

The Search for Bacterial Chemotaxis towards Hydrogen

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Abstract

Bacterial chemotaxis towards O_2 has been studied since the onset of chemotaxis research, yet there are many other gases that serve as substrates for bacterial metabolism. H_2 is one such example. H_2 is utilized by a diversity of microbes in their energy metabolism, including aerobic H_2 oxidizing knallgas bacteria, sulfate reducing bacteria, anoxygenic photoautotrophs, acetogens, and methanogens. But H_2 levels in many of the environments that these organism inhabit are low, spatially and/or temporally. This study seeks to establish the behavior of bacterial chemotaxis towards H_2 . Chemotaxis towards H_2 is hypothesized to be a selective advantage for H_2 utilizing bacteria living in environments characterized by H_2 gradients over non-motile counterparts. Capillaries assays for H_2 chemotaxis under aerobic conditions were performed on freshwater sediment slurries collected from School Street Marsh, Woods Hole, MA. CARD-FISH was used to compare the relative numbers of *Eubacteria*, α -, β -, and γ -*proteobacteria* in capillaries containing sterile sample liquid saturated with air, air and N_2 , or air and H_2 . Results indicate that β -proteobacteria were significantly enriched in H_2 treated capillaries. This is consistent with the phylogeny of known aerobic H_2 utilizing organisms such as *Ralstonia eutropha*, a bacterium whose genome contains a sensory hydrogenase as well as all the genes necessary for chemotaxis. Further studies are needed to establish H_2 chemotaxis in pure cultures as well under anaerobic conditions.

Introduction

The ability to sense and respond to environmental cues underlies much of microbial behavior in nature and is integral to survival when environmental conditions are changing. One of the best-studied responses to a changing environment is the regulation of motility resulting in movement towards a beneficial chemical or away from a toxic chemical in a process called chemotaxis. Bacterial chemotaxis towards light, organic compounds, carbon dioxide, and oxygen has been shown to be important in environments characterized by temporal and/or spatial gradients of these substrates (Berg

1975; Taylor et al 1999; Armitage 1992; Wadhams and Armitage 2004). Chemotaxis towards gases other than oxygen has been less well studied, especially those important in anaerobic environments such as H₂. H₂ gas is a key intermediate in the anaerobic degradation of organic matter and serves as an energy source for important metabolic guilds such as the acetogens, methanogens, and sulfate reducers. In the presence of suboxic levels of O₂, knall-gas bacteria utilize H₂ as an electron donor for chemolithoautrophic growth. The importance of H₂ as an electron donors results from its favorable redox potential (E_o' -420 mV) however its low concentration in many environments (~ 1 Pa in sediments) often limits its usage to those organisms capable of sensing and metabolizing the lowest concentrations of H₂ (ie the H₂ threshold) (Lovely et al 1982). An advantage in these substrate-limited situations would be the ability to chemotax. Chemotaxis may allow organisms with higher H₂ thresholds to place themselves in a regime in which substrate concentrations would be high enough for them to sense and thus use. This may help them outcompete non-motile organisms with lower H₂ thresholds. Chemotaxis has been linked to the higher survival rates of NO₃⁻ chemotactic *Pseudomonas fluorescens* over non-motile strains under aerobic and anaerobic conditions (Kennedy and Lawless 1985) on solid medium despite their displaying no growth rate differences in liquid medium. Pilgram and Williams (1976) also observed chemotactic *Proteus mirabilis* overgrowing motile but non-chemotactic mutants in semi-solid media even though both strains had the same growth rates in liquid. It is not unlikely that chemotaxis towards H₂ would give a selective advantage in gradient

environments such as anoxic-oxic transition zones, sediments, and hydrothermal vents among others. The heterogeneous nature of the microbial mats, marshes, and anaerobic sediments found around Woods Hole, MA, make these good target environments to search for those organisms capable of H₂ chemotactic behavior.

Methods

Samples:

Anaerobic sediment samples and overlying sediment water were collected on June 17, 2008 from a freshwater swamp area at School Street Marsh in Woods Hole, MA, and stored separately at room temperature in sealed glass jars until use on July 15, 2008. Upon use they were exposed to air. Portions transferred into sterile glass Meclats bottles or Falcon tubes for capillary assays.

Capillary Assays:

Two types of assays for H₂ chemotaxis were developed based on the techniques presented by Overmann (2005). One assay used Vitro Dynamics 0.4 x 4 mm flat rectangular microslide glass capillary microslides attached to plastic tubing loaded under normal atmosphere conditions (Figure 1a). To create a 100% H₂ headspace in these capillaries, a capillary attached to tubing was flushed with 100% H₂ for 10 seconds with a 10 ml syringe connected to a 100% H₂ tank. The capillary, tubing and syringe (filled with 100% H₂) were disconnected from the tank after flushing. Then while continuously expelling H₂ through the capillary with the 10 ml syringe to prevent O₂ from entering, the open end of the capillary was dipped into sample sediment slurry water. The capillary was loaded with sample by sucking up slurry water until $\frac{3}{4}$ of the glass capillary was filled. Then, with the capillary end still in the sample, the tubing was clamped and syringe removed. The open end of the capillary was sealed with plasticine. In this set up, the sample would presumably be exposed to a headspace of 100% H₂ in the capillary and is analogous to capillary assays for aerotaxis. Control capillaries were filled with N₂ or air. Up to five of H₂, N₂, and air filled capillaries were set up for a particular sample.

A second assay technique using 75 mm x 1.35 mm round glass capillaries (Biological Research Inc) and glass Meclats bottles was developed to look at chemotaxis towards H₂ under aerobic conditions. Capillaries were filled with 0.2 um filtered sediment water that was saturated with air (control for a gradient resulting from sample filtration and processing), air and N₂ (control for microaerophile aerotaxis behavior), or air and H₂ (H₂ chemotaxis). These were sealed at one side with plasticine and the other end exposed to sediment slurry

sample held within a Meclats bottle adapted for capillary assays (Figure 1b). For the experiment with sediment from School Street Marsh, 4 capillaries were air saturated, 4 were N₂ and air saturated, and 8 were H₂ and air saturated. These were split evenly between two Meclats bottles. Each Meclats bottle was filled with 30 ml sediment slurry diluted 1:2 with 0.2 um filtered sediment water. All assays were allowed to proceed for 12 hours at room temperature on the bench before capillaries were disturbed for sampling and microscopy. Sampling consisted of removing any visible bands in the capillary with 100 ul calibrated glass pipets (VWR) pulled under a flame so that the tips could fit within capillaries. The anaerobic chamber was not used to prepare capillaries or for the assay itself since the headspace in the chamber contained significant amounts of H₂ (5%). This would H₂ saturate samples before being loaded into capillaries and eliminate the H₂ gradient required for chemotaxis.

Community analysis in capillaries by 16S rDNA PCR and CARD-FISH

Bands of motile bacteria in capillaries of the same type (air, N₂ + air, or H₂ + air) were sampled and pooled into 1.5 ml sterile Eppendorf tubes. 10 x 6 ul spots of each unfixed sample were made onto 0.2 um GTTP filters in preparation for community 16S analysis by filter PCR. The filter spots (~ 3mm diameter) were cut out from the filter and placed into 40 ul NP-40 and subjected to a boil at 102 C for 5 minutes to lyse cells on the filter. 3 ul of this was used for 16S PCR amplification using 8F and 1492R primers in 100 ul reactions running 40 cycles with an annealing temperature of 45 C. The rest of the samples were fixed to 1% formaldehyde at 1 hr room temperature and then spotted (6 ul per spot) onto 0.2 um GTTP filters in preparation for CARD-FISH using methods described by Pernthaler et al (2002) and Ishii et al (2004). The Cy3 labeled CARD-FISH probes used were EUB338-I-III for all Eubacteria, Non338 as a control for background fluorescence, Bet42a for beta-proteobacteria, Alf968 for alpha-proteobacteria, and Gam42a for gamma-proteobacteria (Ishii et al , 2004; Amman and Fuchs 2008). These probes were chosen since aerobic H₂ oxidizing bacteria were anticipated under the experimental conditions and these generally fall in the phylum proteobacteria, particularly alpha and beta subclasses. For CARD-FISH counts, the number of cells of each type spotted on the filter was estimated using the square of the diameter of the spot (in terms of number of counting grids) x average cell number per grid (count at least 10 grids across all of the spot) x pi/4 to account for a circular spot. Microscopy was performed on a Zeiss Axioplan microscope with DAPI and Cy3 fluorescence filter sets.

Results and Discussion

The capillary assay technique using tubing connected to flat microslide capillaries did not work well since controls containing rezasurin indicated O₂ contamination in the supposedly 100% H₂ headspace as well as N₂ headspace capillaries, most

likely because tubing was gas permeable. All bands forming in these capillaries were most likely a result of aerotaxis since they formed consistently a few mm below the gas liquid interface. This is typical of microaerophile behavior. Thus this technique was abandoned in favor of the second in which sterile sample liquid was saturated with air, air + H₂, or air + N₂ prior to being loaded into capillaries which were then placed into School St. Marsh sediment slurry. The advantage of the second technique is that there is no worry about air contamination since all samples are already air saturated so that any differences in bacterial communities in the capillaries would indicate taxis towards the second gas used for saturation rather than towards air. Bands in N₂ + air and H₂ + air were visible within 12 hours of assay on School St. Marsh sediment slurry (Fig 2) but no bands were visible in the air saturated controls. All capillaries with obvious bands were sampled for 16S and CARD-FISH analysis. Air saturated control capillaries were sampled even though no bands were seen to get an idea of the number and type of bacteria entering the capillaries by random chance.

16S PCR did not yield any product for any of the 10 spots of each sample. This was mostly likely because no freeze thaw cycles were used for cell lysis and the template concentration was too low. Filter spots should have been used directly as template in the PCR reaction.

CARD-FISH did however work well on 6 ul spots from capillaries. Fig. 3 shows DAPI stained cell (panel a) and the same cells binding the EUB probe within the

H₂ + air saturated capillaries (panel b). Very small cells can be seen in the DAPI stain but not with the Eub probe suggesting that they are either Archaea or viruses. The scale bar would suggest that they are small cells rather than viruses and thus Archaea. Cell counts are shown in Fig. 4 and indicate that about half the population of cells stained by DAPI in all treatments are Eubacteria, leaving the other half of most likely Archaeal classification. No statistically significant differences were seen in the abundances of eubacteria, alpha or gamma proteobacteria amongst air, N₂ + air, and H₂ + air saturated capillary samples. The sum of all proteobacteria probe counts are fairly close to the total Eubacteria probe count indicating that most all of the bacteria in the capillaries were proteobacteria. The only significant difference in cell counts for different probes was with the Bet42a probe targeting beta proteobacteria. H₂ + air saturated capillaries had the greatest enrichment of beta proteobacteria relative to the air saturated control. N₂ + air saturated capillaries showed an enrichment as well but about 10% less than in H₂ saturated capillaries. The difference in beta cell counts between the H₂ and N₂ capillaries suggests that enough chemotaxis towards H₂ has occurred in the H₂ capillaries to show up against the signal of aerotaxis which is expected in N₂ and air saturated capillaries. These results indicate that under aerobic conditions H₂ chemotactic bacteria in sediments from School St. Marsh belong to the beta-proteobacteria subclass. This is consistent with the phylogeny of known aerobic H₂ utilizing chemolithoautotrophic knallgas bacteria (ex *Ralstonia eutropha*, *Hydrogenophilus*) (Aragno and Schlegel 1992). *Ralstonia eutropha* harbors a cytoplasmic sensory Ni-Fe hydrogenase HoxBC,

which is hypothesized to act as a H₂ receptor in concert with a sensor kinase HoxJ to regulate hydrogenase gene transcription (Kleihaus et al 2000). Moreover, all genes necessary for chemotaxis are found in the genome of *R. eutropha* (Pohlmann et al 2007). Nutrient supply and the state of polyhydroxybutyrate accumulation have also been shown to influence flagellar synthesis (Raberg et al 2008).

Further experiments with pure cultures are needed to determine H₂ chemotaxis in knall-gas bacteria and to rigorously demonstrate H₂ chemotaxis in the environment. Results from this study represent only one experiment and are suggestive of H₂ chemotaxis in the environment but are by no means conclusive. Experimental design issues precluded replicate experiments from being performed but are of course needed. Hydrogenase assays could be performed on samples extracted from capillaries as an additional test for chemotaxis in environmental samples. Capillaries also make for useful enrichment tools for H₂ utilizing bacteria from the environment or could also be used to screen for H₂ chemotaxis from enrichment cultures. Additionally, assays with capillaries loaded with gas saturated sterile sample liquid or flat capillaries containing 100% H₂ headspace could also be performed in a N₂ anaerobic chamber to look for H₂ chemotactic anaerobes. But with the tools and time available, CARD-FISH results from this study are consistent with H₂ chemotaxis by aerobic H₂ chemolithoautotrophs and support the hypothesis that H₂ chemotaxis exists in nature.

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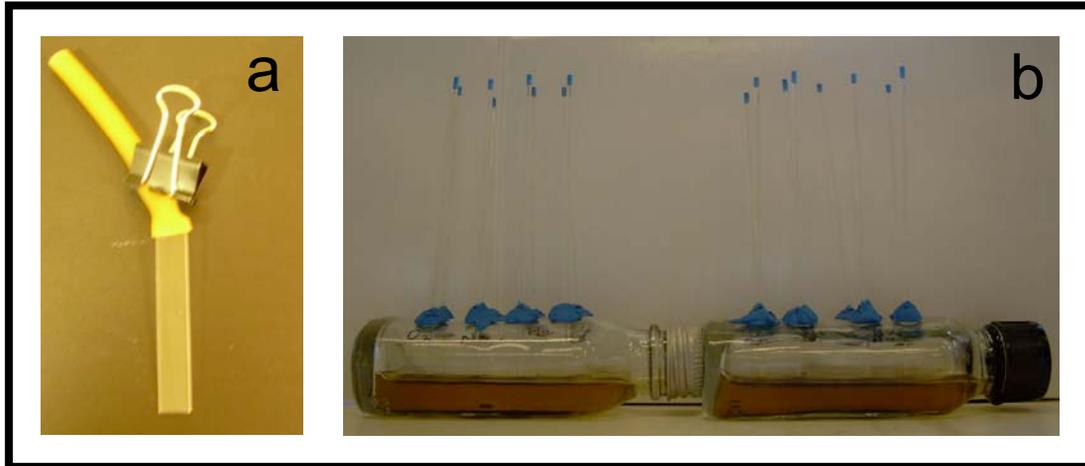


Fig 1. Capillary assays used for H_2 chemotaxis experiments. (a) Assay technique 1 with 0.4 x 4 mm flat microslide capillary tubes in which 100% H_2 is introduced. (b) Assay technique 2 for chemotaxis towards gas saturated liquids.

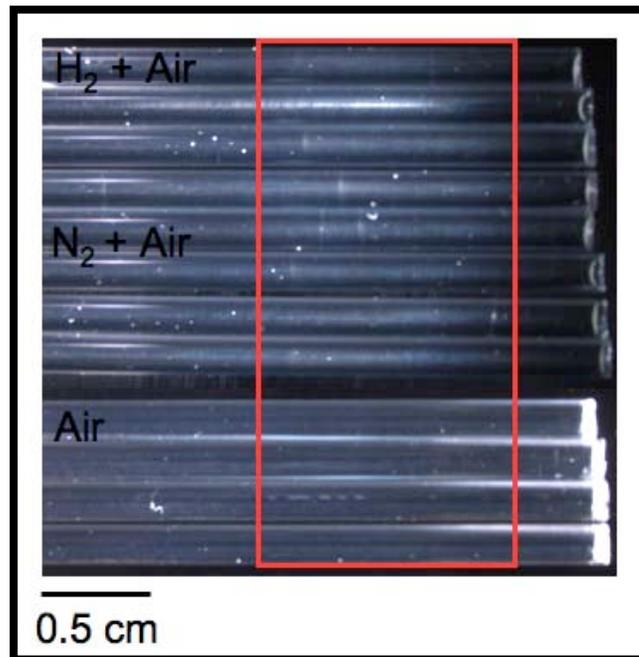


Fig 2. Bands of motile microbes developed in H_2 + Air and N_2 +air capillary tubes within 12 hours of assay.

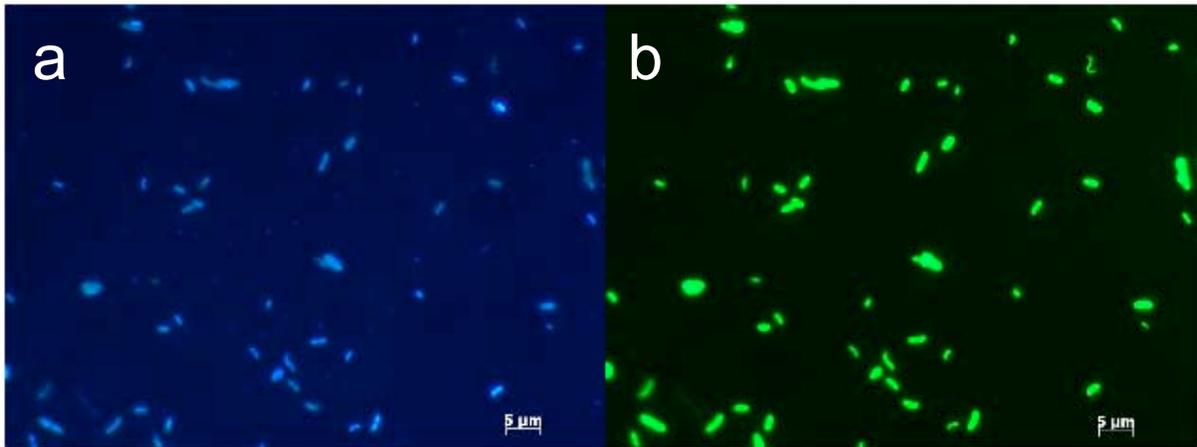


Fig 3. Cells on a 6 ul spt of pooled H₂+Air saturated capillaries. (a) cells under DAPI stain (b) same cells but visualizing cells binding the EUB I-III probe using CARD-FISH

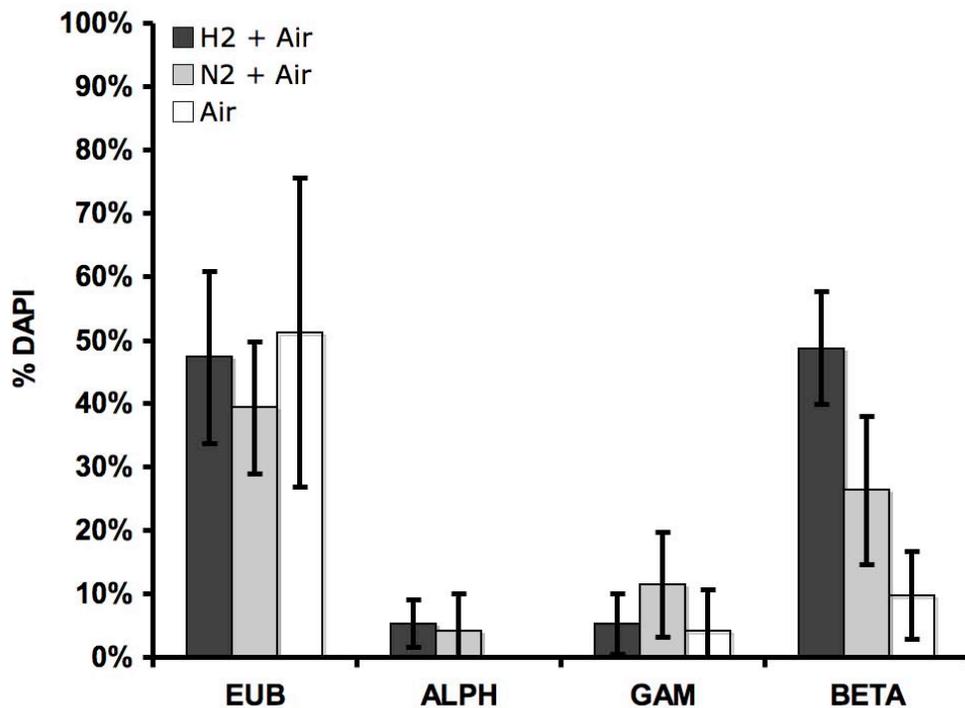


Fig 4. CARD-FISH cell counts normalized to DAPI counts from H₂ + air saturated (8), N₂ + air saturated (4), and air saturated (4) capillaries. EUB = Eub I-II probe, Alph = Alph968 probe, Beta = Bet42a probe (Amman and Fuchs 2008). Error bars indicate 1 standard deviation.