 μ L - 5 μ L) in the a gel electrophoresis. The gel results in Figure 3-3 shows that 2 μ L was the optimum amount of DNA for the sequences of high-affinity NiFe-Hydrogenase because it created a concentrated band that was not smeared and it had a fainter signal for primer-dimer pairs at the end of the gel.

In initial gels, the bands of DNA were smeared and strong signals at the end of the gel, indicating a collection of primer-dimer pairs, were present (as can be seen

Harvard Forest Isolates: NiFe-Hydrogenase PCR Test

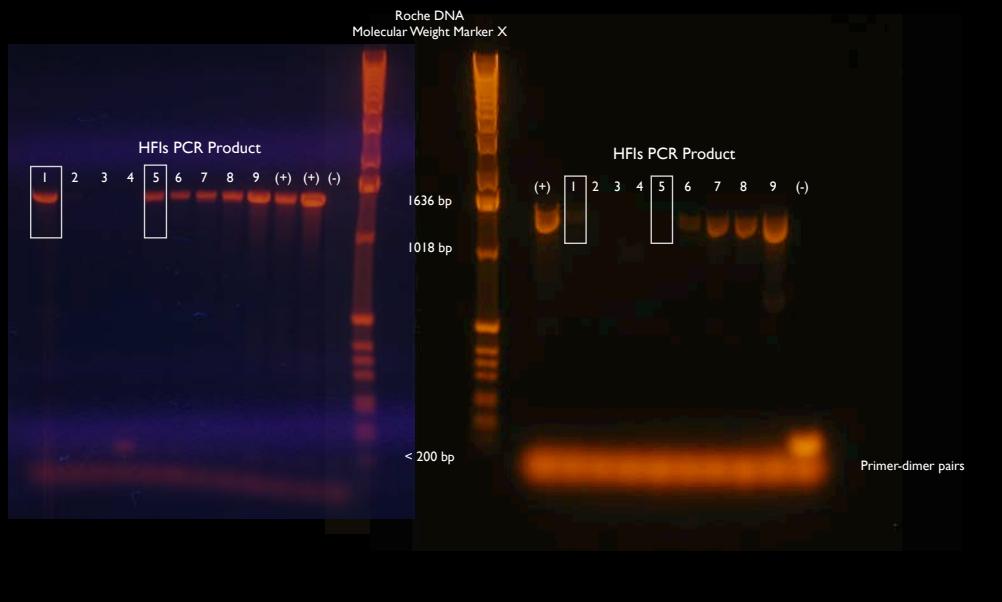


Figure 3-4: A gel (1% agarose, 100 V, 1 hr) was run to determine which of the 9 HFI strains contained a high-affinity NiFe-hydrogenase. The non-smeared gel result on the left clearly shows that HFI 1, 5, 6, 7, 8, and 9 contained a high-affinity NiFe-hydrogenase. For comparison, the gel on the right side was prior to optimizing the PCR protocol for proper DNA primer dilution and annealing temperature.

in Figures 3-2, 3-3, and 3-4). To resolve this problem, the amount of primer was reduced.

Once the ideal concentration of PCR product and optimal high-affinity NiFe-hydrogenase primer annealing temperature were achieved, it was possible to test to the nine HFIs that were suspected to be high-affinity NiFe-hydrogenase containing *Streptomyces*. The PCR product (DNA extract selecting for high-affinity NiFe-hydrogenase gene) of nine HFIs, *S. griseoflavus* (positive control), and a negative control of 25 μ L, were loaded onto a gel. In Figure 3-4, the gel on the left is post-optimizing the PCR protocol and the gel on the right is prior to determining the ideal primer amount and temperature. The results of the gel on the left show that HFIs 1, 5, 6, 7, 8, and 9 contained a high-affinity NiFe-hydrogenase. Although the same strains were loaded into the gel on the right-hand side, the signal was not clear for HFI 1 and 5 because there was too much primer in the gel. HFI 1 and 5 contained a high-affinity



Figure 3-5: Strains HFI 6-9 and *S. griseoflavus* growing on R2A agar plates. Note the different morphology of colony growth as well as pigmentation. *S. griseoflavus* had different colony structure than the HFI strains; its colonies were more rounded, had a smooth surface, and were yellow.

NiFe-hydrogenase and HFI 5 was also *Streptomyces*. For future experiments, only HFI 6-9 and *S. griseoflavus* were used.

HFI strains 6-9 and *S. griseoflavus* were grown in isolation and used as model strains for morphology characterization and testing H₂ uptake. Below in Figures 3-5 and 3-6, are images of the strains growing in plates and low-resolution microscopy images of the strains. Figure 3-5 shows the distinct colony shapes, pigmentation, and growth on an R2A substrate. *S. griseoflavus* was notably different than the HFI strains. It had round, yellow colonies, and no spores or aerial hyphae by day 14 since inoculation, unlike the HFIs. All of the HFIs had fuzzy colony surfaces after inoculation, indicating the presence of aerial hyphae. All HFI colonies produced pigments varying from grey, pink, purple, and brown. Furthermore, aerial hyphae were visible on the surface as the colonies matured.

Figure 3-6 shows images from low-resolution microscopy that revealed unique colony morphologies of the HFI and *S. griseoflavus* strains. *S. griseoflavus* only had mycelia that entered the substrate and no aerial hyphae were observed. In the HFIs, both substrate mycelia and aerial hyphae were observed using low-resolution

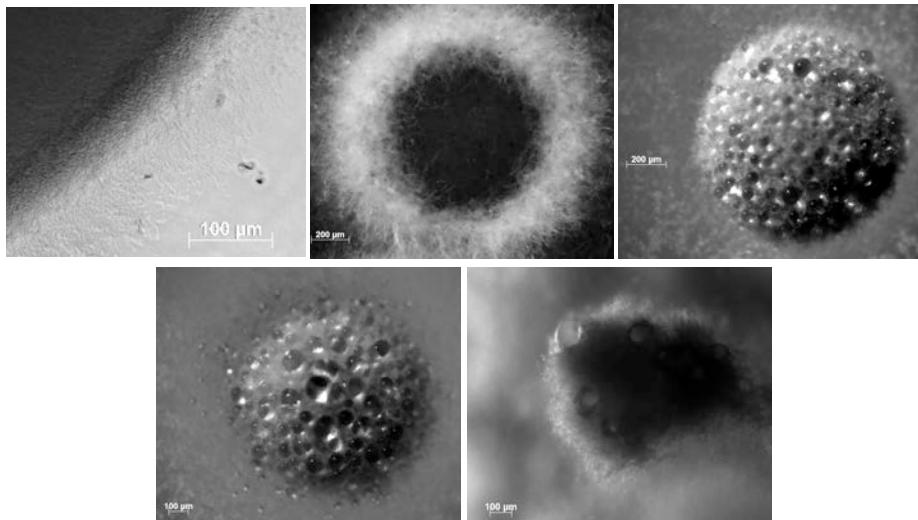


Figure 3-6: Low-resolution microscopy images of HFI 6-9 and *S. griseoflavus* show the three-dimensional structure of a colony. Unique characteristics of each of the strains are clearly visible at this scale. In particular, the aerial hyphae and mycelia are distributed in specific configurations for each strain.

microscopy. HFI 6 had a ring of aerial hyphae. HFI 6-8 had rounded colony structures while HFI 9 had a more amorphous shape, and was densely surrounded by aerial hyphae. HFI 7 and 8 were very similar in their morphologies, their round colonies surrounded by hydrophobic aerial hyphae. The droplets in the microscopy images of HFI 7 and 8 may be condensation droplets of water or perhaps compounds being produced and secreted by the colonies themselves. These various physical traits raise the question: does the morphology of the colony relate to its H₂ uptake behavior?

DNA Sequence Analysis of HFI Isolates and *S. griseoflavus*

The PCR products for both 16s DNA and the high-affinity NiFe-hydrogenase gene of HFI strains 6-9 and the positive control (*S. griseoflavus*) were purified and sent to MIT's Biopolymers Laboratory (76-181 Cambridge, MA 01239) for sequencing. A table of DNA sequencing data containing the 16S rDNA and high-affinity NiFe-hydrogenase reads is in Appendix B for reference. Some DNA sequence reads were of too poor quality to categorize either the high-affinity NiFe-hydrogenase or 16S rDNA sequences.

Phylogenetic Tree Construction Based on High-Affinity Hydrogenases

Using the isolated and sequenced DNA reads of the HFIs and *S. griseoflavus*, a tree of high-affinity hydrogenase amino acid sequences was constructed using reference genomes from the NCBI database and SeaView and Fig Tree to determine the relationship and lineage of the HFI strains high-affinity hydrogenases, using published high-affinity hydrogenases from NCBI genomes. These relationships are based off of initial sequencing results. There are better methods, such as topo cloning, that provide cleaner and more accurate sequences that would result in the construction of a better tree and also of the 16S rDNA. In the future, this method will be used for cleaner sequencing of the HFI strains and to update the tree. The HFI strains 6-9 and *S. griseoflavus* fell into high-affinity NiFe-hydrogenase Cluster 1. The high-affinity NiFe-hydrogenase of the HFIs were more closely related to one another than to *S. griseoflavus*.

3.3 Experiment 1: High Frequency Measurements of H₂ Uptake During Exponential Growth Stage and Spore State

Current, albeit limited, published work concerning *Streptomyces* uptake suggests that H₂-consumption rates are maximal when colonies are approaching and are in the spore state of the life cycle. Given that most *Streptomyces* are in the spore state in soils, understanding H₂ uptake across the life cycle could lead to a deeper understanding of the environmental, microbial soil H₂ sink.

The motivations for this experiment were threefold:

1. To characterize one of the HFI strains at a high frequency in order to test if it utilizes H₂ and, if it does, how strong is its H₂ uptake rate?

2. To determine if H₂ uptake depends on the life cycle state of the microbe. If so, then does maximum H₂ uptake occur during the spore state?
3. If maximal H₂ uptake corresponds to the spore state of the microbes' life cycle, then will H₂ uptake continue at its maximum rate as long the microbes remained in the spore state?

Hypotheses for Experiment 1:

1. If the organism contains the genetic sequence for the high-affinity NiFe-hydrogenase, then we hypothesize that H₂ uptake activity occurs in that organism.
2. We expect that the H₂ uptake rate depends on the life cycle of the organism, specifically that H₂ uptake rate is maximum at the spore state.
3. If Hypothesis 2 was not disproved, then we expected the H₂ uptake rate to remain constant at its maximum rate as long as the strain remained in the spore state.

Why methods were chosen:

Given our resources and time constraints, it was only possible to characterize one strain at high frequency. For this experiment, we analyzed the H₂ uptake behavior and life cycle of strain HFI 8. The imaging and GC methods were used to document life cycle state and H₂ uptake rate of the HFI 8, respectively. The serum vials containing the cultures were photographed to gain an understanding of the amount of microbial growth over the agar and to construct a time series of growth. At the conclusion of the experiment, we will conduct a Protein Extraction and Assay Protocol and use the Thermo Scientific Pierce BCA Protein Assay Kit. Using the protein extract data, we will quantify the amount of H₂ uptake relative to biomass.

3.3.1 Experiment 1: Key Findings

Hypothesis 1: If the organism contains the genetic sequence for high-affinity NiFe-hydrogenase, then we hypothesize that H₂ uptake activity occurs in an organism.

Results: Containing the high-affinity NiFe-hydrogenase gene did predict H₂ uptake for HFI 8.

The H₂ deposition velocity v_d of three replicate vials (A, B, C) of HFI 8 was measured twice a day (high frequency measurements) during the first three days after inoculation (exponential growth phase); then once every second day for one week; then once a week for one month (long-term measurements). The experiment ran for 45 days starting with the initial day of inoculation. Three vials of glass beads (F, G, H) coated in aerial biomass were also measured for H₂ uptake, and will be discussed later in this section.

All three replicates (A, B, and C) had a similar evolution of v_d over the course of both the high-frequency and long-term GC measurements. As seen in Figures 3-7 and 3-8, H₂ uptake was not observed for the first 40 hours. Between day 2 and 4 after inoculation, v_d rapidly increased. The maximum v_d was reached four days after inoculation for all three replicates. After the maximum v_d was reached, v_d appeared to decrease linearly over time. There was significant variation in magnitude between the three replicates. In particular, the magnitude of the deposition velocity of replicate C was statistically different than that of A and B; however, there was a similar pattern of v_d change over time among the three replicates. Replicate C had the highest percent area coverage observed using serum vial photography, and the reason for the consistent difference in replicate C remains unknown. It can be concluded from these results that an H₂ uptake occurred and an overall pattern of H₂ uptake exists, and perhaps depends on the organism's life cycle.

Hypothesis 2: We expect that the H₂ uptake rate depends on the life cycle of the organism, specifically that H₂ uptake rate is maximum at the spore state.

Results: H₂ uptake rate does depend on the life cycle and was maximal at the spore state

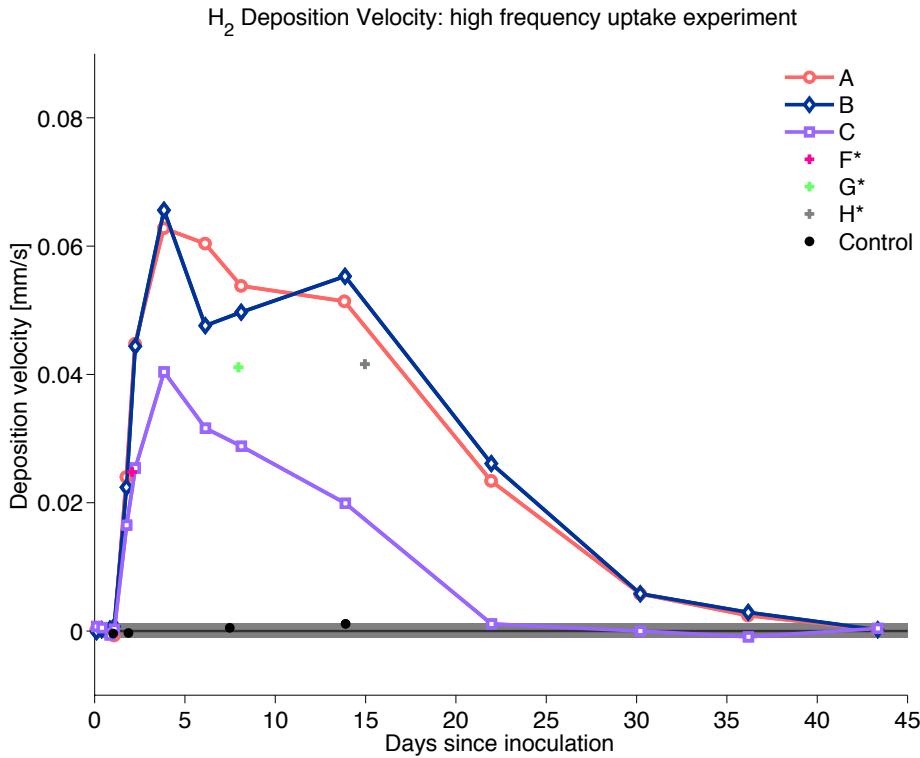


Figure 3-7: A plot of both high-frequency and long-term measurements of HFI 8's H_2 deposition velocity vs. time, shown in days since inoculation. Among all three HFI replicates, there is a general trend of rapid H_2 uptake increase followed by a slowing consumption of H_2 over time. Even though replicate C has a consistently different magnitude of v_d , it follows the same pattern of H_2 uptake evolution. The grey shading indicates the limit of detection for v_d .

The life cycle of *Streptomyces* was compared with the H_2 uptake measurements during the first four days after inoculation. Figure 3-8 is a zoomed in view of Figure 3-7. Observing v_d between day 0 and day 5, there was minimal, almost zero, H_2 uptake in the first 24 hours of growth. The deposition velocity rapidly increased during the transition from the original inoculum spores to new generations of colonies and spores. By day 2, v_d had increased to 0.024 mm/s and continued increasing until a maximum at day 4, when it began to steadily decline.

The increase in v_d over the first four days since inoculation of the vials directly corresponds to changes in the microbe's life cycle—most importantly, the formation of spores. Figure 3-9 below is a time series of microscopy images taken during the high frequency measurements of HFI 8, taken every day with GC measurements for v_d .

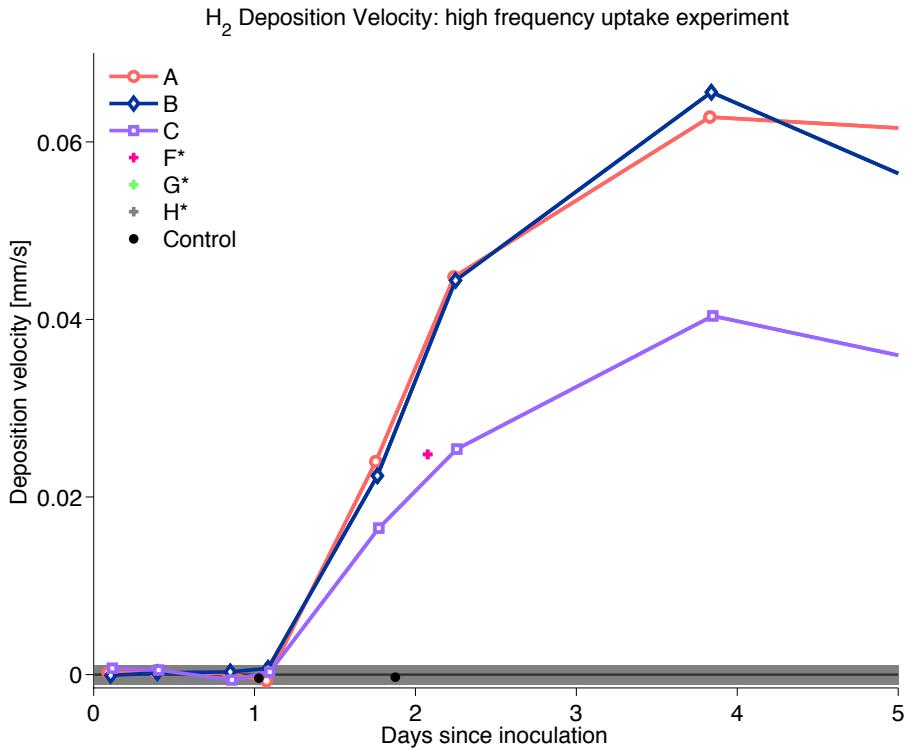


Figure 3-8: A plot of H_2 deposition velocity of HFI 8 over the first five days of GC measurements. The replicates' serum vials were measured twice a day for the first two days since inoculation and once on the 3rd and 4th days.

Images A and B correspond to the first two days after inoculation. Between A and B, there is increasing aerial hyphae formation, elongation, and the beginnings of differentiation into spores. By C (day 1.8 since inoculation), the hyphae had differentiated into distinct segments, called spores. Image C captured a hyphae that is metamorphosing into individual spores. The spores formed but were not fully separated yet. By the second round of GC measurements on day 2.9 (D), more sporulation had occurred. Day 3.8 (E) contained many free spores. Spores existed throughout the remainder of the observation period. Images F-L correspond with day six through twenty-one of GC measurements. H_2 uptake dropped after Day 3.8, when spores were already formed in the colonies. As we collected samples for microscopy, it was possible that the substrate mycelia that were growing in the agar were also picked up with the spatula—perhaps explaining their appearance in the later images (E, F, G) but not a corresponding increase in the H_2 consumption rates.

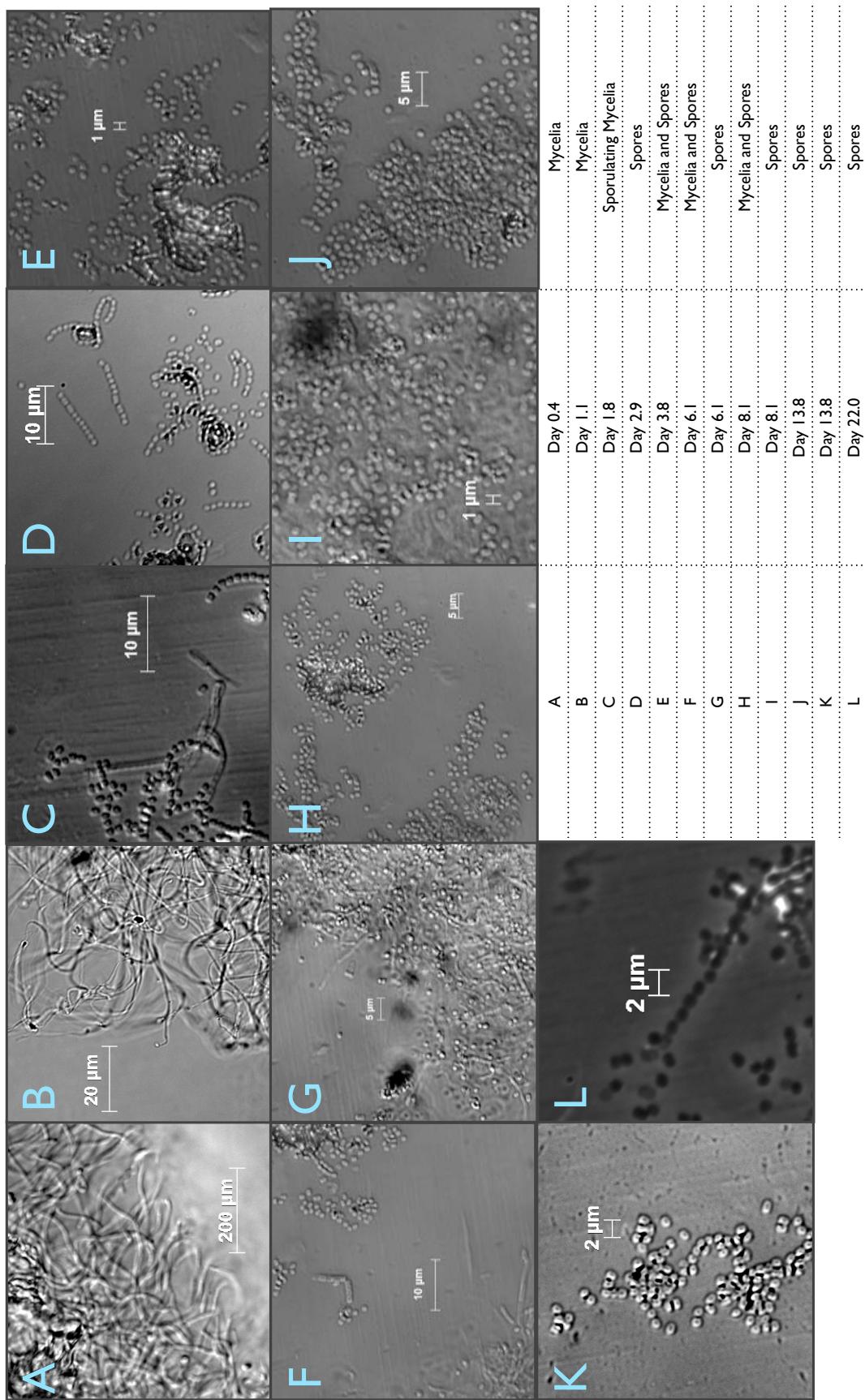


Figure 3-9: A three week time series of microscopy plates chronicling the life cycle of HFI 8 at high frequency. The time is annotated in days since the time of inoculation of the serum vials.

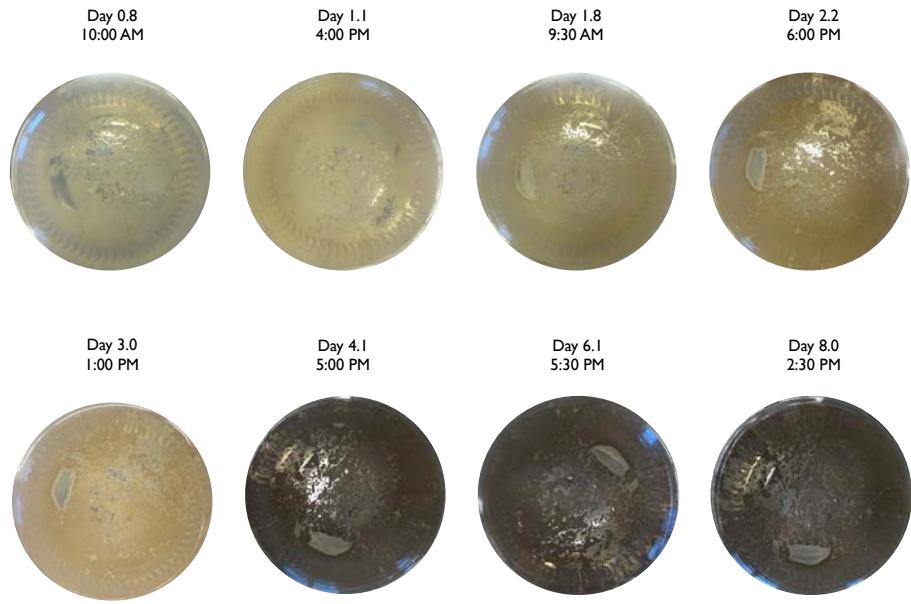


Figure 3-10: A time series of HFI 8 serum vials over a week-long period. The percentage of area cover of the R2A agar did not change much over time. Note: The darkness of the last three images is primarily due to photography, not due to colony growth.

As seen in Figure 3-10, it is important to note that microbial serum vial area coverage did not change drastically over the course of exponential growth. This is due in part to *Streptomyces*' development. They inhabit a region of agar by growing horizontally with substrate mycelia and then begin to grow vertically with their aerial hyphae, so the percentage of area cover is not a complete indicator of the colonies' vertical growth. Although the serum vial images show growth covering most the area of the agar by about day 1.8, maximum H₂ uptake does not occur until later when the colonies approach their spore state through sporulation. There was rapid increase in the H₂ uptake rate when the mycelia started differentiating into spores. H₂ uptake does not appear to be limited by biomass, it is instead linked to the life cycle.

Hypothesis 3: If Hypothesis 2 was not disproved, then we expected the H₂ uptake rate to remain constant at its maximum rate as long as the HFI 8 remained in the spore state.

Result: A maximal H₂ uptake rate did not persist even though the colonies remained in the spore state.

Glass Beads and Spore H₂ Uptake

Over time, H₂ uptake in replicates A-C decreased to zero despite the colonies' existing presence as spores. To test whether or not H₂ uptake would persist in isolated spores, glass beads were used to remove the aerial biomass (hypothetically the aerial hyphae containing differentiated spores) at three stages of HFI 8 growth (over three consecutive weeks) and placed into new serum vials. The serum vials containing glass beads did not contain agar because the spores were the subject of observation and we did not want them to undergo a full life cycle. Without media as a nutrient source and growth substrate, the spores should not grow again. The spores were attached to the sterile glass beads, as seen in Figure 3-11. It should be noted that not 100% of spores were removed from the original vials and that some may have remained or been dispersed onto uninhabited regions of agar.

Vials F, G, and H were inoculated with HFI 8 at the same time as HFI 8 A, B, and C. F was the first vial to be disturbed by glass beads to remove aerial biomass, in the hopes of isolating spores. Vial F was transferred approximately two days after inoculation of the original vials, when grey specks began appearing on top of colonies containing sporulating mycelia. Nine days after inoculation, the aerial biomass was collected from replicate G and moved into a new serum vial via glass beads G. Sixteen days after inoculation, the aerial biomass was collected for a final time and moved to glass beads H. These original serum vials, remaining biomass and medium, and glass beads with transferred spores were measured for H₂ uptake activity.

Figure 3-12 depicts the H₂ deposition velocity of the serum vials containing only glass beads coated in HF1 8 biomass. After the glass beads were transferred, v_d was measured in two vials: 1) the leftover media in the original vial and 2) in the vial with the transferred spores on glass beads. After transferring biomass via glass beads, the total H₂ uptake rates were not conserved; i.e. the sum of the v_d in the leftover media and glass beads did not equal the total original observed v_d . Over time, all glass bead

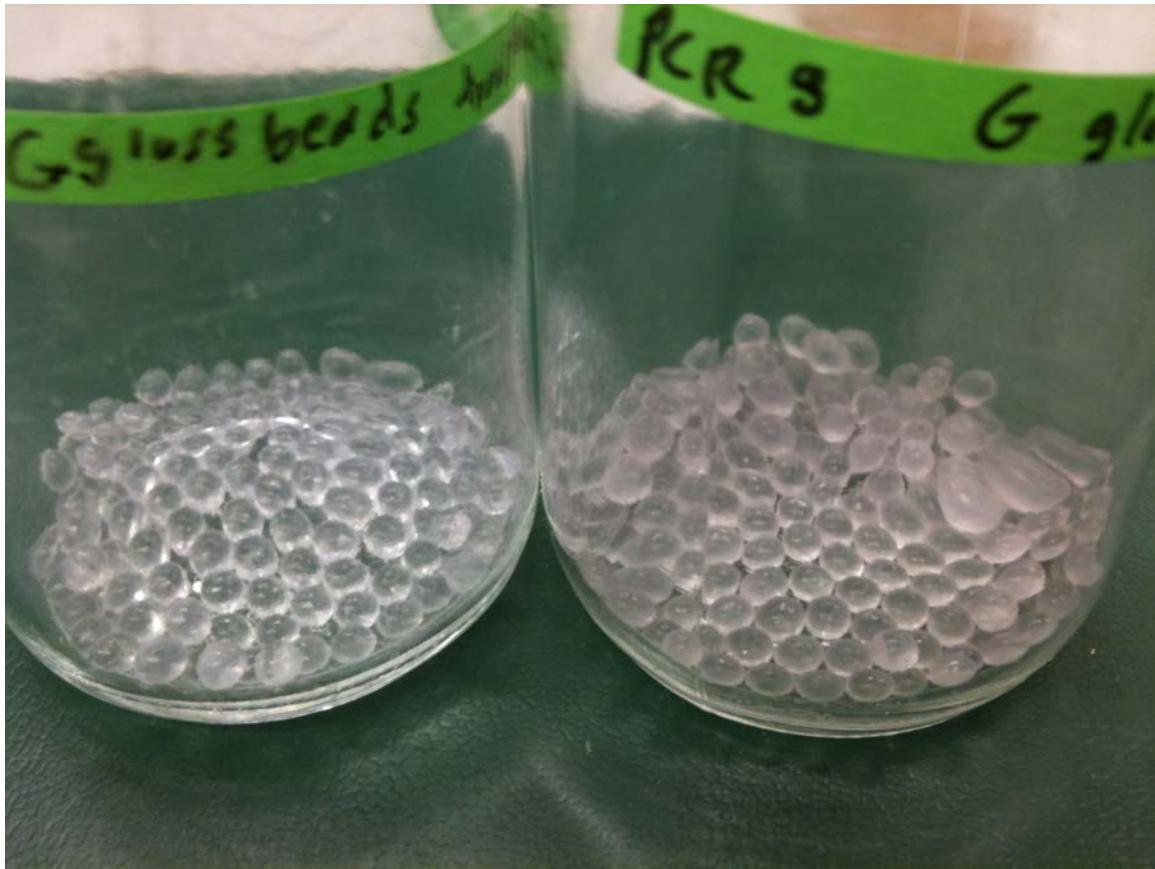


Figure 3-11: A photo of collected aerial biomass coating glass beads which were rolled on top of HFI 8 cultures. The serum vial on the right contains more collected biomass, as indicated by its more pink color, because it was collected at a later time during the culture's growth than the serum vial on the left.

v_d approached zero and did not persist.

3.3.2 Discussion

In Experiment 1, it was found that HFI 8's containing the high-affinity NiFe-hydrogenase gene predicted H₂ uptake activity in the colonies. Furthermore, the rate and change of H₂ uptake over time varied with the organism's life cycle. Lastly, the H₂ uptake rate did reach a maximum point corresponding to spore production, agreeing with the results of Constant et al. (2008) who only observed that their *Streptomyces spp* PCB7 had maximum H₂-uptake activity at the terminal sporulation state of the life cycle. Constant et al. (2008) hypothesized that their strain's high-affinity NiFe-hydrogenase

H₂ deposition velocity of biomass removed using glass beads

compared to original vial and biomass remaining on media after transfer

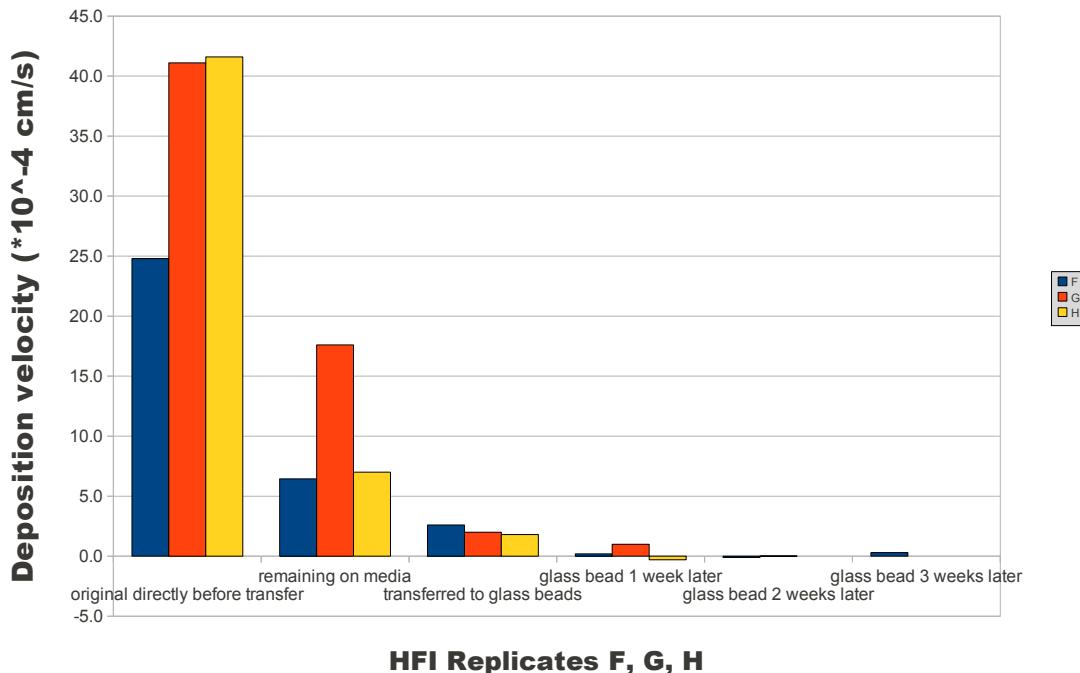


Figure 3-12: Glass beads were used to remove the aerial biomass from the inoculated HFI 8 serum vials. When the aerial biomass was removed from the growing colonies, H₂ uptake on the remaining media diminished and H₂ uptake continued at a low rate on the glass beads. However, the combined H₂ uptake of both the remaining mass in the serum vial and the transferred mass to the media did not sum up to the original measured vials and fell to zero H₂ uptake. The small amount of H₂ uptake in glass beads declined to zero over time.

purpose was either for spore metabolism or was somehow released into the environment, inferring an explanation of free soil hydrogenase H₂ uptake. However, our results show that H₂ uptake rates did not remain at a maximum despite the colonies continuing to be in the spore state. In fact, H₂ uptake rate diminished to zero over the course of a one-month period.

The extinction of H₂ uptake to zero over time is in contrast to our original hypothesis (3) that spores' H₂ uptake will persist over time. Our results question whether spores may be responsible for long-term H₂ uptake. The life cycle transition from old to new spores seems to consume the largest amount of H₂, as seen by the fast increase in H₂ uptake activity during sporulation. One of the original questions of

this research venture was to help answer the question: Why would dormant spores be utilizing H₂? Given these results, it seems like another important question is, “Is H₂ important for the production of aerial hyphae and the differentiation of spores?”

The diminished H₂ uptake, after a culture of spores was transferred to glass beads, hints at the possibility that the three-dimensional structure of the colony could be an important factor contributing to the overall H₂ uptake rate. As structure often denotes function in biology, perhaps the colony scaffolding—in particular the aerial hyphae—provides greater surface area exchange for H₂ gas to interact with the high-affinity NiFe-hydrogenase. The HFI *Streptomyces* strains all have mycelia and aerial hyphae growth. Is there perhaps an integral colony structure that was destroyed during the removal of biomass and spores via glass beads, thereby diminishing H₂ uptake?

In the next two experiments, other strains will be observed to see how broadly these results can be extended.

3.4 Experiment 2 Results: Comparison of H₂ Uptake and Life Cycles among HFI Strains and *S. griseoflavus*

The motivations for this experiment were threefold:

1. To determine if containing the high-affinity NiFe-hydrogenase is a predictor of H₂ uptake activity by observing whether H₂ uptake occurs in HFIs 6-9 and *S. griseoflavus*.
2. To determine if H₂ uptake depends on the life cycle state of the microbes. If so, then compare and contrast the individual H₂ uptake rates' dependence on life cycle state.
3. If H₂ uptake does occur, then what are the average individual H₂ deposition velocity (v_d) rates of each strain per unit biomass?

Hypotheses for Experiment 2:

1. We hypothesized that HFI 6-9 and *S. griseoflavus* should utilize H₂ since all strains contained the high-affinity NiFe-hydrogenase sequence.
2. Since HFIs 6-9 and *S. griseoflavus* belong to the genus *Streptomyces*, we hypothesize similar growth patterns and uptake dependency on the life cycle, just as HFI 8 in Experiment 1.
3. We decided to test the hypothesis that each of these five *Streptomyces* strains should uptake the same amount of hydrogen per unit biomass.

Methods

The H₂ uptake rate and life cycles of HFIs 6-9 and *S. griseoflavus* were documented using the Imaging and GC methods, which are described in the Methods section. The five strains were observed over a three-week (22 days) period, with GC H₂ uptake measurements starting three days after the strains were inoculated in the serum vials. H₂ uptake of the strains was measured every two days. Data points obtained during a period of measurement with a leaky syringe were excluded from these results.

At the conclusion of the H₂ uptake measurements, we conducted a Protein Extraction and Assay Protocol and use the Thermo Scientific Pierce BCA Protein Assay Kit. Using the protein extract data, we quantified the amount of H₂ uptake relative to biomass.

3.4.1 Results

*Hypothesis 1: We hypothesized that HFI 6-9 and *S. griseoflavus* should utilize H₂ since all strains contained the high-affinity NiFe-hydrogenase sequence.*

Result: HFIs 6-9 did utilize H₂, as predicted by their containing a high-affinity NiFe-hydrogenase gene. However, *S. griseoflavus* was not observed to have significant H₂ uptake despite having the high-affinity NiFe-hydrogenase gene.

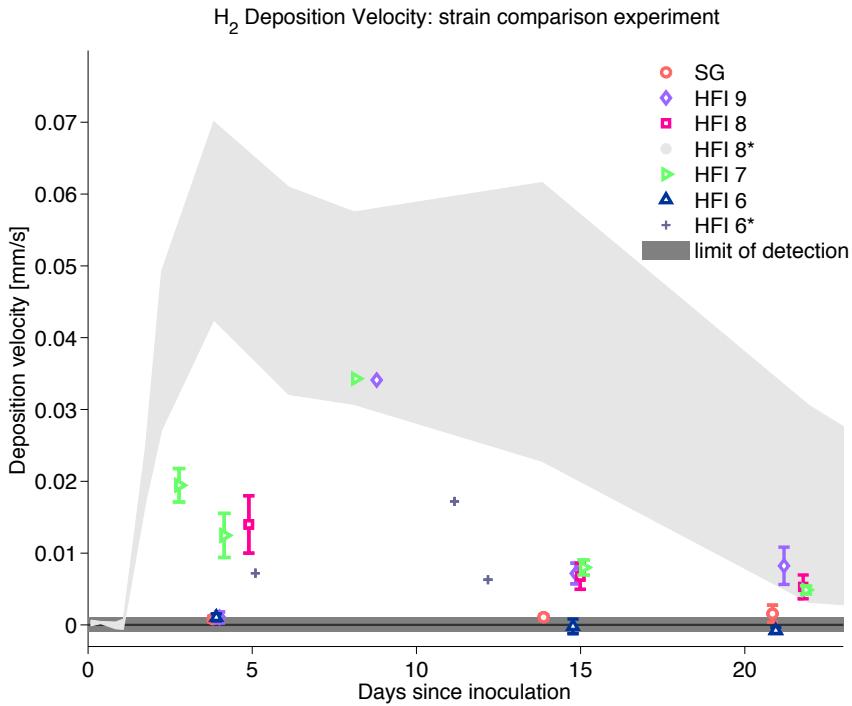


Figure 3-13: The HFI strains and *S. griseoflavus* were inoculated at day 0 and measured for three weeks using gas chromatography for H_2 uptake. The signal for HFI 8* represents the v_d data from Experiment 1, and HFI 6* represents v_d from a previous pilot study, plotted here for comparison with the HFI 7-9 strains from Experiment 2

Figure 3-13 is a plot of H_2 uptake measurement by the strains. HFIs 7, 8, and 9 had significant H_2 uptake above the GC's limit of detection, which is the minimum amount of uptake we can confidently see based on our control measurements. However, in the limited measurements, HFI 6 and *S. griseoflavus* were not observed to have significant H_2 uptake above the limit of detection. For this reason, Figure 3-13 was supplemented with previous H_2 uptake measurements of HFI 6 (HFI 6*) using the same methodology from a pilot study. Similarly, HFI 8* (the H_2 uptake results of Experiment 1) were included to show that the magnitude of H_2 uptake varied between two measurements of the same strain.

GC measurements of the five strains began three days after inoculation, so initial H_2 uptake during the exponential growth phase of the strains was not captured in this set of measurements. HFIs 9 and 7 had the greatest amount of relative H_2 uptake among these five strains. As in Experiment 1, the overall pattern of HFI H_2 uptake

occurred, beginning with the steep rise in v_d after inoculation, followed by a decline approaching zero around days 15 and 20 since inoculation. Maximum H₂ uptake for HFI 7, 8, 9 was observed 8, 5, and 8 days (respectively) since inoculation, but with such sparse data the exact point of maximum uptake is unknown. Containing the gene for high-affinity NiFe-hydrogenase did predict H₂ uptake for HFIs 6-9, but not for *S. griseoflavus*.

Hypothesis 2: HFIs 6-9 and S. griseoflavus belong to the genus Streptomyces but the wild HFI strains had more similar growth patterns amongst themselves than compared to S. griseoflavus.

Result: The strains' H₂ uptake correlated to their life cycles, just as HFI 8 in Experiment 1.

Figure 3-14 is a life cycle analysis compilation of high-resolution microscopy images taken over a two-week period of HFIs and *S. griseoflavus*. The photos detail the life cycle changes among the HFI and *S. griseoflavus* strains for comparison with the GC H₂-uptake measurements. On day 0, the strains were freshly inoculated into the serum vials. The images captured on day 0 are of the original spores, all of which were approximately 1 μm in length. By day 1, all five strains had considerable mycelia growth that penetrated into the media. By day 3, HFIs 7, 8, and 9 had chains of cells in the aerial hyphae that were metamorphosing into spores; individual mature spores in HFI 7 were visible. HFI 6 and *S. griseoflavus* developed slower than HFIs 7, 8, and 9. HFI 6 was slow to produce spores but had them on day 14. Still on day 14, *S. griseoflavus* did not have any visible spores. In summary, the HFIs varied in the duration of their individual life cycle maturation: HFIs 7-9 had relatively fast growth and HFI 6 and *S. griseoflavus* had slower growth. The wild HFI strains had similar life cycles to one another but were distinct from the lab-rat strain, *S. griseoflavus*.

Hypothesis 3: We expected that each of these five Streptomyces strains should utilize the same amount of hydrogen per unit biomass.

Result: Protein-weighted H₂ uptake for HFIs 7-9 revealed that the strains have similar uptake rates per unit biomass. Sufficient H₂ uptake data could not be gathered for HFI 6 and S. griseoflavus

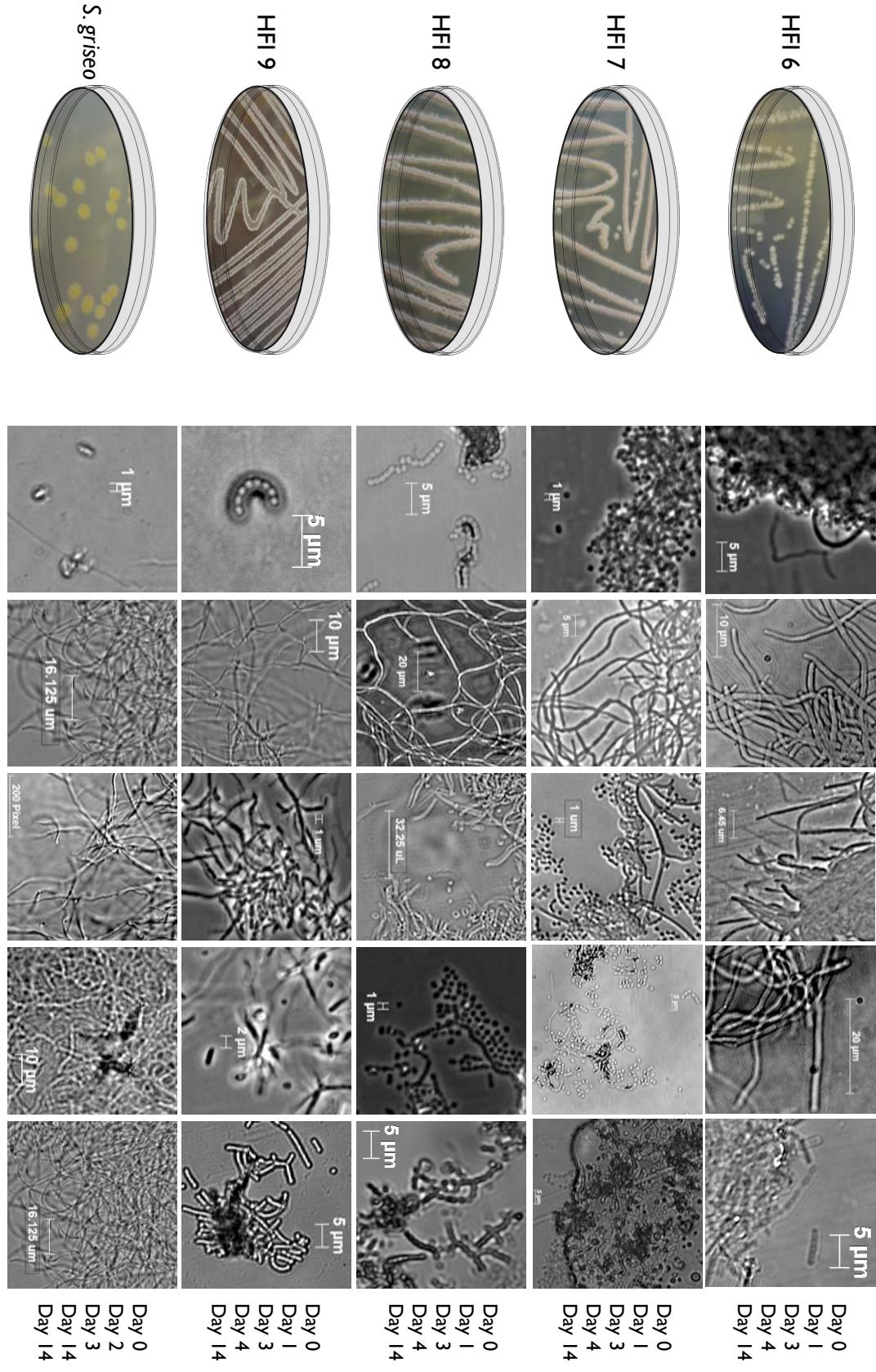


Figure 3-14: A two-week time series of high-resolution microscopy photos capturing the transitions between life stage cycles of the HFIs and *S. griseoflavus* strains. HFIs 6-9 visibly went through the cycle of aerial hyphae formation and spore differentiation. HFI 6 and *S. griseoflavus*. The formation of spores was not observed using microscopy for *S. griseoflavus* over the two-week timeframe.

Figure 3-15 below is a compilation of serum vial images over the course of five days since inoculation. The percent area covered was used as a proxy for biomass growth. For reasons, discussed in Experiment 1, serum vial percent area covered is not a complete indicator of the colonies' growth. It is clear that biomass increased in HFI 6 and *S. griseoflavus* between day 2 and day 5 because the exponential growth phase of their life cycle was observed using microscopy over this time period. HFIs 7-9 had a faster growth rate and their biomass had covered a large amount of the serum vial agar by day 2; between days 2 and 5, the biomass visible via this method of photography did not change. The final average protein concentrations among the three replicates are included next to the serum vial images, as well as the standard deviation of the protein concentration for all three replicates.

The final biomass was collected from the surface of the R2A agar in the serum vials of each replicate of each strain on the final day of GC measurements. A low-concentration-protein-assay standard curve was created to convert absorbance to protein concentration. Note that the final average protein extraction ($\mu\text{g/mL}$) for *S. griseoflavus* and HFI 6 are very low in comparison to HFI 7-9. This can be explained by the difficulty of collecting biomass from the surface of the colonies for these two strains and is not just due to a lack of biomass for HFI 6. Their final average protein concentration is not representative of the complete amount of biomass in the serum vials for all five strains; however, the biomass from HFI strains 7-9 were more effectively collected. Furthermore, with more total biomass, incomplete collection was a relatively smaller error. Comparing the three successful protein quantifications, HFI 9 had the most protein on average (290.80 $\mu\text{g/mL}$). The higher biomass corresponds to HFI 9's dense array of aerial hyphae and pigmentation, seen in low-resolution microscopy as seen in Figure 3-6.

Protein weighting of the HFI strains' H_2 uptake painted a different picture of relative H_2 uptake strengths than originally thought. Only HFI 7, 8, and 9 were used for the protein-weighted H_2 deposition velocity analysis (Figure 3-17). An inadequate and non-representative amount of protein was extracted from both *S. griseoflavus* and HFI 6; Thus, their H_2 uptake weighted against their collected biomass would likely

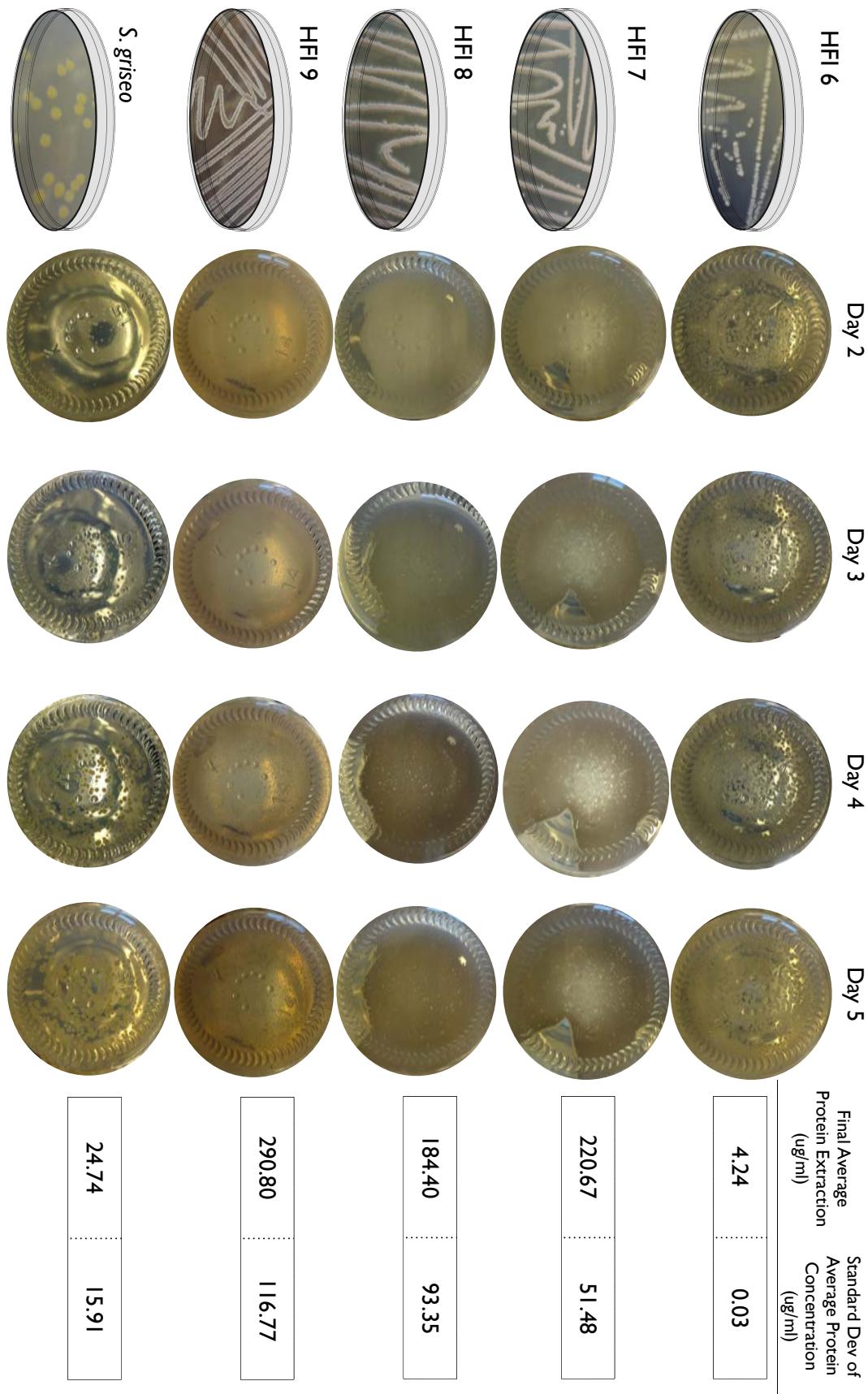


Figure 3-15: A time series of serum vial photos chronicling the amount of growth and area coverage for strains *S. griseoflavus* and HFI 6-9 over the course of five days after inoculation. Final biomass was collected 22 days after inoculation.

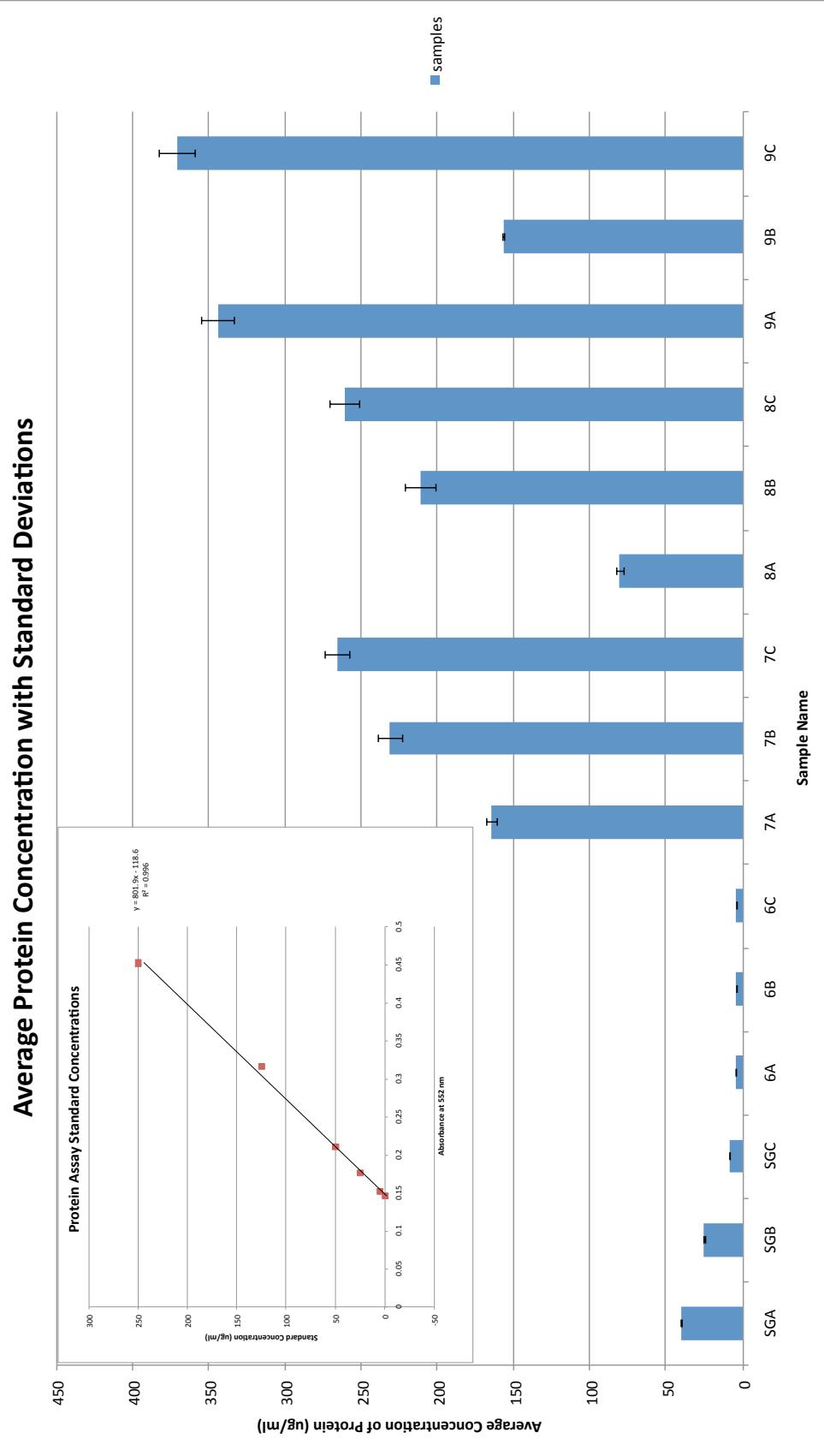


Figure 3-16: Average protein concentration collected from the five strains along with the standard deviations. In the top left-hand corner is a low-concentration-protein standard curve created for our samples.

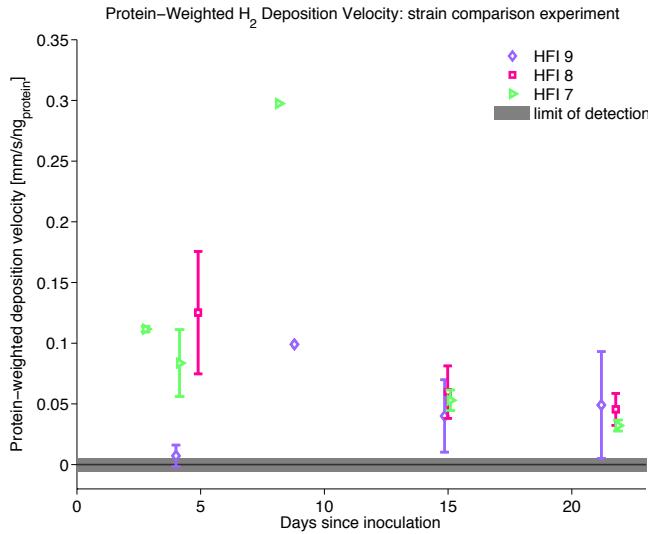


Figure 3-17: The protein-weighted deposition velocity results show that HFI 7-9 have roughly the same magnitude of H_2 uptake.

appear to be much higher. Protein extraction attempted to remove as much of the biomass from the agar substrate as possible. However, embedded mycelia and other fragments of the colonies were definitely lost in the process. It is certain that HFIs 6-9 contained more biomass than *S. griseoflavus*.

Weighted by protein, HFI 7, 8, and 9 appeared to have roughly the same H_2 uptake per unit biomass. Although HFI 9 and 7 had higher biomass overall, their H_2 uptake relative to its biomass was smaller than HFI 8's H_2 uptake. Relative to its biomass, HFI 9's H_2 uptake (including error bars) was still significant. The uptake was roughly equal for HFIs 7-9 once weighted with protein concentration. On most days, H_2 uptake weighted by protein is the same within the error bars for HFIs 7-9. The results for HFI 6 and *S. griseoflavus* are uncertain.

3.4.2 Discussion

H_2 uptake was observed among all HFI strains but was not observed in *S. griseoflavus* on the days tested. So, containing high-affinity NiFe-hydrogenase was a predictor for H_2 uptake activity in the wild HFIs. Since HFIs 6-9 and *S. griseoflavus* belong to the genus *Streptomyces*, we expected similar growth patterns and uptake dependency

on the life cycle, just as HFI 8 in Experiment 1. Overall, the pattern of H₂ uptake correlated with the individual life cycles of the microbes.

Upon comparing the life cycles of the HFI strains, a clear pattern emerged concerning H₂ uptake in HFIs 7-9. Data for HFI 6 from a previous experiment were used and lifecycle analysis not available for this dataset. As the colonies began producing a network of substrate mycelia and later aerial hyphae, H₂ uptake quickly increased. H₂ uptake was maximal on days corresponding to sporulation of the aerial hyphae. Over time, although HFIs 6-9 continued to remain in the spore state, H₂ uptake steadily declined and approached zero uptake. Does the changing morphology of the *Streptomyces* colony relate to its H₂ uptake potential? Both aerial hyphae and substrate mycelia were present in HFI 6-9, the strains with oxidized H₂. But only substrate mycelia were observed in *S. griseoflavus*, the only strain that did not uptake H₂ above the limit of detection within the experiment time period. However, some aerial hyphae must have existed because spores were observed after the first two week of H₂ uptake measurements. Compared to other strains, fewer aerial hyphae were produced.

One potential explanation for the results of H₂ uptake, concerning morphology, would be that increased surface area of the aerial canopy around the colony enhances gas exchange, and therefore facilitates more H₂ uptake. Another potential reason for H₂ uptake during this life-cycle transition is that H₂ is somehow necessary for creation and metabolism of aerial hyphae, which may help explain why, after the differentiation of aerial hyphae to spores, H₂ uptake steadily declines to almost zero. These longer-term measurements disagree with one of our original hypotheses that the semi-dormant spores are responsible for long-term H₂ uptake.

A major question concerning *S. griseoflavus* remains. If *S. griseoflavus* contained the gene for high-affinity NiFe-hydrogenase, why was little or no uptake observed? Comparing the life cycle observations and morphology of *S. griseoflavus*, it had much smaller biomass on average, grew slower, and had no observed aerial hyphae. Future work is required to draw conclusions about the nature of *S. griseoflavus'* H₂ uptake.

In Experiment 2, the initial steep increase in H₂ uptake was not captured, since

all five strains were measured starting three days after inoculation. However, we can paint an overall pattern of H₂ uptake over the 22-day period. Future work can be done to further analyze the differences between *S. griseoflavus* and the wild HFI strains. Increasing the resolution of H₂ uptake measurements and capturing the exponential growth phase of these strains would be useful information to help understand when these organisms would contribute to the environmental soil sink.

3.5 Experiment 3: Other High-Affinity NiFe-hydrogenase containing organisms

A Low (L) frequency set of measurements designed to preliminarily test whether or not H₂ uptake occurred, above the detection limit of the GC, in other high-affinity NiFe-hydrogenase containing organisms.

As seen in Figure 1-2, three distinct clusters of high-affinity NiFe-hydrogenases exist. The HFI strains and *S. griseoflavus*, which were tested at high and medium frequencies in Experiment 1 and Experiment 2, belong to Cluster 1, and are closely related hydrogenases. By examining both the morphological characteristics and high-affinity NiFe-hydrogenase categorization, two novel soil microbes were chosen to be tested for H₂ uptake using GC methods. From Cluster 1, *Rhodococcus equi* (*R. equi*), was chosen for uptake measurements because it contains a similar high-affinity NiFe-hydrogenase as the HFIs and *S. griseoflavus* but did not form spores and had a different life cycle from *Streptomyces*. From Cluster 2, *Streptomyces cattleya* (*S. cattleya*) was chosen because although it should have a similar morphology and life cycle to the HFI strains and *S. griseoflavus*, its NiFe-hydrogenases was more distantly related.

The motivations for this experiment were twofold:

1. Does the presence of a high-affinity NiFe-hydrogenase gene (from Cluster 1) highly similar to HFIs and *S. griseoflavus* in a soil microorganism (*R. equi*),

- that does not form spores, also predict H₂ uptake?
2. Does the presence of a more distant high-affinity NiFe-hydrogenase (from Cluster 2) in a strain of *Streptomyces* (*S. cattleya*), with a highly similar life cycle and morphology, also predict H₂ uptake?

Hypothesis:

1. We expected in both cases that the presence high-affinity NiFe-hydrogenase would predict H₂ uptake in these two strains *R. equi* and *S. cattleya*.

Method:

A total of three cultures were tested for H₂ uptake activity. Both *S. cattleya* and *R. equi* were grown on R2A agar and one more culture of *R. equi* was also grown on TSB media in serum vials. As in Experiments 1 and 2, H₂ uptake was measured using GC procedure detailed in the Methods section.

3.5.1 Results

Hypothesis 1. We expected in both cases that the presence high-affinity NiFe-hydrogenase would predict H₂ uptake in these two strains *R. equi* and *S. cattleya*.

Result: The presence high-affinity NiFe-hydrogenase predicted H₂ uptake, above the detection limit of the GC, in these two strains *R. equi* and *S. cattleya*.

Figure 3-18, below, shows the H₂ uptake of these two high-affinity NiFe-hydrogenase containing organisms. As predicted, there was significant H₂ uptake above the detection limit of the GC in both of these new strains. *S. cattleya* was expected to have a similar life cycle and H₂ uptake pattern to the *Streptomyces* HFI strains. Maximal uptake was observed to have occurred 14 days after inoculation out of the days tested. In comparison to the other HFIs, *S. cattleya* had a slower exponential growth phase and transition into spore state. As observed in the other HFIs, H₂ uptake after reaching a maximum started decreasing towards zero. *R. equi* had H₂ uptake on both

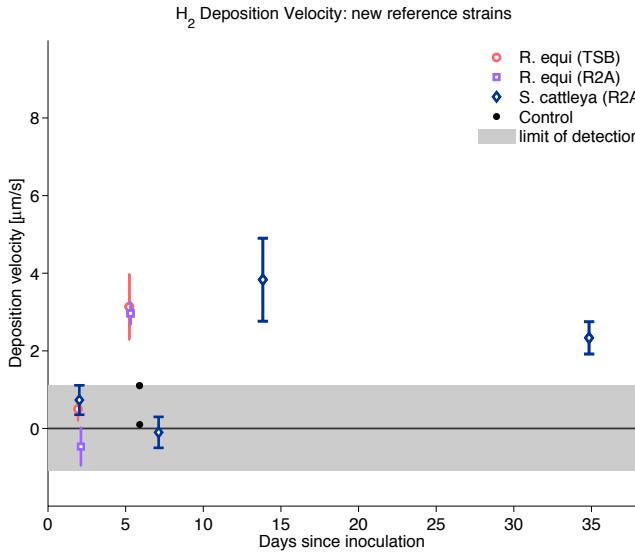


Figure 3-18: The H₂ v_d of new reference strains, *R. equi* on R2A agar and TSB, and *S. cattleya* on R2A. H₂ uptake above the detection limit of the GC was observed in all three cases.

R2A and TSB media. *R. equi* does not sporulate like *Streptomyces* do. This suggests that H₂ uptake may have a different biological purpose for *R. equi* than it does for *Streptomyces*, since H₂ uptake in the *Streptomyces* corresponds to certain stages of its life cycle.

3.5.2 Discussion

These preliminary results raise interesting prospects for future research. Finding that *R. equi* had significant H₂ uptake despite being a non-spore forming organism opens the door for testing other high-affinity NiFe-hydrogenase containing organisms with dissimilar life cycles compared to *Streptomyces*. Perhaps H₂ uptake corresponds to a certain growth stage of *R. equi* as well. Furthermore, finding significant uptake in a Cluster 2 high-affinity NiFe-hydrogenase containing *Streptomyces* engenders more questions about the nature of high-affinity NiFe-hydrogenase and what percent of soil-dwelling *Streptomyces* contain Cluster 1 vs. Cluster 2 hydrogenases and if there is a difference in functionality.

On a grander scale, putting these results into an environmental context has the

potential to further demystify the microbial-mediated H₂ soil sink. If a strong understanding of the relationship between environmental conditions, microbial life cycle, and the H₂ uptake is constructed, perhaps we can one day better understand large-scale H₂ soil fluxes. Metagenomic data of various ecosystems, like the Harvard Forest LTER, contain information about microbial species diversity. In the future, by testing H₂ uptake across a variety of soil microbes containing high-affinity hydrogenases, a regional environmental model of H₂ soil deposition can be constructed and extrapolated to a global model.

Chapter 4

Conclusion

The modern biosphere is a product of the coupled evolution of life and its environment. Across the globe, microbial activity strongly controls land-atmospheric gas exchange. A prime example of this is the microbial-mediated soil uptake of H₂, which accounts for about 80% of the tropospheric H₂ sink. The true ecological importance of H₂-oxidizing organisms is still unknown, as are the environmental and biological triggers that dictate their H₂-uptake activity.

This research began by exploring several major questions concerning the function of high-affinity NiFe-hydrogenase in isolated strains from the Harvard Forest LTER and its correlation to the organisms' life cycles. We observed that containing the gene for high-affinity NiFe-hydrogenase was a clear predictor of H₂ uptake in the wild isolated *Streptomyces* strains. Overall, the wild strains had very similar patterns of H₂ uptake. The rise in their H₂-oxidation activity corresponded to the production of aerial hyphae and spores. However, H₂ uptake declined to zero over a few weeks despite the presence of spores in the serum vials. This result suggests that spores may not be largely responsible for environmental H₂ uptake, despite the fact that *Streptomyces* are typically found in the spore state in the environment. Future steps to build on this research primarily include testing known H₂-oxidizing strains under projected climate change conditions. In particular, testing these characterized strains under varying H₂ levels, temperature, salinity, pH, and moisture extremes and across different soil types.

Several interesting questions were raised through this research and research conducted in soils instead of on agar. In particular, questions concerning the connection between biological colony structure of the microorganism and their H₂ uptake capacity, such as comparing the density of aerial hyphae to H₂ uptake strength among the isolated strains. The removal of aerial biomass using glass beads resulted in severely diminished H₂ uptake soon after disruption of the colony. Furthermore, the intertwining of substrate mycelia observed in microscopy suggest the potential for signaling between the colonies through an exchange of organic compounds. Does colony-to-colony signaling affect H₂ uptake in a community of soil microbes?

Soil samples from different ecosystems could be analyzed in the same method presented in this thesis to find *Streptomyces* strains across a variety of environmental conditions that also oxidize H₂. Using both metagenomic data and H₂ flux measurements, the regional density of H₂-oxidizing microorganisms can be compared to tropospheric H₂ concentrations. This can potentially provide valuable data for climate models about the distribution and relative strengths of H₂ uptake. Our results showed that other organisms beside *Streptomyces* contain a functional gene for high-affinity NiFe-hydrogenase. In *R. equi* and *S. cattleya*, the gene predicted H₂ uptake function just as in the HFI strains. Future work must be done to construct a deeper ecological understanding about the diversity of tropospheric level H₂-oxidizing soil microorganisms. Furthermore, other trace gases may also have sources and sinks correlated with the life cycles of other microbes. In the future, perhaps correlating microbial atmospheric exchange with organismal life cycle analysis can illuminate deeper ecological patterns that help control the composition of the Earth's atmosphere.

Appendix A

Library of Protocols & Materials

A.1 Logistics

The majority of laboratory research was conducted at MIT. Microbiological lab work was conducted in the Bosak Lab and gas chromatography measurements were conducted in the Ono Lab, both located in E25-650B/C at MIT.

The initial isolation of *Streptomyces* microbe and optimization of PCR protocol Harvard Forest (HF) Long Term Ecological Research Site (LTER) located at 324 North Main Street, Petersham, MA (<http://harvardforest.fas.harvard.edu/>).

A.2 *Streptomyces griseoflavus Tu4000*

The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) blastn function was used to search the reference genome database for a NiFe-hydrogenase containing *Streptomyces* strain. Reference genome *Streptomyces griseoflavus Tu4000* (Accession no. NZ_GG657758) was found to contain a highly similar hydrogenase (E value = 0 indicating no chance for accidental match, 99% query coverage, genome positions 436748-438034). *S. griseoflavus* was kindly lent to this study by genome authors Michael Fischbach (University of California, San Francisco) and John Clardy (Harvard University via Joshua Blodgett). The strain was used as a positive control for PCR amplification of its sequenced hydrogenase

gene.

A.3 R2A Agar

Table A.1: Formula per liter

Ingredient	Amount (g)
Yeast Extract	0.5
Protease Peptone No. 3	0.5
Casamino Acids	0.5
Dextrose	0.5
Soluble Starch	0.5
Sodium Pyruvate	0.3
Dipotassium Phosphate	0.3
Magnesium Sulfate	0.5
Agar	15.0

1. Suspend 18.2 g of R2A Agar powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.
4. Pour agar into sterilized container when autoclaving is complete.

A.4 Soil Actinomycete Spore Enrichment Protocol

1. Air dry soil and sift to a fine powder or dry in a 55°C oven for 3-4 hours.
2. Mix 1 g soil and 1 g of calcium carbonate (CaCO_3) with a mortar and pestle.
3. Spread a thin layer to the bottom of the petri plate and insert a moist Whatman paper so that it is suspended in the lid without touching the soil.

4. Wet Whatman paper and maintain plates in a humid environment for 2-10 days at 28°C.
 - (a) Do not wet soil and CaCO₃ mixture.
5. Once the soil has settled for a few days mix with 100 mL of H₂O and agitate in a vortex.
6. Let mixture settle for 30 minutes.
7. Immediately plate 100 µL of soil suspension to a fresh agar plate or create serial dilutions of the original soil suspension and then plate.
8. Incubate plates at 30°C.
9. Check plates for growth for 3-5 days.

A.5 Selecting for *Streptomyces*

Microbial growth on the plates were assessed for four indicators of *Streptomyces* morphology:

1. A zone of clearing produced by the colony secreting antibiotics and thereby inhibiting neighboring growth.
2. A fuzzy top coating of the colony that is distinctive of aerial hyphal stage of the *Streptomyces* lifecycle.
3. A pigmentation produced by the colony.
4. The distinctive scent of geosmin.

A.6 Procedure to add cycloheximide directly to pre-made plates

Cycloheximide is an antibiotic produced by *Streptomyces griseus*, and acts as an inhibitor of protein synthesis in eukaryotes but not prokaryotes. Cycloheximide's selectivity makes it active against yeast and fungi but tolerated by most bacteria. This feature has allowed brewer's to suppress the growth of culture yeast and detect unwanted bacteria. Cycloheximide is also used to induce apoptosis in HL-60 cells, T cell hybridomas, Burkitts lymphoma cells, and a variety of other cell types. Additionally, plant researchers have shown it to stimulate ethylene production in fruit and leaves. In molecular biology applications, it is used in the selection of wild type CAN1, CYH2 and LYS2 genes.

Cycloheximide is added to plates to inhibit fungal growth [**edit?**]. To make 40 mL of 2.5 g/L cycloheximide solution:

1. Weigh out 0.1 g of cycloheximide.
2. Dissolve in 40 mL dH₂O.
3. Filter sterilize into sterile falcon tube.
4. Add 700 μL to each non-innoculated plate, spreading the antifungal agent evenly.
5. Allow to dry for 1 hour with medium side down until no longer runny.

A.7 Fresh Soil Serial Dilution

1. Add 0.4 g of soil to 40 mL of sterile H₂O.
2. Transfer 0.1 mL to a new vial containing 10 mL of H₂O, thereby creating a 10⁻² solution.
3. Continue process to make all desired concentrations.

A.8 Testing for 16S and NiFe Hydrogenase DNA Sequences

Resuspend oligo primers in TE to create a 100 uM concentration

- Forward Primer NiFe Hydrogenase: 29.6 nmoles
- Reverse Primer NiFe Hydrogenase: 26.6 nmoles
- Forward 16S Primer: 1.626 nmoles
- Reverse 16S Primer: 1.56 nmoles

Table A.2: Hydrogenase Properties

Direction	Primer	Sequence	T _m (°C)	Anhyd. Mol. Wght. (g/mol)
Forward	NiFe-244f	5 - GGG ATC TGC GGG GAC AAC CA -3	62.2	6192.1
Reverse	NiFe-1640r	5 - TGC ACG GCG TCC TCG TAC GG -3	64.6	6110.0
Forward	16S rRNA	5 - AGA GTT TGA TCC TGG CTC AG -3	55.20	6148.0
Reverse	16S rRNA	5 - ACG GCT ACC TTG TTA CGA CTT -3	57.41	6372.2

PCR Prep

- 12.5 μL × Promega MasterMix × 2
- 2 μL × forward primer
- 2 μL × reverse primer
- 6.5 μL nuclease free H₂O
- 2 μL DNA template of sample
- 25 μL total reaction amount
- Negative control is 25 μL of Promega MasterMix × 2

PCR Thermocycler Profile (Run Cycle)

*Using BioRad MyCycler

Table A.3: Temperature pattern (16S)

Step	Temperature (°C)	Time
Initial Denaturation	95	5 min
Denaturation of Template	95	30 sec
Annealing of Primers	50	30 sec
Extension	72	1.5 min
Final Extension	72	5 min
Hold	4	∞

Table A.4: Temperature gradient for NiFe Hydrogenase

Step	Temperature (°C)	Time
Initial Denaturation	95	5 min
Denaturation of Template	95	30 sec
Annealing of Primers	60.7	30 sec
Extension	72	1.5 min
Final Extension	72	5 min
Hold	4	∞

Gel Electrophoresis

- Gel Prep for PCR Products
- 1% agarose
- 5 μ L red dye (GelRed Nucleic Acid Stain 10,000 \times in H₂O, BIOTUM)
- pour 50 mL of gel into mold
- load gel with: 8 μ L ladder and 1-2 μ L of loading dye (Fermentas 6 \times loading dye solution #R0611) plus 5 μ L of PCR sample
- 100 volts
- 1 hour

- Gels were imaged using UVP MultiDoc-It Digital Imaging System (Software Doc-ItLS)

DNA sequence submissions were prepared following the MIT Koch Institute Biopolymers and Proteomics Facility instructions .

(<http://web.mit.edu/biopolymers/www/DNA.html#dnass>)

A.9 Glycerol Bacterial Preservation

1. Start a fresh culture of the microorganisms in 4 mL of broth
2. Grow overnight or until late exponential growth phase is reached
3. Prepare a sterile 50% glycerol solution
4. Add 1 mL of 50% glycerol to the broth and culture
5. Transfer to a labeled 10 mL sterile falcon tube
6. Freeze at -80°C until needed

A.10 *Streptomyces* Spore Suspension

H₂ uptake in spore suspensions

1. Select one or more concentrated spore suspensions from both methods for spore harvesting (glass beads/cotton swab)
2. Pour each spore suspension into a small serum vial and crimp close with rubber stopper
3. Pressurize vial with at least 20 mL lab air through sterile syringe
4. Measure changes in H₂ in headspace over one hour; mix vial on a shaker while not being sampled from to facilitate H₂ exchange between liquid and headspace

Calibration curve of spore abundance vs. absorbance

1. Pipette a specified amount¹ of each spore suspension into microplate reader wells
2. Pipette a specified amount of sterile H₂O into a control well
3. Read absorbance at a specified wavelength
4. Prepare dilutions of cell suspension at a specified levels
5. Count number of spores by microscopy of dilutions
6. Create calibration curve of spore number vs. absorbance

A.11 Protein Extraction and Assay

1. Harvest sporulating aerial mycelia aseptically using a metal spatula and transfer into 1.5 mL tubes containing 0.3 g glass beads (0.1-0.2 mm diameter) and 0.7 mL water
2. Disrupt the cells first by vortexing for 5 minutes at 2000 rpm and then cool on ice
3. Further disrupt the cells using the sonication wand with three 30 s bursts and 1 min intermittent cooling on ice
4. Centrifuge at 12,500 rpm for 15 min at 4°C to obtain the soluble protein fraction
5. **Optional:** Remove residues of membranes and nucleic acids by transferring 0.5 mL to the spin-X filter system inner filter and centrifuge at 7000×g for 15 min at 4°C
6. Determine the protein concentration using the BCA protein assay kit (see kit instructions) using the microplate reader in the Bosak lab

¹According to microplate reader instructions.

A.12 Overview of Setup for Experiments 1-3

- Serum vials: 4 replicates (a-d) of each strain, 2 controls
 - 3 replicates for GC measurements (a-c)
 - 1 replicate for microscopy specimens (d)
 - 1 control for GC, 1 control for protein assay
- Plates:
 - large petri: 1 of each strain for CFU
 - small petri: 3 of each strain for colony photographs
- Use R2A medium in vials

Overview of Experiment

1. Day Zero
 - (a) Inoculate serum vials (a-d)
 - (b) Plate cell dilutions to count CFU (enumeration of initial viable bacteria)
 - (c) Plate small petri dishes
 - (d) Image source of inoculum: plate and cells
2. Course of Experiment – every two days
 - (a) (vials a-c) H₂ uptake rates by GC
 - (b) (vial d) Image cells by microscopy
 - (c) (vials a-d) Photograph vials
 - (d) Record room temperature
3. End of Experiment
 - (a) Final GC, microscopy and photo (consider waiting extra week to make sure there is still strong uptake)

(b) Extract protein from vials (a-c) and do a protein assay

Vial Inoculation

1. Prepare R2A medium (1 L)
2. Autoclave serum vials, stoppers, cotton, big swabs, and eppendorfs
3. Pipette 10 mL R2A into each serum vial
4. For each strain, pick colony and suspend in 1 mL sterile H₂O, then vortex
5. Label vials and plates
6. Inoculate serum vials with 200 µL of cell suspension each, swirl to coat agar
7. Add 200 µL of sterile water to control vial
8. Cap vials with sterile cotton or breathable cap
9. Spread 100 µL of cell suspension on plate of R2A - CFU plate
10. Spread 30 µL of cell suspension on small R2A plates for colony imaging

Cell Quantification

1. Thermo Scientific Pierce BCA Protein Assay Kit Count CFU on plates
2. Find percent area coverage of cells on plate from photographs

GC H₂ uptake rate analysis

1. Run standard tank one time in between sets of three vials
2. Gather three vials for analysis plus the following materials
 - 3 autoclaved stoppers
 - 3 crimps
 - 3 needles

- 3 syringe filters
 - 1 10 mL syringe
3. Enter the sample info for the first vial
 4. Cap the first vial with the stopper and crimp
 5. Inject 20 mL of sterile lab air into first vial to pressurize by sterilizing through syringe filter
 6. Remove 4-5 mL of headspace air with syringe and inject into GC sample loop
 7. Repeat steps 3-6 for second and third vials
 8. Continue to measure three vials in order every 12 minutes
 9. Measure each vial 3 times
 10. Repeat for next three vials from step 1
 11. Measure all sample vials and the GC control vial

Photography

1. Plate surfaces - to show colony morphology
2. Vials - photograph from below to get idea of percent area coverage
3. Vials - photograph from above to look at colony morphology and color

Microscopy

1. Prepare microscope slide with one sample from each strain (vial c)
 - Pick a new colony each time
 - Mark colonies with date after pick
2. Start with 10x dry and work up to 100x oil

3. Capture images that show the status of the cell life cycle
4. Save images

A.13 Gas Chromatography

Molecular hydrogen (H_2) mixing ratios were measured for these laboratory-based studies using gas chromatography with a Helium Pulsed Discharge Detector (HePDD). A gas chromatograph (GC) (Model 2014, Shimadzu Co., Kyoto, Japan) configured with a thermal conductivity detector (TCD) and dual flame ionization detectors (FID) was retrofit with a HePDD (D-4-I-SH17-R Model, Valco Instruments Co. Inc. (VICI), Houston, Texas) for measuring H_2 at atmospheric levels (~ 530 ppb) to high precision. The instrument is shown in Figure A-1.

The retrofit HePDD was mounted above the GC oven. The HePDD signal was collected through the left FID electrometer and detector temperature was controlled by an auxiliary GC heater. Carrier gas flow was controlled by pressure regulators external to the GC to satisfy the manufacturers specifications for permissible fitting types and materials to contact the gas stream.

For timely and targeted measurements, a backflush-to-vent configuration was created using 6- and 8-port 2-position VICI sampling valves as shown in Figure A-1. Research grade helium was sent at 60 psig through a VICI helium purifier and the stream was split at a ‘T’ to 1) a crimped tube delivering a 30 mL/min purge flow to the detector and 2) to pressure regulator to control the carrier gas pressure. Needle valves were used to mimic the resistance of the pre-column, and pressures were adjusted (~ 38 psig) to achieve approximately 15 mL/min of column flow regardless of valve position.

Air samples were loaded via 1/16 in. tubing or luer lock syringe port into a 1 mL sample loop mounted on the 6-port sampling valve before being injected via the 8-port backflush valve onto the somewhat coarse mesh packed pre-column (4 m \times 1/8 in. OD stainless steel Hayesep DB, 80/100 mesh, Chromatographic Specialties Inc. (CSI), Brockville, Ontario Canada). Neon and H_2 were the first constituents of atmospheric

samples to exit the pre-column, after which time the backflush valve was switched (at 2.2 min) to reverse the carrier gas flow direction to purge the remaining bulk and trace gases from the detection stream. Neon and H₂ were then further resolved in a finer mesh packed main column (4 m × 1/8 in. OD stainless steel, Hayesep DB, 100/120 mesh, CSI) until reaching the HePDD for analysis. The total run duration was 12 minutes to allow adequate time for the pre-column to be cleansed. The oven was run isothermally at 40°C and the detector at 100°C.

Chromatograms were recorded and peaks were integrated using the Shimadzu GCSolutions 2.30.00 SU6 software. Neon and H₂ eluted at approximately 3.3 and 3.7 min, respectively. These trace gas peaks are centered upon a raised stair-step patterned baseline. The pattern likely results from small differences in pressures and leakiness of the two carrier gas streams (the detector is extremely sensitive to small leaks). Likewise, the peaks bracketing the staircase pattern are either due to pressure fluctuations or leaks that occur during valve switching and should not be attributed to any specific trace gas species. No purged housing was available for the valves in this system. The GC-HePDD achieves a 2 ppb one-sigma precision on repeat calibration standard runs for H₂.

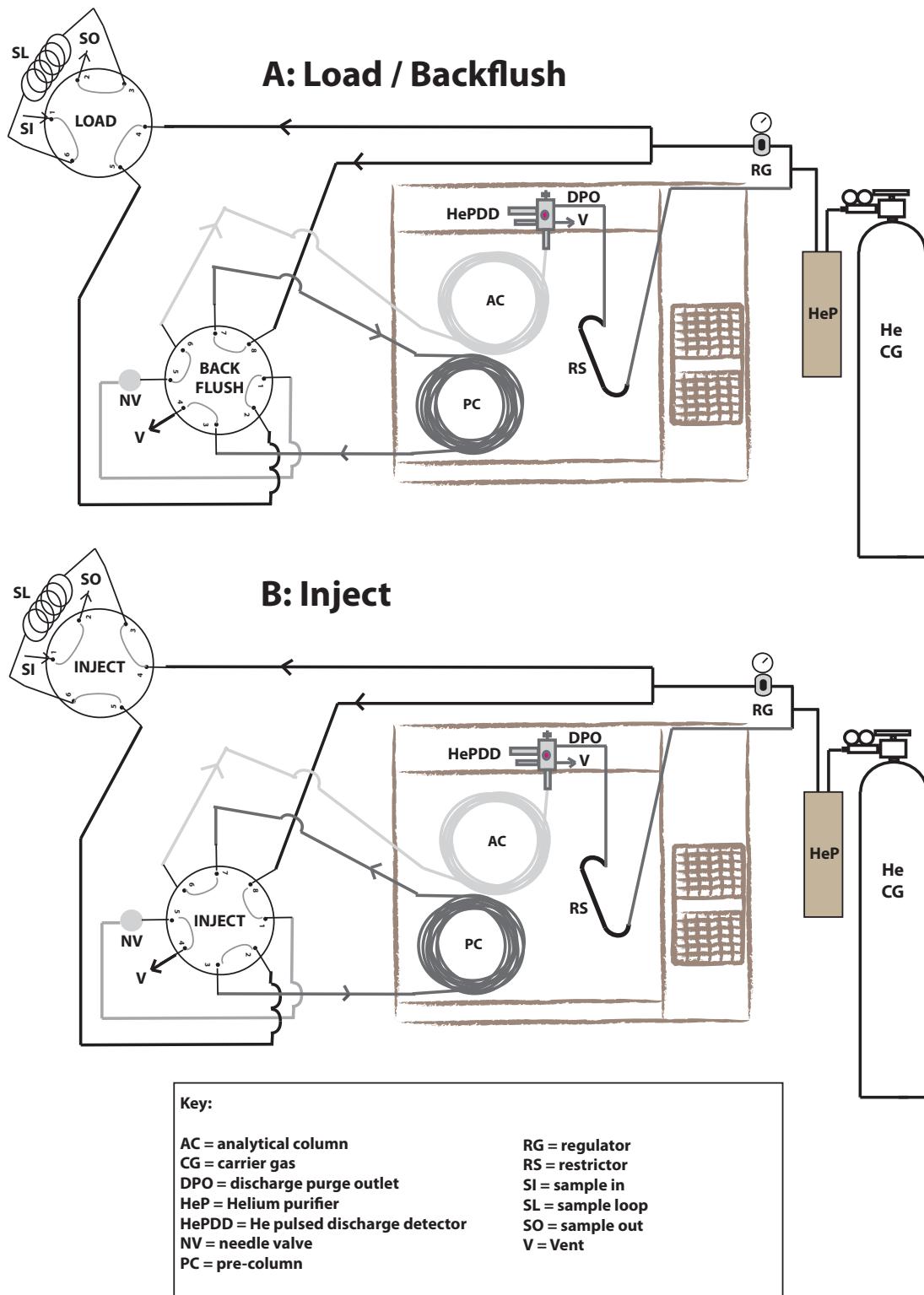


Figure A-1: Schematic of the GC-HePDD system constructed for the laboratory measurements of H₂.

Appendix B

DNA Sequencing Results

Table B.1: DNA Sequencing Results

Continued on next page

Table B.1 – continued from previous page

Table B.1 – continued from previous page

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