

ECOPHYSIOLOGY OF *METHANOCOCCUS MARIPALUDIS* AND *DESULFOVIBRIO*
VULGARIS: THE ROLE OF STRUCTURE IN RELATION TO FUNCTION

by

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DEDICATION

To Harold and Helen Gould- who did not get to see this day for themselves, but would have never doubted that it was possible.

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ABSTRACT

Sulfate-reducing bacteria (SRB) and methanogenic archaea are known to interact in anaerobic environments under a range of conditions, and the nature of the interaction is dependent on the concentration of available carbon and energy sources, electron acceptors and the metabolic potential of the specific genera present. In the absence of sulfate, SRB can participate in a mutualism by product inhibition with hydrogenotrophic methanogens. SRB reduce protons to form hydrogen gas as a methanogenic substrate, and methanogens prevent inhibition of hydrogenase activity in the SRB by consuming the evolved hydrogen, making the interaction beneficial to both parties. This type of interaction can occur in nature when organisms are free-swimming or attached to a surface as biofilm. The impact of structured biofilm on ecosystem function at various scales is becoming increasingly clear, as this growth mode concentrates biomass which can increase the capacity for compound immobilization, affect hydrodynamic flow paths, and leave cells in altered physiological states. In spite of this, few studies have systematically characterized mutualistic interactions within the biofilm state using model organisms. Syntrophic continuous culture of *Desulfovibrio vulgaris* Hildenborough and *Methanococcus maripaludis* was monitored from inoculation through steady state for biofilm and planktonic community structure and function in terms of biomass production, lactate oxidation, and methane production. This biofilm was structurally distinct from monoculture biofilms grown under the same conditions and yield of biomass per lactate mass flux or methane produced was much higher when biofilm was present under lactate limitation. The results suggested that biofilm helped optimize carrying capacity of the syntrophic culture.

Observations in coculture biofilm of attraction by *M. maripaludis* to a surface in the presence of a hydrogen-producing biofilm indicated a tactic response in the archaeum. Movement toward favorable conditions, or chemotaxis is a strategy employed across all three domains of life, yet chemotaxis in archaea is still poorly described, and no previous work has demonstrated taxis toward a hydrogen source, like a syntrophic partner, in spite of its role in electron flow in anaerobic communities. Here we present the first direct observation of taxis toward hydrogen, or “hydrogenotaxis” in the archaeum *M. maripaludis*.

CHAPTER ONE

INTRODUCTION

Anaerobic Community Ecophysiology

Anaerobic microorganisms are important players in a wide range of ecosystem processes including biogeochemical cycling, bioremediation, metal pipe and ship hull corrosion, oil souring, greenhouse gas emission, wastewater treatment, and bioenergy. Approaches at varying scales can be taken to gain an understanding of how these community members interact, and what effect their interactions have on ecosystem function and community structure or stability (Fuhrman 2009). These methods include at one extreme, meta-surveys of community members before and after some disturbance, over time, or along geochemical gradients, and seek to find patterns in occurrence of specific guilds or species that can be correlated to a function. For this type of study, it is not always necessary to cultivate the community, since informational or functional molecules such as DNA, RNA, and proteins can be extracted from whole communities and then compared to extensive databases to determine the community composition and potential function. On the opposite end of the spectrum, single organisms are cultivated in the lab with painstaking effort to keep other organisms from contaminating the culture. This approach is very valuable for testing physiological attributes of an organism under controlled conditions. It is also a methodical way of learning about the function of specific proteins for which genes have been annotated as “unknown function” or “hypothetical” in organisms with available sequenced genomes (Editorial 2012).

However, few if any microorganisms function in isolation, and community function is most likely a conglomerate of multiple, interacting populations. Therefore, an understanding of microbial interactions at different scales (*e.g.*, community, population, cellular) will provide great insight into ecosystem function.

The scope of this dissertation is a step beyond a pure culturing approach – two interacting organisms are cocultured. The two organisms were evaluated under various conditions, described herein, that are environmentally relevant, in an attempt to understand community function in a simplified, controlled laboratory system. The coculture pairing was the sulfate-reducing bacterium (SRB) *Desulfovibrio vulgaris* Hildenborough and the hydrogenotrophic methanogen *Methanococcus maripaludis* S2.

Sulfate-Reducing Microorganisms

Sulfate-reducing microorganisms (*Bacteria* and *Archaea*; SRB and SRA) include Gram-negative δ -*Proteobacteria*, *Euryarchaeota*, *Crenarchaeota*, deeply-branching *Thermodesulfobacterium* spp., Gram-positive *Firmicutes*, and *Nitrospira*. This diverse functional group is characterized by the ability to use sulfate as a terminal electron acceptor and often other compounds including fumarate, S⁰, thiosulfate, sulfite, polysulfide, nitrate, dimethylsulfoxide, Mn (IV), Fe(III), O₂, and U(VI) (Barton & Hamilton 2007). SRB and SRA, have been observed at all biologically favorable temperatures ranging from cold to hydrothermal systems and can use a variety of electron donors including H₂, lactate, acetate, propionate, formate, pyruvate, butyrate, succinate, malate, ethanol, longer chain fatty-acids, alkanes, and benzoic acid (Barton & Hamilton 2007; Pester et al. 2012; Leloup et al. 2009). Evidence indicates their activity on Earth as

early as 3.47 Ga, based on sulfur isotope fractionation in microscopic pyrite crystals depleted in ^{34}S compared to the surrounding sulfate (Shen & Buick 2004). This indicates that dissimilatory sulfate reduction is one of the oldest metabolisms known (Shen & Buick 2004), predating anoxygenic photosynthesis ~ 2.5 Ga (Konhauser 2009).

Given the broad range of substrates by which SRB and SRA can gain energy and divide, it is not surprising that they play vital roles in many ecosystem processes. They affect the redox state of their environment and are a linking point in biogeochemical cycles of sulfur, carbon, and nitrogen (Falkowski et al. 2008). The contribution of SRB and SRA to carbon mineralization linked to sulfate-reduction has been shown to be as high as 50% in shallow marine environments, alongside the total contribution from aerobic community members (Jorgensen 1982). It was also recently shown that CO_2 produced from sulfate-reduction in wetlands could account for 36-50% of anaerobic carbon mineralization and that sulfate-reduction rates were comparable to those observed at higher sulfate concentrations in marine environments (Pester et al. 2012).

SRB are being studied extensively to understand potential roles (biotic and abiotic) in heavy metal/metalloid and radionuclide transformations (Gadd 2010; 2001; Neal et al. 2004). Several contaminants considered important for bioremediation projects can be reduced to an insoluble form through the activity of SRB. These include U(VI) and Cr(VI) enzymatically via cytochrome c_3 (Lovley & Phillips 1994; Lovley et al. 1993) in addition to Tc(VII) and Se(VI)/(IV) via both enzymatic reduction and chemical precipitation as insoluble sulfide metals (Lloyd et al. 1998; Zehr & Oremland 1987). As(V) reduction results in a more soluble species; however, it has been shown that As(V)

reduction with sulfate in SRB results in immobile As(III) sulfide precipitation under acidic conditions (Newman et al. 1997; Rittle et al. 1995; Kulp et al. 2006).

For this work the model SRB *Desulfovibrio vulgaris* Hildenborough was used because of the abundance of previous work that has been done to understand its physiology. *D. vulgaris* has a sequenced genome and has a tractable genetic system for creating knockout mutants (Heidelberg et al. 2004). Several mutants have been created and characterized, including for iron uptake regulation (Δfur), hydrogenases, and a mutant lacking the pDV1 plasmid and motility (Δmp) (Bender et al. 2007; Walker et al. 2009; Clark et al. 2007). The structure of biofilm formed by *D. vulgaris* has been previously described and characterized by transcriptomics and the analysis of extracellular appendages apparently important to biofilm formation (Clark et al. 2007; 2012). *D. vulgaris* is capable of growing at elevated salt concentrations and in the absence of sulfate, as a syntrophic proton-reducer with hydrogen-utilizing organisms, making it an ideal coculture partner for *M. maripaludis*.

Methanogenic Archaea

Biogenic methane production is observed in the *Euryarchaeota* represented by many organisms within five orders of methanogens. Methanogenesis proceeds via reduction of oxidized carbon including CO₂ (with H₂), acetate, methanol, formate, and methylamines under strict anaerobic conditions (intracellular redox potential of approximately -600mV) (Ettwig et al. 2008). Despite variation in the enzymes and mechanisms of energy generation between orders, all methanogens possess the methyl-coenzyme M reductase (Mcr) which catalyzes the final reduction of methyl-coM to

methane and coM-coB heterodisulfide (Thauer et al. 2008). Methanogenesis is also one of the earliest metabolisms on earth, dating to >3.47 Ga, based on isotopic evidence of ^{13}C depleted methane in fluid inclusions of hydrothermal precipitates. The observed $\delta^{13}\text{C}$ values are in the range for methane of microbial origin (Ueno et al. 2006).

Methanogens provide a variety of ecosystem services, both positive and negative to humans. They are important contributors to carbon mineralization in the reduction of CO_2 or acetate to CH_4 , and therefore play an integral role in global carbon cycling. Methane is a potent greenhouse gas that has an estimated global warming potential (GWP) 25-40% higher than CO_2 per molecule. This range of predicted GWP is dependent upon the type of model used, and the highest estimates result from considering gas-phase interactions and chemistry of atmospheric methane with aerosols, sulfate, and nitrate (Shindell et al. 2009). The largest sources of methane as of 2010 were wetlands, with an estimated flux between 143 to 174.5×10^{12} g CH_4/yr ; and the belches of ruminants constituting approximately 116×10^{12} g CH_4/yr ; with emissions from rice cultivation contributing 40×10^{12} g CH_4/yr (Neef et al. 2010; Schlesinger & Bernhardt 2013). These methane sources are the net results of anaerobic community activity in each case and are not the only microbially mediated methane sources. Additional contributions to total flux as a product of microbial metabolism include input from sewage treatment, termites, coal mining, animal waste, and landfills (Schlesinger & Bernhardt 2013; Neef et al. 2010). Methane from these sources is a potent greenhouse gas, but can also serve a useful purpose as a readily available source of energy. Natural gas, which contains up to 75% CH_4 is a major source of energy worldwide and biogas

produced via methanogenesis can be harvested to fill energy demand on cattle farms or in wastewater treatment facilities (Kato & Watanabe 2010). Given these two contrasting ecosystem roles for methanogens, it is important to gain an understanding of physiological response as a result of interactions with other community members, namely SRB.

M. maripaludis was chosen for this work because of its rapid growth at mesophilic, circumneutral conditions and because of the magnitude of previous work that has been done on this model methanogen. A complete genome sequence is available and a tractable genetic system has been developed in *M. maripaludis* for generation of mutants (Hendrickson et al. 2004). Several have been developed thus far including a knockout mutant for six of the seven hydrogenases ($\Delta 6H_2ase$) (Moore & Leigh 2005; Lie 2004; Costa et al. 2013; Jarrell et al. 2011; Blank et al. 1995; Stathopoulos et al. 2001; Sarmiento & Mrázek 2013; Lie et al. 2012; Walker et al. 2012).

Competitive and Cooperative Interactions

The relationship between SRB and methanogens has been studied extensively in various environments, and has been shown to be highly variable with respect to the nature of the interaction. In the presence of sulfate, methanogens are often outcompeted by SRB using hydrogen, formate, and acetate as electron donors for sulfate reduction (Plugge et al. 2011). For example in the human colon several different genera of SRB have been observed in individuals who do not produce methane gas, constituting up to 3.3% of total fecal RNA, while individuals who produce methane gas were shown to have few or no SRB present and sulfate-reduction rates were very low (Barton &

Hamilton 2007). These results highlight one possible scenario where either sulfate-reduction or methanogenesis dominate exclusively.

SRB can alternatively form syntrophic partnerships with hydrogenotrophic methanogens in the absence of sulfate. In the absence of an exogenous electron acceptor, SRB reduce protons to form hydrogen gas. This reaction is kept favorable when hydrogenotrophic methanogens consume the evolved H_2 , keeping the partial pressure low (McInerney et al. 2009; Stams & Plugge 2009).

The nature of SRB-methanogen interactions is complex and often transient based on substrate flux and availability (Plugge et al. 2011; Stams & Plugge 2009; Leloup et al. 2009). For example it has been demonstrated that sulfate reduction rates in freshwater wetlands are higher than originally predicted, often as high as those observed in marine environments. This process was not recognized initially since sulfate pools were kept very low by sulfate reducing activity. Methanogens are found interacting with SRB in these environments, and the relationship can alternate between cooperative and competitive based on sulfate and hydrogen availability. This dynamic has an important effect on carbon mineralization, in that when sulfate reduction dominates, CO_2 is the dominant product. However, when sulfate pools are depleted and methanogenesis is favorable to reduce CO_2 , methane is the primary end-product (Pester et al. 2012). This interplay between sulfur and carbon cycling has important implications for greenhouse gas production since the rates of gas evolution vary for the two types of interactions, and the GWP of CO_2 and CH_4 are drastically different (Shindell et al. 2009). It is predicted that sulfate reduction in freshwater wetlands will become even more important in the

future as the input of sulfuric acid (thus sulfate) in acid rain increases as a result of higher SO₂ emissions from burning coal and fossil fuels (Pester et al. 2012).

Biofilm: A Ubiquitous Interaction Strategy

One strategy employed almost universally in microorganisms across both domains is biofilm formation, or growth on a surface. The ability to form biofilm is an ancient strategy as evidenced by the diversity of microorganisms that are capable of forming biofilm, including deeply branching phyla, like *Aquificales* (Hamamura et al. 2013). Evidence also exists in the fossil record as early as 3.25 Ga for biofilm streamer communities, like those found in terrestrial hot springs today (Hall-Stoodley et al. 2004). Biofilm cells are often enclosed in a matrix of extra-polymeric substances (EPS) that can consist of carbohydrates, protein, membrane vesicle, nanowires, and extracellular DNA or RNA (Stewart & Franklin 2008; Hall-Stoodley et al. 2004; Flemming & Wingender 2010). EPS production and biofilm formation offer a combination of protective measures and advantages to communities including protection from stresses like pH, UV radiation, salt, dehydration, antimicrobials, and metal toxicity (Hall-Stoodley et al. 2004; Poli et al. 2010). EPS also plays a role in protection from metal toxicity by immobilizing and precipitating metal cations, and this is likely an important strategy for biofilm communities at sites contaminated with metals and metalloids (Halan et al. 2012; Pal & Paul 2008).

Structure-Function Relationship

The structures of biofilms have been studied intensely through the use of high-resolution imaging techniques like confocal laser scanning microscopy (CLSM) combined with staining with fluorescent dyes. An enormous variety of stains are available that differentially stain components of the EPS including lectins for carbohydrates, and nucleic acids stains to visualize cells or extracellular nucleic acids (Flemming & Wingender 2010). Additionally, fluorescent stains that are indicative of membrane integrity (propidium iodide and Syto 9) or respiratory potential (tetrazolium salts like CTC) can be used to determine the physiological state of biofilm cells (Stewart et al. 1994; Stewart & Franklin 2008; Gruden et al. 2003; Ullrich & Karrasch 1999). In addition, fluorescently-labeled oligonucleotide probes are hybridized (FISH) to organisms in biofilm communities with a potential degree of phylogenetic specificity ranging from domain-level to strain-level (Daims et al. 2006; Congestri 2008; Malic et al. 2009; Schaudinn et al. 2009; Thurnheer et al. 2004). This is a powerful tool for examining cell localization in natural communities without the need to culture individuals. FISH is frequently used to characterize structure of biofilms including flocs from wastewater treatment and oral communities associated with teeth surfaces (Zijnga et al. 2010; Guggenheim et al. 2001; Jakubovics 2010; Lee et al. 1999; Juretschko et al. 1998). Microbial ecologists strive to understand the interplay between these observed structures and their effect on community function, and/or predict structural aspects based on function. Advances in molecular-based imaging techniques are allowing resolution of community function at nanometer-scales, and thus single cells, through nanometer scale

secondary ion mass spectrometry (nanoSIMS) and FISH combined with microautoradiography or Raman spectroscopy (Orphan 2009; Milucka et al. 2013; Wagner et al. 2009; Hatzenpichler et al. 2008).

Recent work has shown that the structure of a biofilm community is dependent upon the nature of the interactions (*i.e.*, cooperative or competitive) and that the degree of intermixing of two-member communities is greater when they act cooperatively versus competitively (Momeni et al. 2013). This dependence of structure on function has also been demonstrated in another dual culture system that could be grown in a commensalism or ammensalism. Commensal biofilm cells interacted closely in mixed microcolonies, while ammensals formed separate non-interacting biofilm micro-colonies (Nielsen et al. 2000).

Motility and Chemotaxis

Briefly consider how different your life would be as a sessile being, incapable of movement toward food, or away from danger, and you will quickly understand the advantages a microorganism could incur from being able to move toward a more favorable location. It is intuitive therefore, that nearly all life has evolved a method for moving.

The first description of motility via cellular appendages was made by Anton van Leeuwenhoek in 1675 when he observed the movement of ciliated protozoa (Gibbons 1981). Tactic response were observed as early as 1881 by Theodore Engelmann when he was characterizing photosynthetic response to light of varying wavelengths by observing

the aerotactic response of bacteria toward chloroplasts (Drews 2005). This observation was repeated by Beijerinck in 1893, where he observed aerotactic cells swimming toward the liquid-air interface of a test tube (Adler 1966). Since then, bacterial motility and chemotaxis have been extensively studied, now including a strong interest in archaea (Bardy 2003; Thomas et al. 2001).

The Archaeal “Flagella”

The primary structure for movement in microorganisms, the flagellum, is analogous between all three domains of life, but not homologous. The flagella of Eukaryotes are true to the Latin meaning, “whip”, whereby unicellular members of this domain move via a whip-like motion of their flagellum (*e.g.*, sperm) (Gibbons 1981; Armon et al. 2012). Both the archaeal and bacterial flagella operate, in contrast, via a rotating motor, but that is the end of the similarity. The two structures are so unique that Ken Jarrell (2012) has suggested the name “archaellum” be used to prevent further propagation of the idea that the two structures are similar. The flagellum is made of a hook, rod, and rings; and has a hollow central channel where the primary filament proteins, or flagellins, are secreted via a Type III secretion system and added to a growing distal tip. In contrast, the archaellum is presumably formed from the base by the addition of archaellin proteins, initially as preproteins until the signal peptides are removed by a class III signal peptidase. Genetically, the archaeal flagellum has several homologs to Type IV Pili in bacteria including the similarities in prepilin peptide processing (Jarrell & Albers 2012; Ellen et al. 2010; Ng et al. 2006; Streif et al. 2008; Thomas et al. 2001). The archaellum from *Halobacterium salinarium* has been shown to

be ATP dependent (Streif et al. 2008), in contrast to bacterial flagella that are powered by proton or sodium motive force (Ellen et al. 2010). The driving force for the archaeellar motor has not been determined for any other archaea thus far. Finally, an interesting difference that is rarely discussed explicitly in the literature is the left-handedness of the flagellar helix (with a few known exceptions) and the right-handedness of the archaeellar helix, which presumably arise from the different base and structural proteins (Fu et al. 2012; Thomas et al. 2001).

Recent advances have been made in microscopy setups that allow for monitoring swimming behavior of archaea with more extreme requirements. A thermal-microscope was employed by Herzog and colleagues (2012) to characterize swimming behavior in seven species of archaea, at temperatures up to 95°C. Interestingly, they found that two of the Euryarchaeota in the study, *Methanocaldococcus janaschii* and *Methanocaldococcus villosus* were the fastest organisms studied to date with speeds up to 400 and 500 body lengths per second (bps). This measure factors in an organisms size so that comparisons can be made for speeds of drastically different sized organisms. For example a cheetah runs at 113 km/h, or 20 bps, making these archaea 20 and 25 times faster (Herzog & Wirth 2012). Speed is one strategy that may be important in environments that can experience rapid, drastic changes, and another strategy is directed movement or taxis.

Taxis in Bacteria and Archaea

Archaea tactically respond to a wide range of stimulants including light, acetate, leucine, isoleucine, arginine, betaine, phosphate, and oxygen (Migas et al. 1989; Sment &

Konisky 1989; Scharf & Wolff 1994; Lewus & Ford 1999; Kokoeva et al. 2002; Wende et al. 2009). Despite the difference between archaeal and bacterial flagellar proteins, the system that interacts with the flagella to relay the chemotactic response is homologous between the domains. A two-component signal transduction system interacts with the flagellar switch to change the direction of the motor (Schlesner et al. 2009; Lacal et al. 2010). The motor can turn in one direction, causing the bundle of flagella, or single flagellum to turn together and propel the cell forward in a run, or it can stop or change direction (based on the species) causing the cell to change direction or momentarily pause and be moved by Brownian forces, known as a tumble. Cells normally alternate between runs and tumbles, but in the presence of a chemoattractant, cellular receptors known as methyl-accepting chemotaxis proteins (mcp) interact with the histidine kinase CheA to reduce autophosphorylation activity. This ultimately results in inhibition of autophosphorylation of the response regulator CheY, which in bacteria interacts directly with the flagellar switch FliM (Schlesner et al. 2009; Thomas & Jarrell 2001; Porter et al. 2011). The mechanism of CheY interaction has yet to be described in archaea. Phosphorylated CheY causes the motor to switch more often, resulting in more tumbles, but when autophosphorylation is inhibited by a chemoattractant, the cell experiences more long runs (Masson et al. 2012; Soyer & Goldstein 2011; Terasawa et al. 2011; Porter et al. 2011). This signal transduction system is conserved in *Euryarchaeota*, and the response is the same for any number of chemoattractants. The difference lies in the receptors which are specific for one or a few chemicals and are found either as membrane proteins or inside the cytoplasm (Lacal et al. 2010).

It has been demonstrated that archaeella are required for attachment and presumably biofilm formation in *M. maripaludis*, *Pyrococcus furiosus*, *Methanocaldococcus villosus*, and *Sulfolobus solfataricus* (Ellen et al. 2010; Jarrell et al. 2011; Herzog & Wirth 2012). Given this role, in concert with the role of chemotactic responses in archaea, it can be inferred that archaea have evolved mechanisms of moving toward more favorable locations, and for remaining there. This is an important ecological strategy that allows organisms to maximize available resources in rapidly changing environmental conditions, in the presence of concentration gradients.

Modeling for Microbial Ecology

Four different types of models are described in this dissertation, each for making predictions about some aspect of community interactions. On a whole, modeling is a powerful tool for microbial ecologists to find patterns in large datasets, and to quantitatively compare concepts (Prosser et al. 2007). For example one cannot directly compare the nature of a competition between species of frogs and a competition between strains of yeast by describing how competitors interact on a macro-scale. Competition models, however, can help quantify the nature of the interaction and predict patterns of population growth and stability (Boucher 1988; Stilling 2003). Modeling is therefore a logical accompaniment to microbial ecology-based experiments.

Thesis Objectives

I hypothesized that interactions between cooperating community members would reveal alternative physiological strategies not previously described in pure cultures.

Alternative growth modes (*i.e.*, biofilm and planktonic) were predicted to be important for community level function and ultimately ecosystem service. Through this research I sought to characterize the structure and function of a reduced community (*i.e.* coculture) to gain insight into how interacting community members would affect overall ecosystem function, specifically in terms of electron and carbon flow. While SRB and methanogens are known to interact in different environments, little work has been done to understand these interactions within a biofilm. In more general terms, a simplified system of two populations is the next level of complexity above a monoculture. To accomplish these tasks, a tractable anaerobic biofilm continuous culture system had to be developed with strict gas phase control and monitoring, and sampling methods that would not disturb the system despite low flow rates, relatively slow growth rates, and low biomass yields.

After countless iterations, the modified system resulted in consistent and highly reproducible results that could be used to characterize the community. Chapter 2 is a detailed description of the structure and effects on function observed when the coculture was grown under environmentally relevant nutrient-limited conditions as a biofilm with an associated planktonic phase. This base-case was subjected to two different perturbations, namely, increased loading rate that caused a nutrient excess, and alternatively, removal of the biofilm phase during nutrient limitation. A reaction-diffusion (biofilm accumulation) model (Wanner & Gujer 1986) was constructed and

compared to the experimental results and population distribution and structure was related to function (*i.e.*, methanogenesis).

Observations made during coculture experiments where *M. maripaludis* did not form biofilm in monoculture, and only in the presence of a hydrogen-producing biofilm of *D. vulgaris*, suggested that the methanogen was capable of a chemotactic response toward H₂ gas that had not previously been reported. A second objective of this work was to determine if this response was truly chemotaxis, by observing swimming behavior in the absence of the SRB and in the presence of a controlled hydrogen gradient. The result was the first direct observation of hydrogenotaxis in any domain of life, as described in Chapter 3.

The final objective of this research was to build on the observations made through biofilm coculture and response of *M. maripaludis* to hydrogen, by characterizing the changes in the proteome of these two organisms in response to a range of applicable culturing conditions, via High-Throughput Tandem Mass Spectrometry (HT-MS/MS). Proteomics are a powerful tool for determining the actual state of an organism versus the metabolic potential, as inferred from a genome or even transcriptome. Methods and preliminary results of this work are presented in Chapter 4 of this dissertation.

While the tasks associated with these thesis objectives were being performed, two highly interdisciplinary projects were simultaneously pursued. In Appendix A, the results of a collaborative effort between students and faculty in the Integrative Graduate Education Research Traineeship (IGERT) program are presented. The objective of this project was to develop a community-level metabolic flux model, based on the genomes of

key members of a microbial mat system in Octopus Spring in Yellowstone National Park (Taffs et al. 2009). Appendix B presents the results of a collaboration with Dr. Wenying Shou and Dr. Babak Momeni, who constructed a fitness model that could predict differences in structure of a mixed-community biofilm based on the type of interaction between members (*i.e.*, competitive or cooperative) (Momeni et al. 2013). The structure of the cooperative coculture from this thesis was compared to the model predictions by the collaborators, as an example of cooperation in a less synthetic consortium, and observed to match the predicted arrangement and intermixing patterns. These two projects demonstrate ways of integrating the study of microbial community ecophysiology across disciplines, and the power of modeling as a tool for microbial ecologists. These methods are a common thread throughout this dissertation.

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CHAPTER TWO

BIOFILM GROWTH MODE OPTIMIZES CARRYING CAPACITY OF
INTERACTING POPULATIONS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Developed experimental design, performed experiments, analyzed data, wrote and revised the manuscript.

Co-Author: Laura B. Camilleri

Contributions: Developed experimental design, performed experiments, analyzed data, revised the manuscript.

Co-Author: Matthew W. Fields

Contributions: Developed experimental design, analyzed data, wrote and revised the manuscript.

Manuscript Information Page

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Abstract

Sulfate-reducing bacteria can be syntrophic in the absence of sulfate, particularly with hydrogenotrophic methanogens by the consumption of inhibitory products (*i.e.*, H₂). The presented work uses a dual-culture approach to examine the structure and function of a syntrophic biofilm formed by the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough and the methanogenic archaeum *Methanococcus maripaludis*. Under the tested conditions, monoculture *D. vulgaris* formed biofilm, but monoculture *M. maripaludis* did not. Under syntrophic conditions, a methanogenic biofilm formed and reached steady-state in approximately 7 days. Confocal laser scanning microscopy of intact biofilm confirmed the role of *D. vulgaris* in forming a scaffold for the methanogenic biofilm, while intermediate and steady-state images revealed that *M. maripaludis* was recruited to the biofilm as both single cells and large micro-colonies interspersed throughout the framework of *D. vulgaris*. *M. maripaludis* produced more carbohydrates (uronic acid, hexose, and pentose) as a monoculture compared to coculture biofilm, suggesting an altered carbon flux in the coculture biofilm. In the presence of biofilm, biomass yield per methane produced was increased (2.7-fold) and biofilm community members were shown to be active by staining with the redox dye CTC. The biofilm rapidly responded to an increased lactate loading rate, and subsequently increased the methane production rate (3-fold). The results indicate that the structure of cooperative interactions between a bacterium and archaeum positively impacted the efficiency of lactate oxidation and methane production per unit biomass under nutrient limitation, and the biofilm optimized carrying capacity for both populations in terms of biomass generation and maintenance.

Introduction

Syntrophy, and specifically mutualism, is widespread throughout the biosphere with well-studied examples in and across all three domains of life (Moissl-Eichinger & Huber 2011; Boucher 1988; Gokhale & Traulsen 2012; Sieber et al. 2012; Plugge et al. 2011; Kato et al. 2011). Macroecologists have modeled mutualisms to gain insight into population dynamics using modified Lotka-Volterra equations that describe cooperation rather than competition, with resulting predictions of stability under only very specific density-dependent conditions (Boucher 1988). Mutualisms between different pairs of microorganisms have been cultured in controlled lab conditions, and both macro- and micro-ecological field studies present inherent challenges in repeatability and control over variables (Jessup et al. 2004). Our recent study has shown that cooperative interactions can have specific patterns of growth and population intermixing when compared to other types of associations (*e.g.*, competition) (Momeni et al. 2013). Other experiments with mutualistic microbes have revealed many adaptations to syntrophic life, including alternative electron-transport pathways, increased biomass production, rapid evolution resulting in optimized syntrophic biomass production, and differences in gene expression patterns in the presence of a syntrophic partner (Walker et al. 2009; 2012; Bernstein et al. 2012; Hillesland & Stahl 2010; Lawrence et al. 2012; Plugge et al. 2011; Sieber et al. 2012; Shimoyama et al. 2009). Syntrophic-specific physiology plays an important role in the environment, where organisms experience fluctuations in nutrient availability and stress in which cellular interactions are thought to provide stability. The inhabitants of anaerobic ecosystems, by nature of the metabolisms, often function at the

thermodynamic limit for energy generation and biomass production (McInerney et al. 2009; Kato & Watanabe 2010; Thauer et al. 2008; Bryant et al. 1977). When one metabolism is obligately coupled to another through interspecies hydrogen, formate, or electron transfer, both organisms must often persist by sharing the overall free energy of the reaction (Kato & Watanabe 2010). Little is known; however, about how microbial population structure can impact interactive ecosystem function (*e.g.*, biomass generation versus respiration).

Sulfate-reducing bacteria play crucial roles in many anaerobic environments where they link the carbon, sulfur, and nitrogen cycles via carbon-oxidation and sulfate reduction (Purdy et al. 2002; Canfield et al. 2010) and also contribute to redox gradients of microbial ecosystems via the production of sulfide compounds (Moreau et al. 2010; Xie et al. 2013). Methanogenic archaea are responsible for a vast majority of biogenic methane production and form the basis of most anaerobic environments (natural and man-made) that convert CO₂, H₂, and/or acetate/methyl-groups to methane which can escape back to aerobic environments (van Groenigen et al. 2012; Thauer et al. 2008). The transiently obligate mutualism between these two clades is of special interest because of their crucial roles in anaerobic environments.

It is well accepted that microorganisms can exist attached to surfaces and each other, often surrounded by extracellular polymeric substance (EPS) (Hall-Stoodley et al. 2004; Stewart & Franklin 2008; Gross et al. 2007). Biofilms have been described from countless environments where there is a liquid-surface interface including terrestrial and deep-sea hydrothermal features, riparian zones, ship hulls, metal pipes, contaminated

soils, and the human body (Hall-Stoodley et al. 2004). Much work has been done to identify the driving force and genetic control over biofilm formation through pure culture studies (Stewart & Franklin 2008; Clark et al. 2012; Gross et al. 2007; Perez-Osorio et al. 2010). Additionally, structures of biofilms from several environments have been characterized with confocal scanning laser microscopy (CSLM) using fluorescence *in situ* hybridization and immunofluorescence (Zijnga et al. 2010; Jakubovics 2010; Al-Ahmad et al. 2009; Stams & Plugge 2009; Møller et al. 1998; Hall-Stoodley et al. 2006). Despite the high likelihood that a majority of microbial populations exist naturally as biofilms, little work has been done to understand how the biofilm growth mode affects mutualism, and more specifically how biofilm structure impacts cooperating or competing microbial populations (Nielsen et al. 2000; Bernstein et al. 2012; Brenner & Arnold 2011).

The purpose of this work was to characterize the structure and relative function of biofilm formed by a sulfate-reducing bacterium and a hydrogenotrophic methanogen when cultured syntrophically under environmentally relevant conditions of nutrient limitation. A system was developed for anaerobic continuous culture where biofilm and planktonic growth phases could be monitored together and individually to determine the difference in biomass yield of the mutualism when (i) both biofilm and planktonic growth phases were present (base case), (ii) biofilm was removed leaving only a planktonic phase, and (iii) the loading rate was increased after a period of nutrient-limitation when both growth phases were present.

Materials and Methods

Culture Conditions

Desulfovibrio vulgaris Hildenborough and *Methanococcus maripaludis* S2 were continuously cultured in modified 1L CDC reactors (BioSurface Technologies Corp., Bozeman, MT) for anaerobic biofilm growth. Biofilm coupon holders were modified to hold glass microscope slides cut to 7.6 cm x 1.8 cm. Both monocultures and cocultures were grown in Coculture Medium (CCM) (Walker et al. 2009), a bicarbonate buffered, modified basal salt medium without choline chloride. Monoculture *D. vulgaris* medium was supplemented with 25 mM sodium sulfate, or grown in standard lactate-sulfate medium (LS4D) with 30 mM lactate and 25 mM sodium sulfate as previously described (Clark et al. 2006). Headspace (290 mL) was sparged at 20 mL/min with anoxic 80% N₂: 20% CO₂ for coculture and monoculture *D. vulgaris* or 80% H₂: 20% CO₂ for monoculture *M. maripaludis*, through a 0-20 SCCM mass controller (Alicat Scientific, Tucson, AZ). Reactors were maintained at 30°C with stirring at 150 rpm. The reactor aqueous phase (375 mL) was inoculated with 20 mL of mid-exponential phase planktonic cultures grown from freezer stocks in 40 mL of CCM in 125 mL serum bottles. Fresh CCM in a 20 L glass carboy was continuously sparged with sterile anoxic 80% N₂: 20% CO₂ and supplied at a dilution rate of 0.01 h⁻¹ starting after 48 hours of batch growth. Batch monoculture *M. maripaludis* was grown in Balch tubes in 5 mL of CCM, with 30 mM acetate in lieu of lactate, prepared under 80 % N₂: 20% CO₂, then pressurized after autoclaving to 200 kPa with 80% H₂: 20% CO₂.

Gas Chromatography

Permanent gas measurements were made by automated 250 ms injections of reactor headspace via a 16-port stream selector (Vici-Valco Instruments Co. Inc., Houston, TX) to a 490microGC (Agilent Technologies Inc., Santa Clara, CA) equipped with dual channels and dual thermal conductivity detectors. Molsieve5A and PoraplotQ (both 10 m) columns were run with Helium carrier gas at 145 kPa and 80°C with injectors at 110°C and heated sample line at 40°C. The CDC reactor lids were fitted with stainless steel fittings (Swagelock, Idaho Falls, ID) to accommodate 1/16" PEEK tubing to the stream selector. Scotty calibration gases were used as standards (AirLiquide Specialty Gases, Plumsteadville, PA).

Fluorescence in situ Hybridization (FISH)

Whole biofilm was fixed in 4% paraformaldehyde for 3 hours, then scraped into a well on a Teflon coated slide (Paul Marienfeld GmbH. and Co., Lauda-Königshofen, Germany). Dried biofilm was dehydrated and hybridized in buffer solution containing 180 μ L 5M NaCl, 20 μ L 1M Tris HCl, 449 μ L double deionized (dd) H₂O, 1 μ L 10% SDS and 350 μ L deionized formamide (final concentration 35%) with 3 ng each of probes EUB338 (GCT GCC TCC CGT AGG AGT) double labeled with Cy3 and ARCH915 (GTG CTC CCC CGC CAA TTC CT) double labeled with Cy5 for 4 hours at 46°C in a humid chamber (Stoecker et al. 2010). Samples were washed in prewarmed washing buffer containing 700 μ L 5M NaCl, 1 mL 1M TrisHCl, 500 μ L 0.5M EDTA and raised to 50 mL with ddH₂O, at 47°C for 10 minutes, then dipped in ice cold ddH₂O and quickly dried with compressed air. Samples were mounted with Citifluor AF1 antifadent

(Citifluor Ltd., Leicester, UK) for CLSM. For 3D-FISH, whole fixed biofilm was embedded in polyacrylamide prior to dehydration (Daims et al. 2006).

Confocal Laser Scanning Microscopy

Fluorescently labeled biofilm was imaged using a Leica TCS SP5 II inverted confocal laser scanning microscope with 488, 561, and 633nm lasers and appropriate filter sets for Cy3 and Cy5. Polyacrylamide-embedded whole biofilm (3D-FISH) and fluorescently stained hydrated biofilm were imaged on a Leica TCS SP5 II upright confocal laser scanning microscope using a 63x long working distance water dipping objective.

CTC Staining

Whole hydrated biofilm coupons were removed in an anaerobic chamber and incubated in freshly prepared anoxic 0.05% 5-cyano-2,3-ditolyl tetrazolium chloride solution for 2 h as in Stewart et al. (1994). The reaction was stopped with 5% formaldehyde and rinsed with ddH₂O. Hydrated biofilm was stained with 1 µg/mL DAPI for 20 min in the dark and rinsed with ddH₂O before CLSM.

Results and Discussion

Biofilm structure and composition

Monocultures of *D. vulgaris* formed biofilm on silica slides under continuous culture conditions when sulfate was provided as an electron acceptor in CCM (Figure 1a and c). Monoculture *M. maripaludis* did not form a biofilm on silica slides when grown in continuous culture in CCM supplemented with hydrogen, as observed via protein assay, light microscopy, and scanning electron microscopy (Figure 1b). Material was

observed on the glass slides but was confirmed to be medium salts via energy dispersive X-ray spectroscopy and not protein or carbohydrate (data not shown).

When *D. vulgaris* and *M. maripaludis* were cocultivated in CCM without sulfate and hydrogen, methane was produced and biofilm was formed. The coculture biofilm

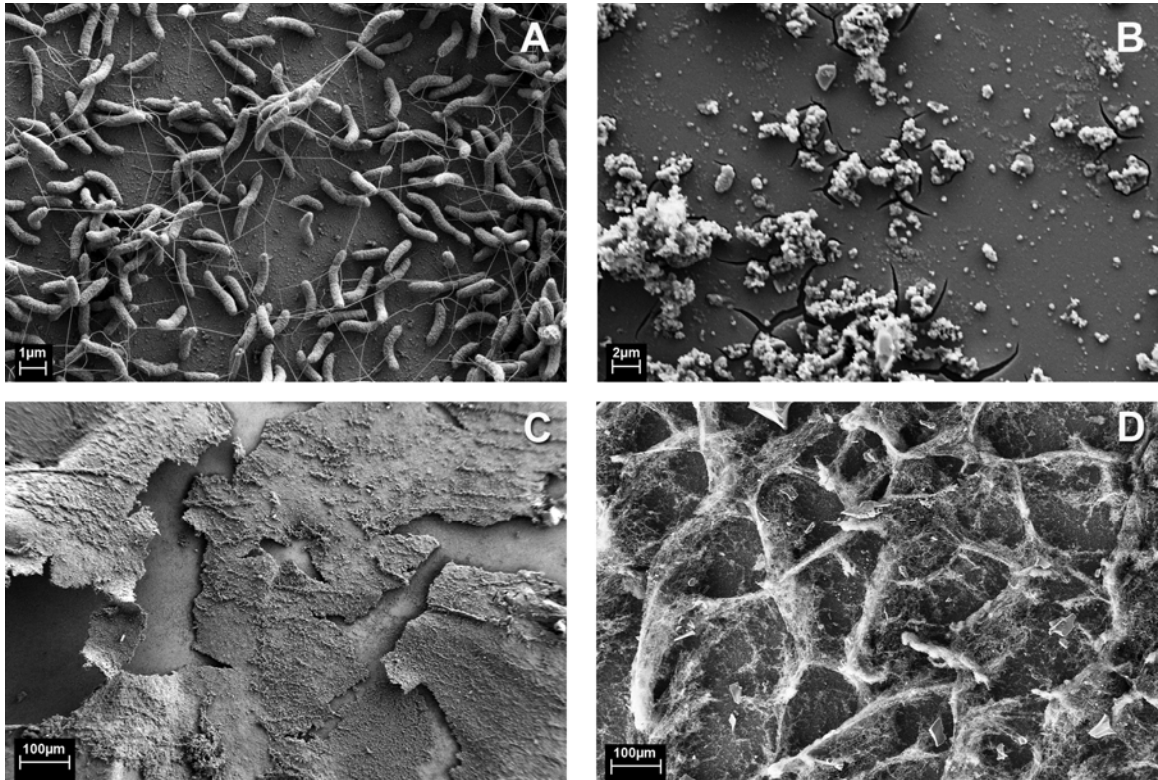


Figure 1. Monocultures of (a and c) *Desulfovibrio vulgaris* biofilm from continuous culture (5 420X and 101X, respectively). (b) Media precipitates on glass slides from continuous culture of *M. maripaludis* (2 810x). (d) Coculture biofilm of *D. vulgaris* and *M. maripaludis* (100X).

had an altered appearance and structure compared to monoculture *D. vulgaris* biofilm (Figure 1c and d) and *M. maripaludis* cells were observed in both the biofilm and planktonic phases. These results indicate that the methanogen was dependent upon *D. vulgaris* under the tested growth conditions to grow in a biofilm state. While it is possible that *D. vulgaris* biofilm merely provided a more suitable surface for attachment

of *M. maripaludis*, it is more likely that hydrogen production attracted *M. maripaludis*. Hydrogen produced by SRB cells in the biofilm also diffuses to the aqueous and gas phases of the reactor, so it is possible for planktonic *M. maripaludis* to scavenge the energy source without joining the biofilm. Based on the abundance of methanogens observed in the biofilm, the data suggest that some benefit is gained by interacting directly or closely with the SRB in the biofilm.

The protein and carbohydrate levels were compared for different growth conditions after cell-associated carbohydrate levels were normalized to biomass. As previously reported, *D. vulgaris* does not produce an extensive carbohydrate-rich biofilm on glass slides (Clark et al. 2007), but in CCM *D. vulgaris* produced slightly increased levels of hexose and pentose equivalents compared to growth in LS4D medium (Figure 2). The uronic acid levels were similar for *D. vulgaris* when grown in LS4D or CCM (Figure 2) under the tested conditions. In the coculture biofilms, the uronic acid levels were similar while hexose and pentose levels were slightly decreased compared to *D. vulgaris* monocultures in CCM. The reported values were lower than previous reports for other monoculture and multispecies biofilms that contained high levels of EPS, which can constitute as much as 90% of the dry mass of a culture (Flemming & Wingender 2010; Poli et al. 2010). Lack of extracellular material might present less mass transfer resistance to hydrogen diffusion and would therefore be beneficial to both organisms. *M. maripaludis* did not form monoculture biofilm under the tested conditions. However, *M. maripaludis* did form a pellicle when grown in static tubes, and under these conditions approximately 10-fold more uronic acid, 7-fold more hexose, and 30-fold more pentose

was produced by the pellicle compared to coculture biofilm (Figure 2). These results suggest that *M. maripaludis* had altered physiology and resource allocation (carbon) in the presence of the SRB biofilm as a result of closer proximity to each other.

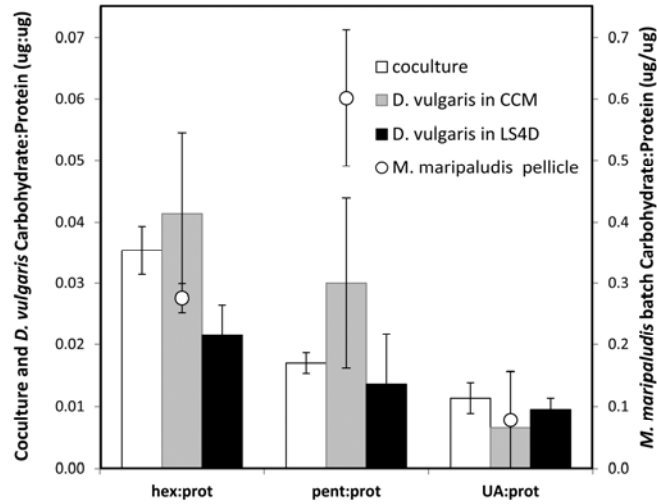


Figure 2. Biofilm carbohydrate composition normalized to biomass for hexose (hex), pentose (pent), and uronic acid (ua) for continuous culture biofilm (bars, primary axis) and batch *M. maripaludis* pellicle (●, secondary axis). Error bars represent 95% confidence interval. Coculture n=6, *D. vulgaris* in CCM n=4, *D. vulgaris* in LS4D slow n=8, *M. maripaludis* batch pellicle n=3.

Coculture biofilm development

The biofilm reactor was inoculated with a coculture that had a cell ratio of approximately 3:1 of *D. vulgaris*:*M. maripaludis* and this is a stable ratio after repeated, batch transfers. Coculture biofilm was initiated by *D. vulgaris*, which formed a monolayer during the initial 48 hours of batch mode in the continuous culture system (Figure 3a and 4a). At this early time point (0 h, initiation of flow) *D. vulgaris* outnumbered *M. maripaludis* in the biofilm 32:1, while the planktonic phase ratios of *D. vulgaris* to *M. maripaludis* were 2.7:1 (Table 1). After 48 hours of continuous culture the biofilm grew in ridges, both normal and parallel to stirring flow (Figure 3b and 4b). Cell

ratios in the biofilm of *D. vulgaris* to *M. maripaludis* decreased rapidly to 3.5:1 after 48 hours as *M. maripaludis* cells were incorporated into the biofilm and grew, while planktonic ratios increased slightly to an average of 3.2:1. After 240 hours, steady-state biofilm cell ratios remained approximately 2.2:1 with similar planktonic ratios of 1.6:1 (Table 1). This is quite different than the published 4:1 ratios observed in a reactor with a planktonic phase only, and our own observation of planktonic-only (Table 1) (Stolyar et al. 2007; Walker et al. 2009; 2012).

	% <i>M. maripaludis</i> Biofilm	% <i>D.vulgaris</i> Biofilm	% <i>M. maripaludis</i> Planktonic	% <i>D.vulgaris</i> Planktonic
Biofilm and Planktonic Early	3% $\pm$ 0.8	97% $\pm$ 0.8	26% $\pm$ 13.7	69% $\pm$ 11.1
Biofilm and Planktonic Intermediate	22% $\pm$ 1.5	78% $\pm$ 1.5	23% $\pm$ 12.1	73% $\pm$ 10.6
Biofilm and Planktonic Steady State	31% $\pm$ 1.4	69% $\pm$ 1.4	36% $\pm$ 10.8	59% $\pm$ 2.2
Biofilm Removed	0	0	13% $\pm$ 1.9	82% $\pm$ 1.9
Increased Loading Rate	18% $\pm$ 1.5	82% $\pm$ 1.5	22% $\pm$ 5.9	73% $\pm$ 3.3

Table 1 Percent of *M. maripaludis* and *D. vulgaris* cells in planktonic and biofilm phases over time in a reactor containing both growth phases, a reactor where biofilm has been removed, and a reactor with an increased loading rate. Error is the 95% confidence interval. The number of images analyzed for each time point is early n=146, intermediate n=269, steady state n=2118, biofilm removed n=140, increased loading rate n=174.

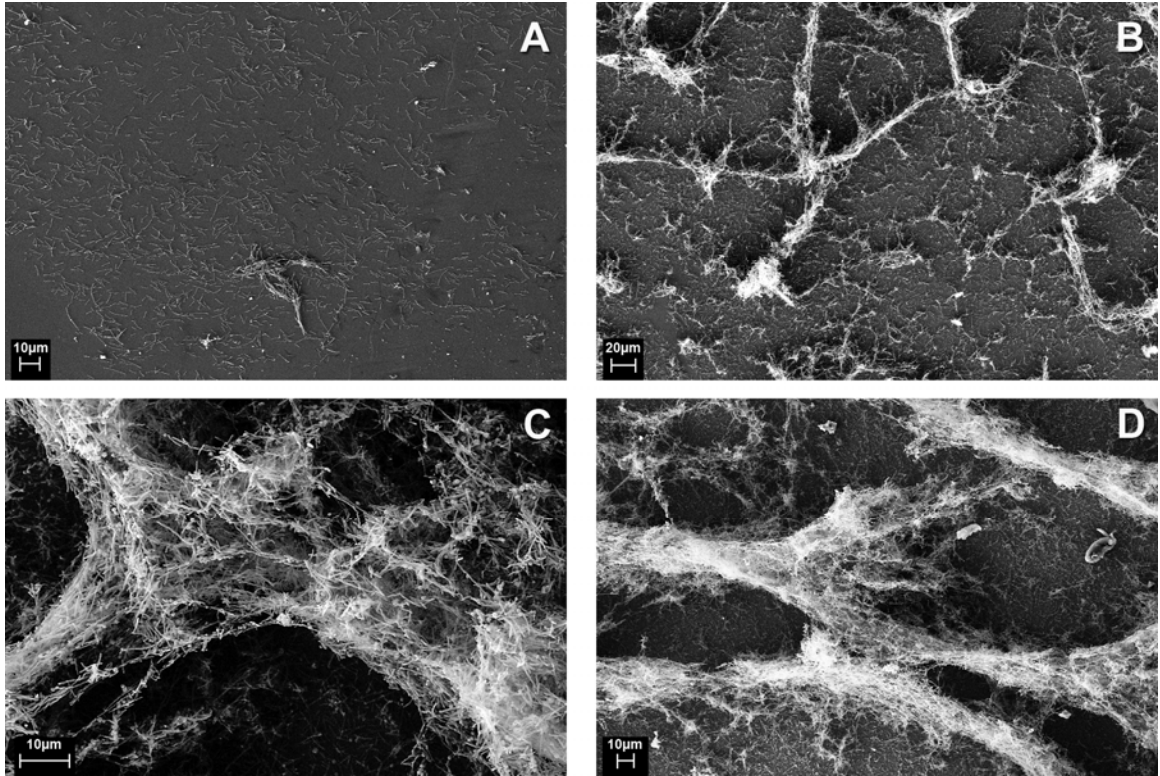


Figure 3. Formation of coculture biofilm over time (a,b,c,d). Electron micrographs of fixed coculture at (a) early (386X, 0 h) (b) intermediate (243X, 48 h) and (c) steady state (1 000X, 240 h) (d) steady state (336X, 240 h) time points.

Coculture biofilm macrostructure was observed with both fixed and hydrated biofilm (Figure 1c, 1d, 3c, and 4c). The structured biofilm included tall ridges and spires with deep valleys, often 300-400 μm tall, but always with at least one dimension not exceeding 50 μm as measured by fluorescence microscopy of intact hydrated biofilm or cryosections of frozen hydrated biofilm (Figure 3c,d and 4c). Notably, this macrostructure was not observed in *D. vulgaris* monoculture biofilms grown in LS4D medium (Clark et al. 2007) nor in CCM (Figure 1a and c). The critical biofilm thickness for diffusion of 30 mM lactate in the reactor system was estimated at 50 μm , as predicted by a 1D biofilm accumulation model (Supplemental Figure 3 and supplemental Table 1).

This result suggests that the multi-species biofilm macrostructure was driven to some degree by lactate mass transport limitation. It is unclear why the coculture biofilm formed spires and ridges and the monoculture did not, but even biofilm at intermediate time points (48 h) already displayed ridges. The macrostructure could have been shaped by a combination of lactate diffusion limitation and biofilm growth in the presence of the methanogen. Similar biofilm structures have been observed in interacting communities and are presumed to be a direct result of interaction type (Nielsen et al. 2000; Molin & Tolker-Nielsen 2003) and such an intermixed structure was recently shown to be a marker of cooperation (Momeni et al. 2013). Future experiments are planned to grow *D. vulgaris* and *M. maripaludis* in a competitive situation (*i.e.*, formate/sulfate; formate/CO₂; sulfate) to determine how a different type of interaction could affect community structure.

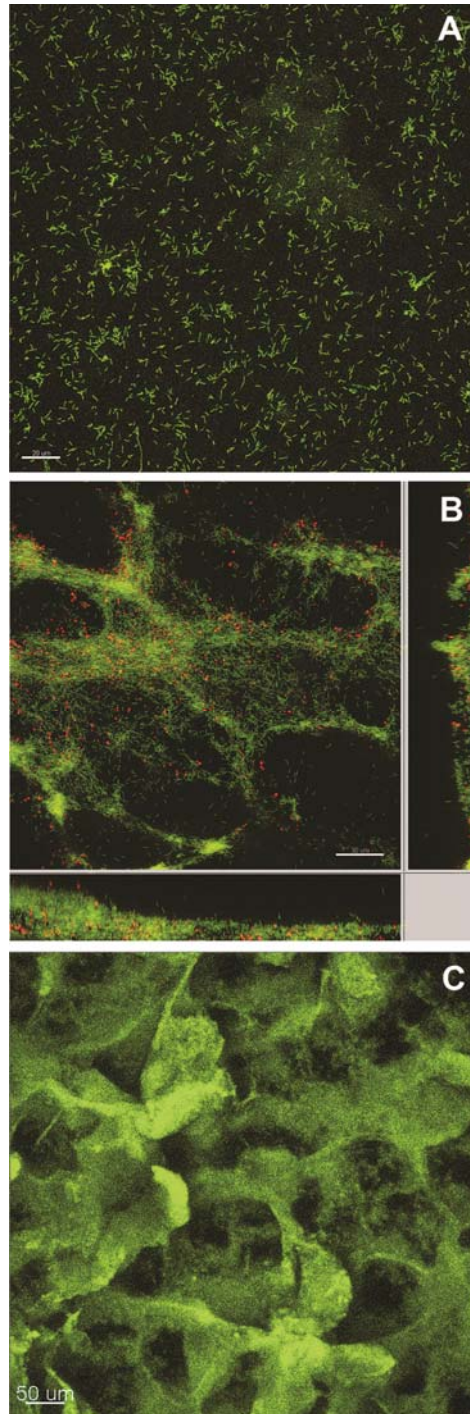


Figure 4 Fluorescence micrographs of coculture biofilm (a and b) embedded in polyacrylamide and hybridized with domain-specific oligonucleotide probes labeled green for *D.vulgaris* and red for *M.maripaludis* at (a) early (0 h) and (b) intermediate (48 h) time points. (c) Intact hydrated biofilm unfixed and stained with Acridine Orange at steady-state (240 h).

Coculture biofilm interactions

A layer of extracellular material was observed by FE-SEM when frozen hydrated biofilm was dried under a vacuum, and likely corresponded to the low level of carbohydrate that was measured (Figure 6). Underneath the layer was an intermixed matrix of *D. vulgaris* and *M. maripaludis* cells in which direct and indirect cell interactions were observed. Cell association was observed to be random (*i.e.*, intermixed), with no pattern of colocalization (Supplemental Figure 1) with microcolonies of *M. maripaludis* cells spread throughout a matrix of *D. vulgaris* cells. Both cell types were observed interspersed as individuals to large colonies throughout the biofilm (Figure 5).

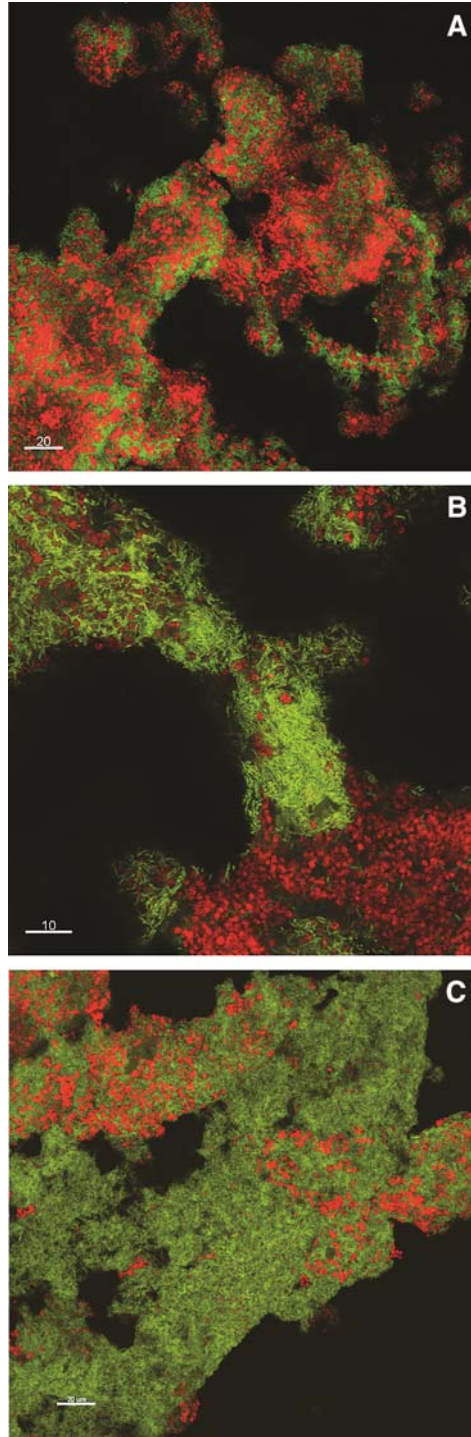


Figure 5. Coculture biofilm cell association (a,b,c) biofilm scraped from the substratum, fixed and hybridized with domain-specific probes for *D.vulgaris* (green) and *M.maripaludis* (red).

Filaments presumed to be flagella and pili were observed on both cell types in scraped biofilm, by TEM (Figure 7b,c) and Atomic Force Microscopy (not shown). In addition to filamentous extracellular structures, cells from the respective populations were also observed to be in direct contact (Figure 7). Structures that appeared to be Gram-negative membrane vesicles were observed in large numbers throughout the biofilm via TEM (Figure 7c). The role of Gram-negative membrane vesicles are becoming increasingly appreciated, and have been hypothesized to play broad roles in microbial communities including interspecies and interdomain communication and redox reactions (Mashburn-Warren et al. 2008; Gorby et al. 2008). More recently, vesicles have been reported in archaea (Šuštar et al. 2012; Ellen et al. 2011), but the potential role of either type of vesicle is not known in multi-species biofilms.

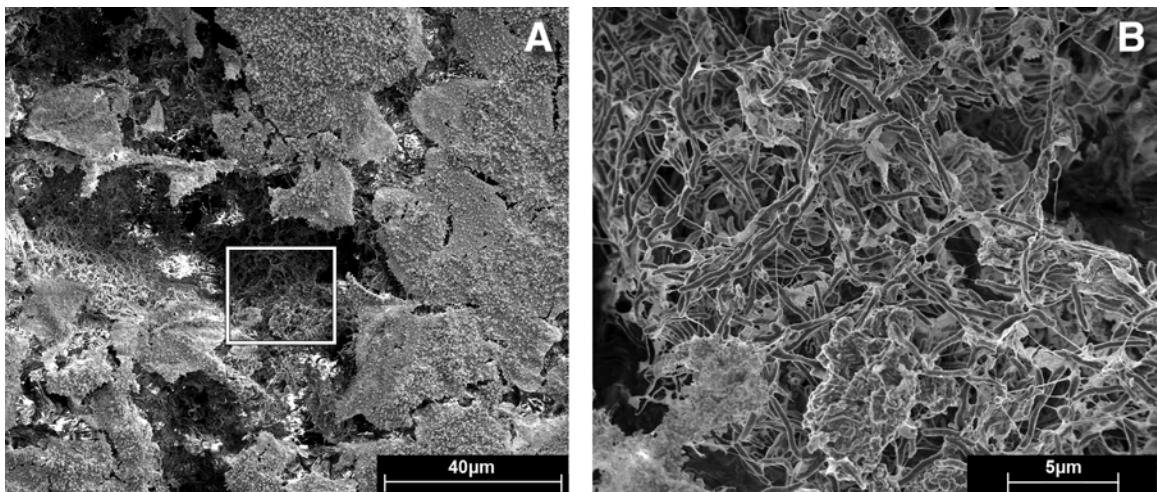


Figure 6. Field emission scanning electron micrographs of coculture biofilm scraped from the substratum (a and b) unfixed and dried under a vacuum by warming to room temperature overnight after being flash frozen while hydrated. (b) Represents 10 000X of white box in (a) 2 000X.

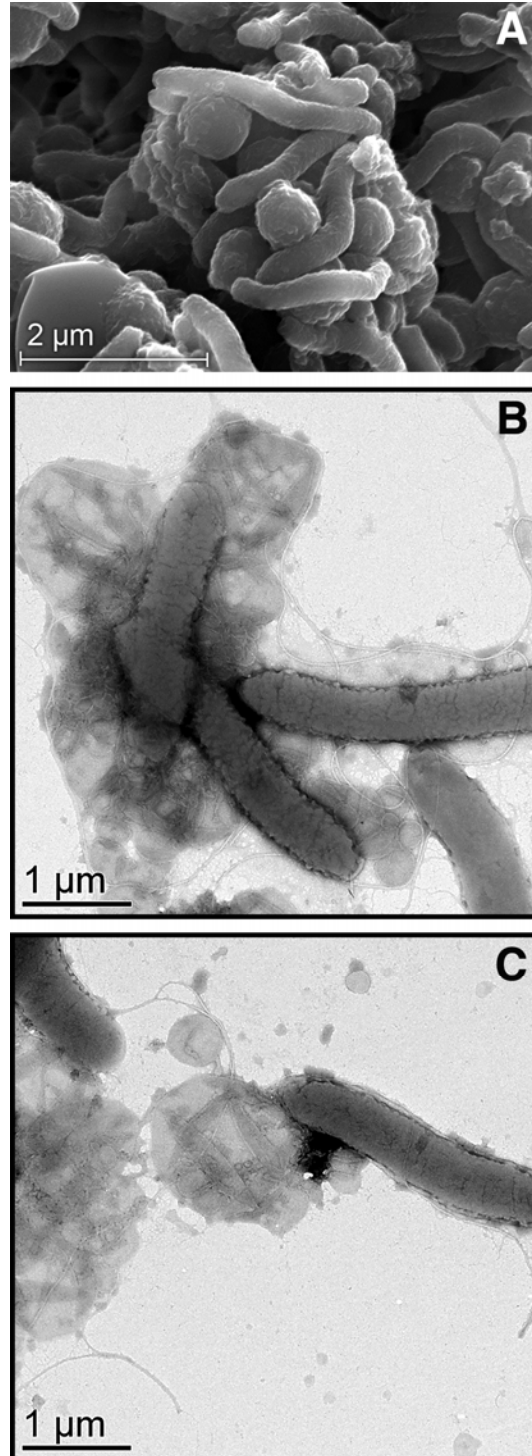


Figure 7. (a) Field emission scanning electron micrograph of fixed and critical point-dried coculture biofilm (b and c) Air-dried whole mount transmission electron micrographs of biofilm scraped from the substratum. Both coculture cell types are visible in addition to appendages and potential vesicles.

Biofilm and planktonic community function: The Base Case

Function and efficiency were determined based on methane and biomass yields, lactate consumption, and community response to perturbation. The base case was a reactor system that contained both a biofilm and planktonic phase, structured as described above. During the first 100 h of biofilm development, methane levels increased as lactate levels declined with an approximate equimolar increase in acetate (Figure 8a). During the increase in methane production and lactate consumption, the biofilm population ratio shifted to consist of 78% *D. vulgaris* and 22% *M. maripaludis* (3.5:1) with similar planktonic population distribution of 3.2:1 (Table 1). When lactate levels were below 5 mM, methane levels peaked around 0.09 mM and declined to a steady-state level of approximately 0.07 mM (Figure 8a). During the first 150 hours, the system approached a steady state where all lactate was consumed, and both organisms in both phases of the reactor increased rapidly in number. The corresponding peak in OD and methane is approximately one retention time of the reactor and occurs when exogenous lactate levels approach zero. These results suggest that biofilm cells were competitive for bulk phase lactate, and the biofilm growth mode may contribute to more efficient multi-species substrate utilization.

During steady-state with regards to methane production, the biofilm structure and composition was consistent with respect to population ratio as well as the carbohydrate to protein ratio. The biofilm remained around 2.2:1 *D. vulgaris* to *M. maripaludis* and the

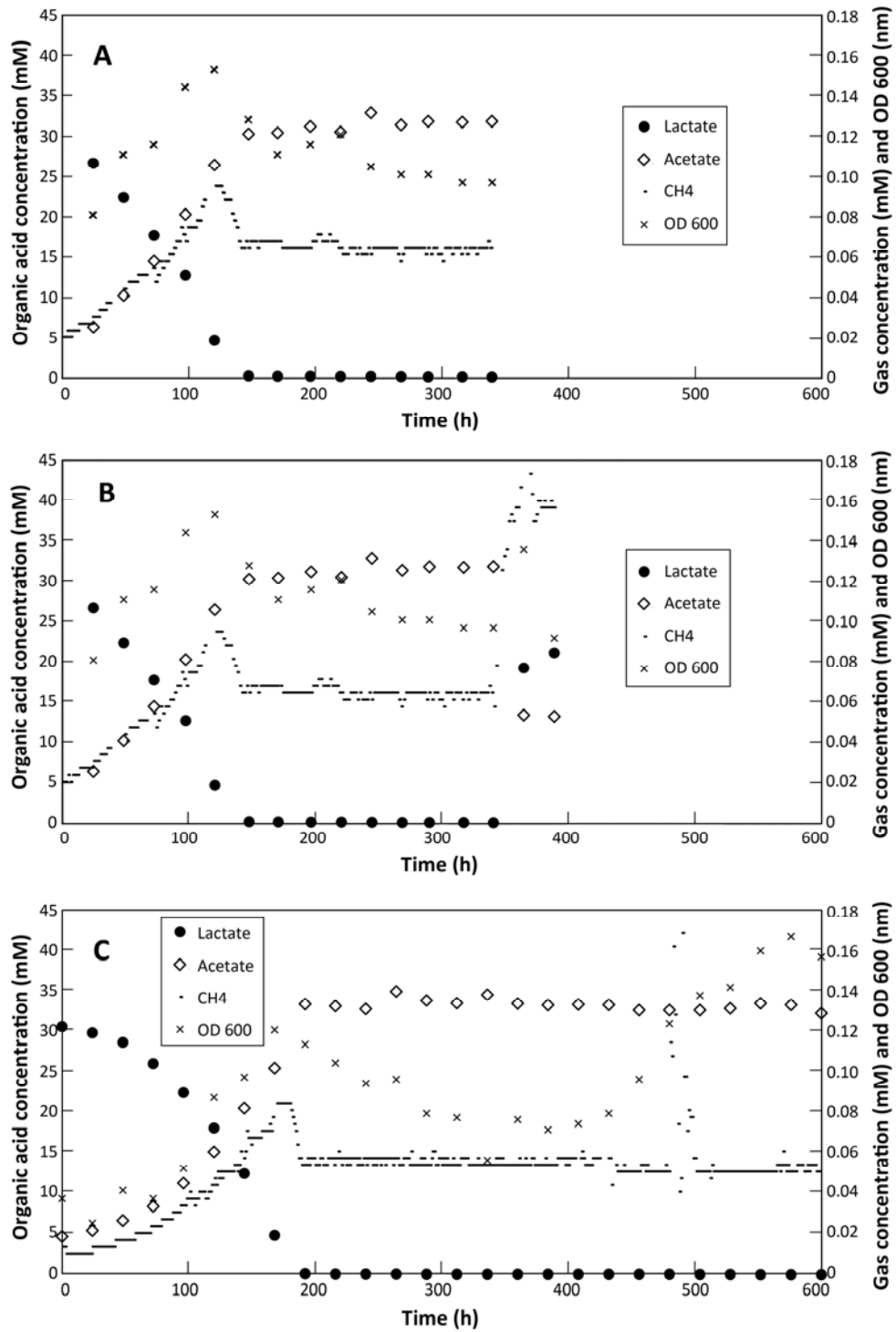


Figure 8. Metabolite concentration over time in coculture biofilm reactors (a) with both biofilm and planktonic phases (Base case), (b) in which loading rate was increased at 341 hours, (c) that had biofilm coupons removed at 432 hr.

planktonic phase was approximately 1.6:1 (Table 1). Hydrogen was not detectable at any point during biofilm formation and growth. Walker (2009) observed a spike followed by a constant low level of hydrogen (less than 10 Pa) at steady state when a planktonic-only system was not limited for lactate. Presumably all hydrogen produced in the biofilm and planktonic reactor was subsequently consumed.

In aerobic biofilms, it has been shown that cells near the substratum can be limited for oxygen with respect to electron donor, and metabolically inactive (Xu et al. 2000). In order to assess the metabolic state of the syntrophic biofilm, an anaerobic incubation of intact 360 h coculture biofilm was done in the presence of CTC (Stewart et al. 1994). The effectiveness of this method for anaerobes has been debated; however, one of the primary concerns is that of abiotic reduction of CTC in the presence of sulfide and cysteine (Stewart et al. 1994; Gruden et al. 2003; Halan et al. 2012). It has been suggested that CT-formazan granules formed abiotically would be poorly localized and would rapidly photo-bleach, while CT-formazan of biogenic origin would be an intracellular granule more resistant to photo-bleaching. CCM contains only 1 mM each of sodium sulfide and cysteine, and biofilm was rinsed prior to staining. In addition, the results were interpreted by direct observation with high resolution CSLM. The incubation showed respiratory potential of the intact biofilm; however, portions of the biofilm were not visible via CSLM most likely due to depth limitations (Figure 10a). When intact biofilm was incubated with CTC, rinsed, and then scraped for visualization, the entire biofilm biomass was stained (Figure 10b and c). In addition, reduced CT-formazan granules could be observed in nearly all individual cells, localized inside the cell with

persistent fluorescence. These results indicate that the entire steady-state biofilm biomass retained respiratory potential under the tested growth conditions and was metabolically active.

The carrying capacity, K , is defined as the maximum potential population size a given landscape is capable of supporting and is a common attribute used to describe population dynamics in ecology (Stilling 2003; Berck et al. 2012). Given faster metabolic rates of microorganisms in both natural and man-made environments, microbial communities are more often studied in the context of function or ecosystem service. Even under lactate-limiting conditions, the biofilm grew and was maintained despite low mass flux of lactate. The results indicate that the biofilm growth mode was advantageous for syntrophic, lactate oxidation to methane, and that both function and carrying capacity contributed to the multispecies biofilm advantage.

Increased loading rate: Effect on biofilm and planktonic community function

When the dilution rate was increased after 341 hours by approximately 6-fold to 0.10 h^{-1} in a biofilm reactor in steady-state, the methane levels increased within one hour and peaked at approximately 0.17 mM after 27 hours (Figure 8b). The optical density increased slightly and then declined to just below steady-state levels on average, with a net 19% decrease of *D. vulgaris* cells and 14% decrease in *M. maripaludis* cells in the planktonic phase, based on cell counts. The system was monitored for four retention times ($RT=9.9 \text{ h}$) and the decrease observed in planktonic biomass is likely a result of washout. The doubling time of *D. vulgaris* in CCM supplemented with sulfate is approximately 20 h while the doubling time of *M. maripaludis* with unlimited hydrogen

in CCM is 5 h. As *D. vulgaris* was washed out of the reactor, hydrogen was not produced at such a rate that would allow the methanogen to divide before the entire reactor was turned over in 9.9 h. This washout situation highlights a complication of mutualistic interaction under flow conditions, where one organism's limiting substrate is produced by another whose specific growth rate is, at best, 4-fold lower. Biomass retention in biofilm and close interaction represent logical ecological solutions to this problem.

The biofilm population structure more closely resembled pre-steady-state population structure with 82% *D. vulgaris* in biofilm, most likely as a result of the increased loading rate for lactate (approximately 3 mM h⁻¹) (Table 1). Despite the altered population ratio, the macrostructure of the biofilm remained similar to the steady-state (lactate loading rate approximately 0.5 mM h⁻¹) structure, a *D. vulgaris* matrix with intermixed *M. maripaludis*. Biomass shifted toward a greater percentage of *D. vulgaris* in both phases with the total *D. vulgaris* biomass in the biofilm increased by nearly 25% from 16.5 mg to 20.0 mg (Figure 9b). However, the total amount of biomass in the reactor did not change with increased loading and it seems that while the planktonic populations began to washout, biomass was replaced by growth in the biofilm (Figure 9b). The carrying capacity thus remained the same, even though the population distribution changed under lactate (carbon and electron) excess conditions.

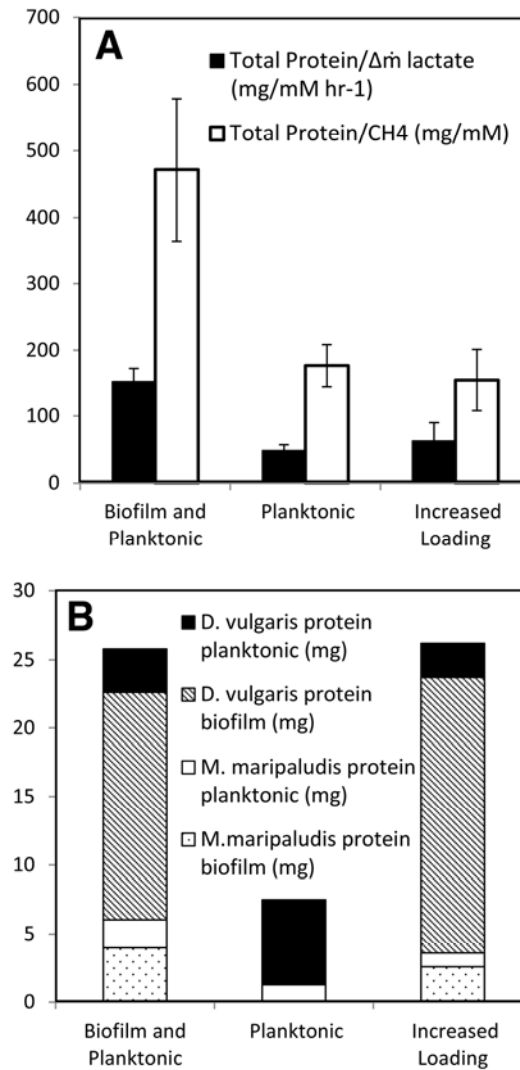


Figure 9 (a) Biomass yield per lactate and per methane for reactors containing coculture with biofilm and planktonic phases (base case) (n=8), planktonic phase only after biofilm was removed (n=14), and running at an increased loading rate (n=2). Error bars represent 95% confidence interval. (b) Biomass distribution of each cell type in biofilm and planktonic phases for the same culture conditions based upon cell volume ratios and cell counts respectively. Unpaired two-tailed t-test results indicate total protein/ $\Delta\dot{m}$ lactate difference is significant between coculture biofilm and planktonic (base case) versus planktonic only ($p < 0.005$) and coculture biofilm and planktonic versus increased loading rate ($p < 0.005$), but that planktonic only versus increased loading rate is not significant ($p = 0.4$). Total Protein/CH₄ unpaired two-tailed t-test results biofilm and planktonic (base case) versus planktonic only ($p < 0.005$) biofilm and planktonic (base case) versus increased loading rate ($p = 0.05$) planktonic only versus increased loading rate ($p = 0.4$).

In spite of lactate excess, hydrogen was still undetectable when the loading rate was increased. This is contrary to published results for excess lactate planktonic only conditions where hydrogen is detected (Walker et al. 2009). These results suggest that the presence of biofilm caused a more efficient transfer and/or utilization of the produced hydrogen gas. A recent study hypothesized that resource allocation and nutrient-use efficiency was a result of distinct growth and uptake activities, and a similar relationship may hold true at higher levels of ecosystem resolution (*i.e.*, interacting populations) (Oskar Franklin et al. 2011).

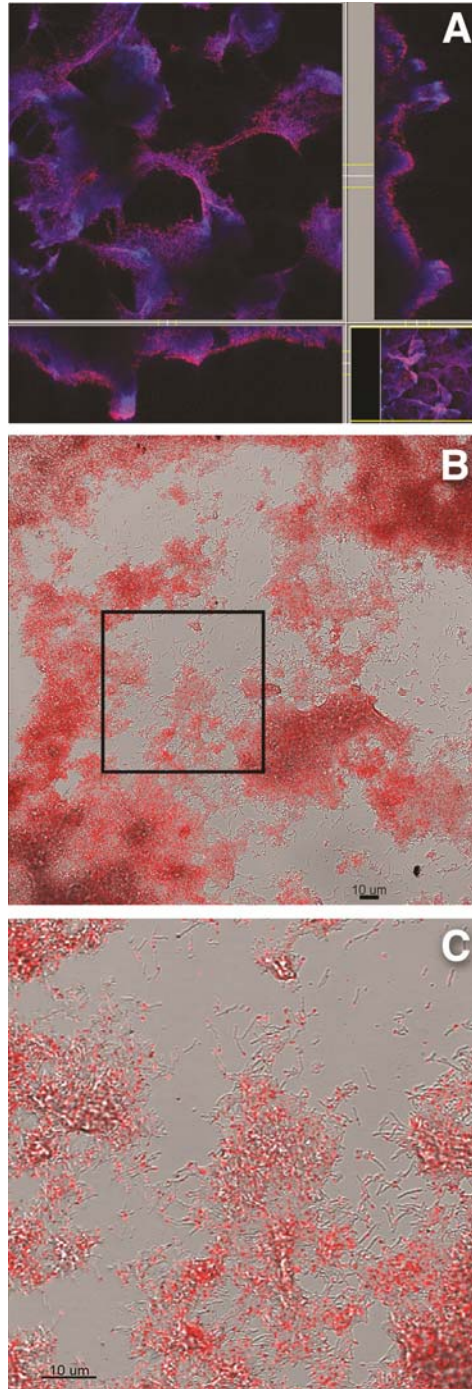


Figure 10. Coculture biofilm incubated anaerobically with CTC. (a) Intact hydrated biofilm stained with CTC and DAPI. (b) and (c) scraped biofilm after CTC staining showing individual grains of red fluorescent CT-formazan in each cell (c) zoomed in from the same field of view in (b).

It is tempting to speculate that the 2.5-fold increase in methane production was specifically from biofilm *M. maripaludis*. While this could not be shown directly, it was observed that approximately 60% overall less *M. maripaludis* biomass produced 2.5-fold more methane when lactate was not limiting. When efficiency is considered in terms of the amount of biomass sustained per methane produced, or per lactate consumed, yields were significantly lower in nutrient excess conditions than in nutrient limited conditions (Figure 9a), and the results demonstrated that the same amount of biomass was capable of being more efficient under nutrient limitation. In addition, an increase in biomass did not cause an equal increase in function (*i.e.*, methane production). These results indicate that the biofilm optimized a carrying capacity that was more efficient and better prepared to respond to nutrient perturbation in terms of both biomass productivity and function.

Biofilm removal: Effect on planktonic community function

In a separate biofilm reactor at steady state, the biofilm coupons were removed after 432 hours (Figure 8c). Upon biofilm removal, the planktonic optical density increased within 24 hours, but methane levels did not increase for 50 hours (Figure 8c). During the 50-hour lag period, methane concentrations appeared slightly lower (an artifact of removing half of the headspace volume that was occupied by biofilm coupon holders), before spiking to approximately 0.16 mM and ultimately declining back to original levels. Interestingly, this spike in methane concentrations corresponds to one retention time ($RT=62.2$ h) in the reactor system. Lactate was not detected during the transition to planktonic-phase only, and the results indicated that similar levels of methane were being produced as the system attempted to reach a new steady-state. The

planktonic-phase only population was 82% *D. vulgaris* but both populations increased in absolute number based on cell counts and OD (Table 1 and Figure 8c). The planktonic only reactor maintained 1.4 times greater biomass than the planktonic phase of the base case (*i.e.*, with biofilm). However, the total reactor biomass was 3.4 times lower than the base case with biofilm. Without a biofilm community, the carrying capacity and stability of the system was significantly reduced.

When considered in terms of efficiency, biomass yield per lactate mass flux was 3 times lower in the coculture reactor with only a planktonic-phase compared to the yield in the base case (*i.e.*, biofilm and planktonic reactor) (Figure 9a). Biomass yield per methane (mg protein/mM methane) was 2.7-fold lower in the planktonic only reactor than the base case (Figure 9a). Therefore, when both biofilm and planktonic growth phases were present and at a steady-state, the coculture reactor ran most efficiently in that the most biomass was supported by the least amount of lactate oxidation and methanogenesis (Figure 9b). This efficiency is likely a result of distribution of function across the community (*e.g.*, more even population distribution) as well as stability incurred by close interactions of syntrophs in biofilm.

In contrast to our reported results for the base case, the planktonic 1.6:1 ratio (*D. vulgaris*: *M. maripaludis*) was not observed in planktonic-only reactors for the same syntrophic pair under lactate excess conditions (Walker et al. 2009; 2012; Stolyar et al. 2007). The planktonic-only reactor in this study reached a 6.3:1 ratio (*D. vulgaris*: *M. maripaludis*) under lactate-limitation. These results indicate that at least 4 times more *D. vulgaris* are typically needed to oxidize enough lactate to generate sufficient hydrogen for

M. maripaludis in the planktonic-phase. In the presence of biofilm, a more even and equal distribution of interacting populations was sustained by increased carrying capacity (*i.e.*, biomass generation and maintenance).

Biofilms help optimize the carrying capacity in terms of equal distribution for both mutualistic populations and biomass yield. It is not surprising that biomass retention (maximized carrying capacity) is a microbial strategy for stability and perseverance under nutrient limitation, given that cell-cell signaling is thought to be biomass concentration dependent, as in the case of quorum sensing (QS) via homoserine lactones (Irie 2008; Allesen-Holm et al. 2006). Several examples of synergistic interactions in biofilm have reported on the importance of QS to biofilm structure (Irie 2008). It was recently demonstrated in *Burkholderia* spp. that secretion of a QS molecule allowed the population to collectively prepare for stationary phase, by triggering a community wide enzymatic pH protection response (Goo et al. 2012). Additionally, QS via acyl homoserine lactones was found to be a strategy of Archaea, specifically the methanogen *Methanosaeta harundinaceae*, to regulate cell assembly and carbon metabolic flux (Zhang et al. 2012). Additionally, in evolved syntrophy, increased biomass production is a rapid result (Hillesland & Stahl 2010; Bernstein et al. 2012). It may be that a biofilm growth mode inherently promotes increased biomass retention and maintenance, and allows microbial communities to optimize carrying capacity to increase stability and resiliency.

Conclusion

When ecosystem function was compared between the biofilm and planktonic cell containing base case reactor at steady state and a planktonic-only reactor, methane production was similar, but total biomass was reduced 3.4-fold. The methane level transiently increased after biofilm was removed, but was not stable and ultimately declined. Methane production in lactate-excess conditions by biofilm and planktonic methanogens together was 2.8-fold greater than the base case with lactate limitation and this elevated methane production commenced within one hour of the increased loading rate. These results suggest a more efficient electron flux from substrate to products (*i.e.*, biomass and methane) in the reactors with biofilm. Taken with the ability of the biofilm to rapidly respond to increases in lactate, and the respiratory potential demonstrated by CTC staining, the biofilm optimizes function when carbon and electron flux are limiting, by maximizing a more even carrying capacity. It is unknown if other forms of mutualistic biofilms would be structured in a similar fashion, although previous work suggests that specific patterns can be expected (Momeni et al. 2013; Nielsen et al. 2000). The presented results show that multi-species biofilm interactions promoted increased and even carrying capacity, and not necessarily increased function (*i.e.*, methanogenesis). Additional work is needed to understand how specific microbial biofilm interactions can impact meso- and macro-scale processes including greenhouse gas production, biogeochemical cycling, and waste conversion.

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Supplementary information is available at ISME Journal's website.

Supplemental Methods

Cell Counts and Biofilm Relative Abundance

1 mL of planktonic phase was fixed in formaldehyde (final concentration 2%) overnight then diluted as necessary and stained for 20 min in the dark with an equal volume of filtered 0.3g/L Acridine Orange. Stained samples were collected through a filter chimney on a black polycarbonate track-etched isopore filter (EMD Millipore Corp., Billerica, MA) and imaged on a Nikon Eclipse E800 microscope with a mercury bulb for fluorescence. At least ten random fields of view were analyzed and cells were counted via integrated morphometry analysis in MetaMorph version 7.6 (Molecular Devices, Sunnyvale, CA).

Biofilm relative abundance was determined using thirty CLSM images per sample, captured from random locations in x, y, and z planes. MetaMorph was used to measure the thresholded area of the two channels in each image.

Protein Normalization

One *M. maripaludis* cell and one *D. vulgaris* cell are not the same shape or volume, so average biomass per cell was determined using monocultures. Biological duplicates of each monoculture were grown to late exponential phase in 125 mL serum bottles. One portion was filtered and dried to determine dry weight per cell. The Lowry protein assay was done in triplicate on each culture to determine biomass weight per volume. Additionally cells were fixed and stained for counting as described above. Twenty fields of view were analyzed for each culture to determine cell number per volume and area per cell using MetaMorph version 7.6 software (Supplemental Figure 2).

1-D Biofilm Accumulation Model

Diffusion in the biofilm was modeled using a Biofilm Accumulation Model (BAM) (Wanner & Gujer 1986) to predict effects of biofilm thickness, inlet substrate concentration, and volumetric flow rate on methane production and cell ratios. Input parameters are listed in Supplemental Table 1. Rate coefficients for substrates were $K_s = 1$ while stoichiometric coefficients were $1/\text{yield}$. Yields were calculated based on Gibbs Free Energies for the associated half reactions normalized to one electron. Aqueous diffusion coefficients (D_{aq}) at 25°C for substrates (Stewart 2003) were corrected to 30°C using $D_{30}/D_{25} = 1.135$. D_{aq} of lactate was calculated as in Wilke and Chang (1955):

$$\frac{D_L \mu}{T_{abs}} = \frac{-7.4 \times 10^{-8} (XM)^{0.5}}{V_b^{0.6}}$$

BAM allows for input of a ratio of the effective diffusion coefficient to the aqueous diffusion coefficient (D_e/D_{aq}) which is then applied to all solutes to account for the decreased diffusion observed in the biofilm matrix compared to water.

Electron Microscopy

Micrographs in Figures 1 and 3 were collected on a Zeiss Supra55VP FE-SEM. Biofilm was fixed in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde and 0.05M Na-cacodylate overnight at room temperature. Coupons were rinsed and stepwise dehydrated in ethanol before being cut and critical point dried on a Samdri-795 (tousimis, Rockville, MD). Glass pieces with dry biofilm were mounted on SEM stubs with double-sided carbon tape and silver then splutter coated with Iridium for 35 s at 35mA.

Micrographs in Figures 6 and 7 were unfixed biofilm scraped directly onto double-sided carbon tape, frozen while hydrated in liquid N₂, sputter-coated with Platinum for 2 min, and imaged using a dual beam focused ion beam (FIB)-FE-SEM (Helios NanoLab, FEI Company, Hillsboro, OR) equipped with a cryostage.

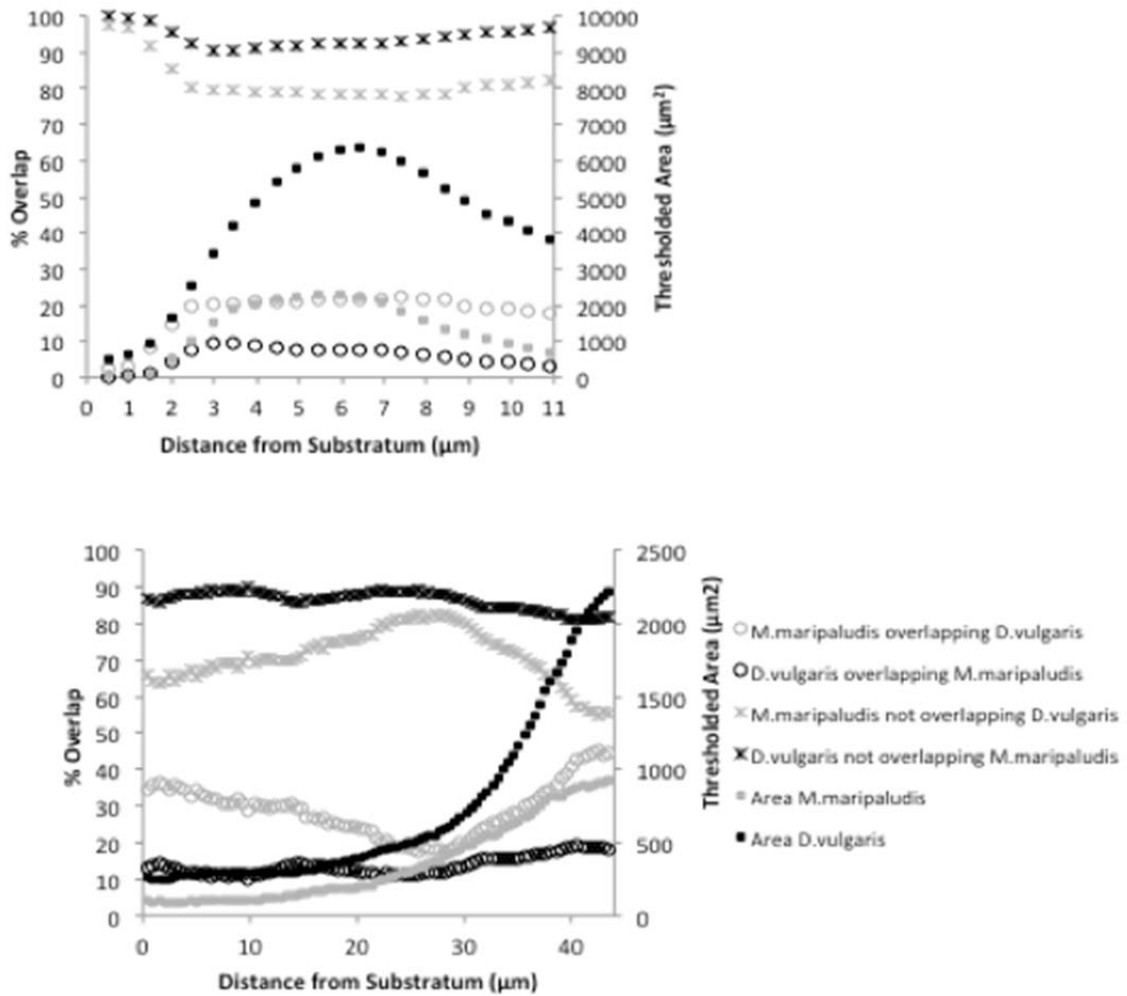
Biofilm for whole mount TEM was rinsed in phosphate-buffered saline and scraped into 1 mL ddH₂O. The sample was gently dispersed by pipetting up and down five times, and then mounted on a slotted TEM grid. Excess liquid was wicked away, and the mount was stained with Nano-W (Nanoprobes, Yaphank, NY) for 30 s. The grid was imaged on a Tecnai T-12 cryo-transmission electron microscope (FEI Company, Hillsboro, OR).

Planktonic Phase Sampling

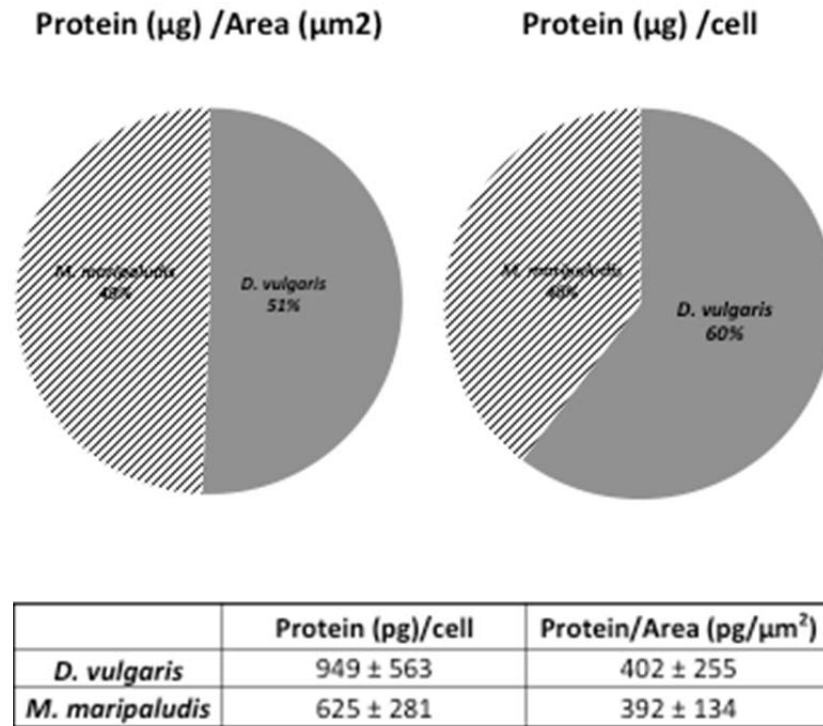
Reactor outflow was collected on ice and measured daily to monitor flow rate. Samples of the planktonic phase were collected for optical density at 600nm (OD), high performance liquid chromatography (HPLC), protein measurements, and direct cell counts. Filtered samples were analyzed in triplicate with a fucose internal standard, for lactate, acetate, and formate concentrations via HPLC (Agilent 1200 series) equipped with a BioRad Aminex HPX-87H column. Lactate and formate concentration were measured with a VWD detector while acetate concentration was measured with an RID detector. Protein concentrations were determined with the Lowry assay using bovine serum albumin as the standard (Lowry et al. 1951). Hexose sugars were measured by the colorimetric cysteine-sulfuric acid method with glucose as the standard. Pentose sugars were measured with a colorimetric orcinol-FeCl₃ assay with xylose as the standard. A

colorimetric carbazole assay was used to measure uronic acid concentration with D-galacturonic acid as the standard (Chaplin & Kennedy 1994).

Supplemental Figures and Legends to Figures



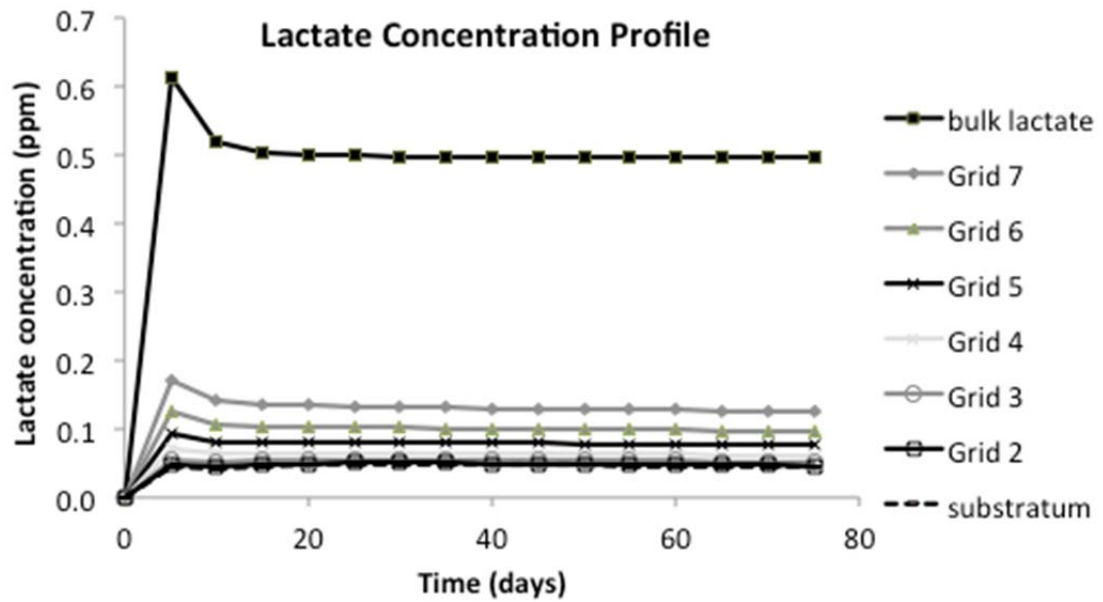
Supplemental Figure 1. Percent of *D. vulgaris* and *M. maripaludis* cells overlapping (O) and not overlapping (*) (primary axis) and total thresholded area of each (□) (secondary axis) in a vertical section of (a) steady state (b) intermediate (48 h) coculture biofilm.



Supplemental Figure 2. Average protein per cell and per thresholded area for monocultures of *D. vulgaris* and *M. maripaludis* and 95% confidence interval. These ratios were used to adjust coculture planktonic protein amounts per organism based on cell counts.

Area	.0591 m ²
Volume	3.5 x 10 ⁻⁴ m ³
Volumetric Flow Rate	.000126 m ³ day ⁻¹
$\mu_{\max}$	.72 day ⁻¹
S _{lactate}	2702.24 g/m ³
S _{CO2}	5000g/m ³
De lactate	1.83 x 10 ⁻⁵ m ² /day
De CO ₂	1.13 x 10 ⁻⁴ m ² /day
De H ₂	2.65 x 10 ⁻⁴ m ² /day
De CH ₄	8.76 x 10 ⁻⁵ m ² /day
Cell Growth	First Order
Substrate consumption	First Order
Substrate production	Zero Order

Supplemental Table 1. Input parameters for Biofilm Accumulation Model.



Supplemental Figure 3 Lactate concentration (ppm) predicted by the Biofilm Accumulation Model in the aqueous (bulk) phase and at multiple depths in the biofilm, including the bottom (substratum).

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CHAPTER THREE

TAXIS TOWARD HYDROGEN GAS BY *METHANOCOCCUS MARIPALUDIS*Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Developed experimental design, performed experiments, critically evaluated the model, wrote and revised the manuscript.

Co-Author: James M. Connolly

Contributions: Developed experimental design, performed experiments, created the model, wrote and revised the manuscript.

Co-Author: Carey Downey

Contributions: Performed experiments and revised the manuscript.

Co-Author: Robin Gerlach

Contributions: Developed experimental design, critically evaluated the model, and revised the manuscript.

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Contributions: Developed experimental design, critically evaluated the model, and revised the manuscript.

Manuscript Information Page

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Abstract: It has been hypothesized that motile microorganisms are capable of taxis toward H_2 , but this has yet to be directly observed. Knowledge of taxis in the *Archaea* is rapidly expanding through identification of novel receptors, effectors, and proteins involved in chemotaxis signal transduction to the flagellar motor. Through the use of a modified capillary assay with anoxic gas-phase control, we report that the average swimming velocity increases in the direction of a H_2 source for the methanogen *Methanococcus maripaludis*. These results were fitted to a Keller-Segel chemotaxis model by estimating three parameters, namely the ligand-receptor dissociation coefficient (k_d), random cell diffusion coefficient (μ), and chemotactic coefficient (χ). The results indicate that *M. maripaludis* couples motility to H_2 concentration sensing - hydrogenotaxis.

Main Text:

Hydrogen (H_2) is a crucial substrate for methanogens as well as a common source of electrons for other organisms in anaerobic environments. Biological methane (CH_4) production from H_2 and carbon dioxide (CO_2) contributes to greenhouse gas emissions and is possibly one of the oldest microbial processes (1). *M. maripaludis* is an anaerobic archaeum that can use H_2 or formate as electron donor to reduce CO_2 to CH_4 and is considered a model mesophilic methanogen. Recently, the swimming behavior of *M. maripaludis* was described as long runs interrupted by slight changes in direction (2), but chemotactic responses have not been shown. Chemotaxis has been demonstrated for other archaea, including two methanogens (3, 4) and *Halobacterium salinarum*. The signal transduction system for chemotaxis in *Archaea* is similar to that of *Bacteria*, where an effector interacts with a cellular receptor (either membrane-spanning or cytoplasmic) which results in a signal cascade that affects the flagellar motor (5, 6). In *Bacteria*, phosphorylated CheY interacts directly with the flagellar motor switch acting either as a molecular brake, momentarily stopping the motor and allowing Brownian motion to randomly affect the position of the cell, or a molecular clutch, changing the motor direction and causing the cell to tumble or reverse direction, dependent on the organism. The control over the flagellar motor has the same result and variability for *Archaea*; however, the switch is not the same and none of the archaeal flagellar proteins have homologs to bacterial flagella proteins (5, 7, 8).

Chemotaxis has been the subject of many mathematical models and the majority have concentrated on reproducing the population-level observation of migrating bands of high cell concentration in swarm plates and capillary experiments (9). Pioneering work in modeling chemotaxis behavior was done by Keller and Segel in 1971 and continues to be the basis of most common continuum models (10). In one dimension, with x being the space variable, the Keller-Segel model can be described as a flux, J , such that

$$J = -\mu \frac{\partial b}{\partial x} + \chi(s)b \frac{\partial s}{\partial x} \quad (a)$$

where μ is the cell diffusion coefficient that takes random, non-directed, movement of cells into account. b is the microbial population density, s is the attractant concentration and $\chi(s)$ is the non-constant chemotactic coefficient. The population flux, J , can be differentiated to yield the more common form

$$\frac{\partial b}{\partial t} = \frac{-\partial J}{\partial x} = \frac{-\partial}{\partial x} \left[-\mu \frac{\partial b}{\partial x} + \chi(s)b \frac{\partial s}{\partial x} \right] \quad (b)$$

and the average cell swimming velocity, v , is calculated by dividing the flux by the population density, or $v = J/b$. Lapidus and Schiller (11) proposed a form of $\chi(s)$ such that

$$\chi(s) = \chi \frac{k_d}{(k_d + s)^2} \quad (c)$$

where χ is the constant chemotactic coefficient and k_d is the receptor-ligand binding dissociation constant. The Lapidus-Schiller $\chi(s)$ term, and variations thereof, have been used widely to describe chemotaxis in bacteria (12).

The goal of the present study was to subject *M. maripaludis* cells to a H_2 concentration gradient and compare swimming behavior to model predictions. Attractant (H_2) transport was modeled by Fickian diffusion and consumption by the population was modeled in the Michaelis–Menten form such that

$$\frac{\partial s}{\partial t} = D \frac{\partial^2 s}{\partial x^2} - \frac{r_{\max} bs}{(k_m + s)} \quad (d)$$

where D is the diffusion coefficient for H_2 , $r_{\max}$ is the maximum consumption rate, and k_m is the half-saturation constant. A modified capillary assay was used in which cells were loaded into a gas-tight anaerobic capillary, and a valve allowed controlled exposure to a H_2 gradient. In traditional experiments, cells are external to the capillary and will enter in the presence of a chemoattractant (13). Here, microscopic swimming behavior of cells inside the capillary was monitored for changes upon exposure to H_2 or an Ar control. Cell movement was measured using time-lapse confocal laser scanning microscopy (CLSM) in the center of the capillary, 0.5 cm from the gas phase (figure S1, supplemental methods). At that point, H_2 concentration would reach the threshold of 10-30 μM at which hydrogenotrophic methanogens are competitive in the environment (14) on a time scale appropriate to the experiment, but not immediately. The diffusion model predicted these concentrations would be reached within 10-15 minutes (figure 1).

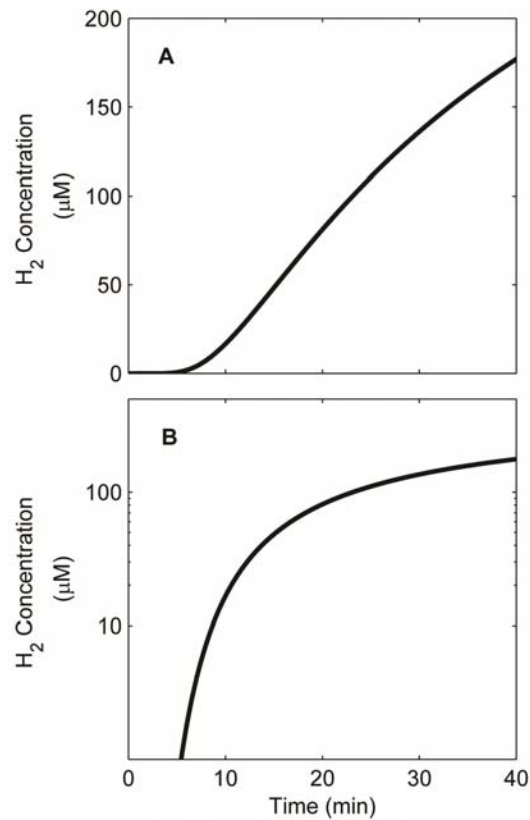


Fig. 1. The predicted hydrogen concentration over time at the observation point 0.5 cm from the gas phase over the course of the experiment shown with **(A)** linear axes and **(B)** log-linear axes. The 10-30 μM threshold is reached at approximately 10 minutes.

When *M. maripaludis* cells were exposed to H_2 , the average swimming velocity was positive toward H_2 within ten minutes (figure 2A). Biased random walk was not observed when cells were exposed in the same way to an Ar gradient (figure 2B), nor was swimming velocity affected normal to the H_2 or Ar gradients (figure 2C and D). A chemotactic response was only observed when cells were first starved of H_2 for 4-5 hours, while cultures that had not been starved did not show an increase in biased swimming (figure S2). This was also observed in *Rhodobacter sphaeroides*, where well-fed cells exhibited weaker chemotactic responses than starved cells (15).

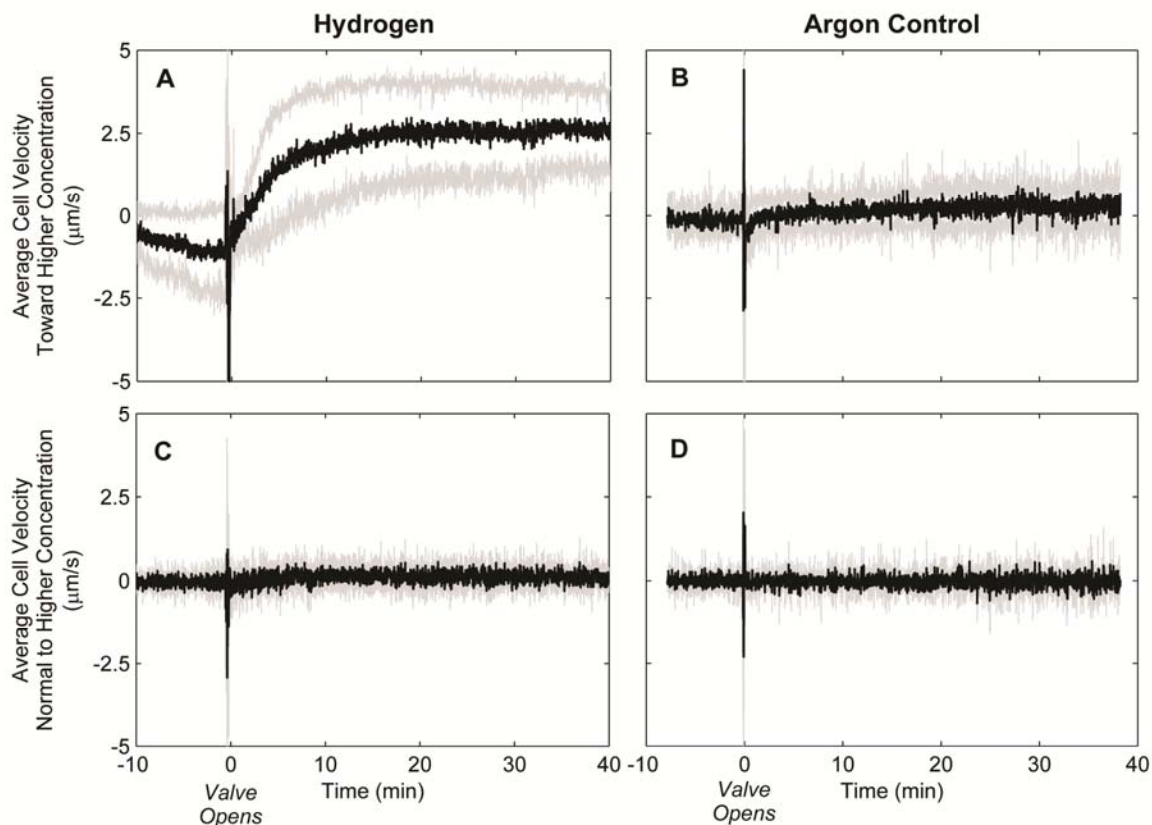


Fig. 2. Average cell velocity (black) with 95% confidence interval (gray) before and after opening valve to gas phase for (A) H₂ and (B) Ar control. Positive y-axis values indicate movement toward the gas phase, negative y-axis values indicate movement away from the gas phase. Average cell velocity (C) and (D) normal to concentration gradient (n= 5 for H₂ and n=3 for Ar).

The starvation period (4-5 h) that was required to induce a tractable response to H₂ was not long enough to cause loss of motility, as in starvation of *H. salinarum* (16). The average swimming speed of starved cells was slightly lower before exposure to H₂ at 2.1 μm s⁻¹ (figure 3A), and increased slightly after exposure to 3.1 μm s⁻¹ equal to non-starved cells and similar to previously reported speeds of 2.5 ± 3.4 μm s⁻¹ (2). Maximum swimming speed averages (calculated from average of maximum speed at each time point) were highly variable between time points, and the average maximum was slightly higher for non-starved cells and lowest for starved cells before H₂ exposure (figure 3B). The absolute maximum observed speed was 91 μm s⁻¹ (figure 3B inner boxes), approximately twice the previously observed maximum speed of 45 μm s⁻¹ (2).

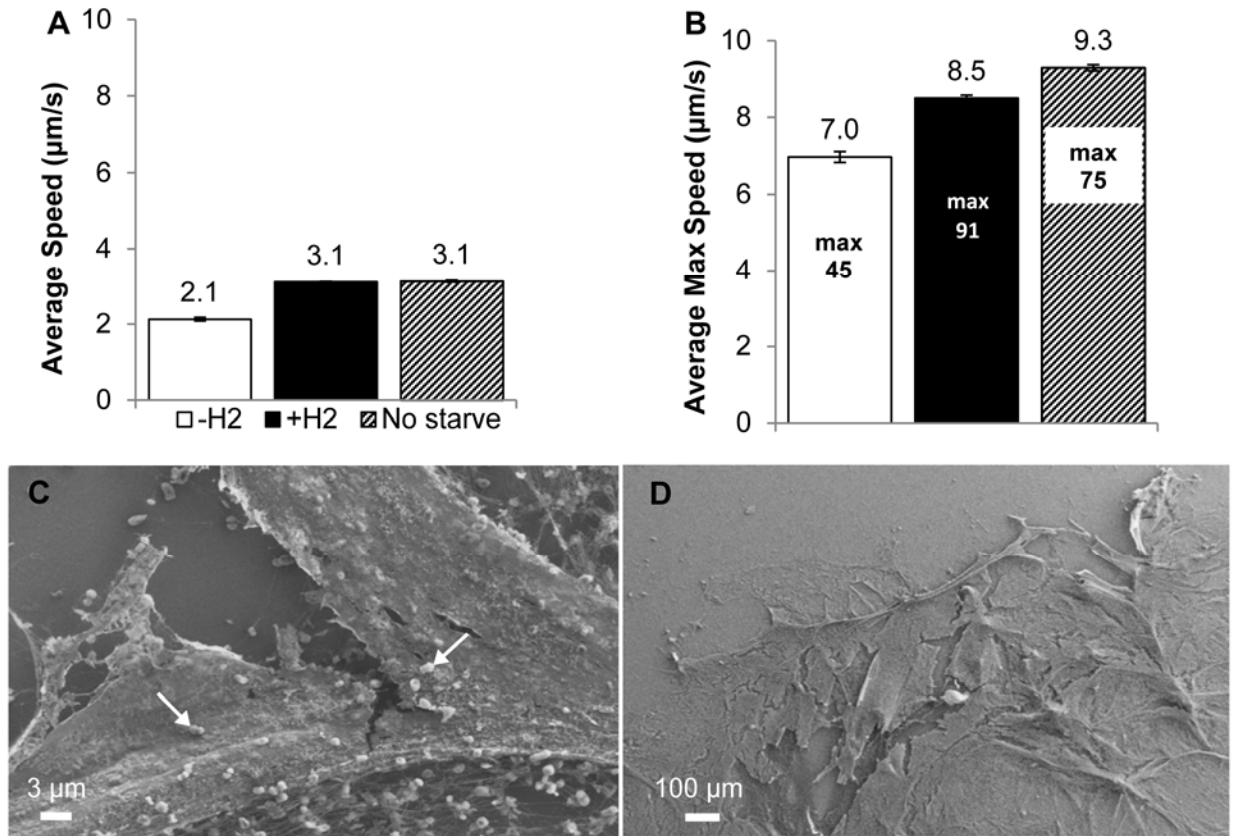


Fig. 3. Swimming speed of *M. maripaludis* cells when starved for 5 hours before H₂ exposure (-H₂ and n=4,725) after H₂ exposure (+H₂ and n=20,189), and when not starved (n=45,001). Values represent the mean and error bars represent 95% confidence interval. **(A)** Average swimming speed. The difference between -H₂ and both +H₂ and no starvation is significant (p<0.05). **(B)** Average maximum swimming speeds are represented by bars, and absolute maximum values inside each bar represent the highest observed speed over all time points for the condition. Differences between all conditions are significant (p<0.05) and p values were calculated with two-tailed t-tests. **(C)** and **(D)** Field emission scanning electron micrographs of *M. maripaludis* pellicle with extracellular material and cells (indicated by arrows in (C)).

In this capillary assay, cells were not observed to accumulate in bands despite the chemotactic response observed. Typical chemotactic bands observed in capillary and swarm plate assays result from cell accumulation as a net result of a biased random walk. Migration of a band then occurs as cells consume the substrate and move collectively to higher concentrations (9). Although there was no bulk flow in the system on a macro-scale, and the cell suspension meniscus did not move during the experiments, it is possible that micro-scale convective forces could prevent cells from accumulating. Thermal convection may have been induced either from focused energy input from the microscope laser or from slight environmental temperature fluctuation. The collective motion of swimming cells may have had an effect on convective flow, and previous work estimates that a force of 0.5 pN is exerted by a cell swimming at 25 μm s⁻¹ (17); however,

the magnitude of this effect was not investigated here and remains poorly understood. The interface between polar and nonpolar fluids (water and gas) has also been shown to cause long range repulsion of charged colloidal particles (18), and presumably net negatively charged microbial cells. It is unknown if charges present at the liquid/gas interface were important to this work but it is a possible explanation as to why cells did not accumulate at the interface where the highest H_2 concentration was predicted. Alternatively, the modification of this capillary assay to start with a homogenous cell suspension inside the capillary, rather than allowing cells to enter the capillary or not, may have changed the nature of the response. It is also possible that there are factors involved in chemotactic band formation that are different between organisms or specific to hydrogenotaxis (*i.e.*, swimming mode, adaption response, quorum sensing).

A banding phenomenon was observed on a larger scale when *M. maripaludis* batch cultures were grown statically with H_2 . Under these conditions a pellicle formed at the gas-liquid interface (figure 3C, D). This is a similar observation to the one made by Beijerinck in 1893, where he observed aerotactic cells swimming toward the meniscus of a test tube (9). When cultures of *M. maripaludis* were grown statically with the soluble electron donor formate, cells grew throughout the liquid medium (not in a pellicle) and had less cell-associated carbohydrate than H_2 -grown pellicle cultures (figure S4). These results suggest that extra-polymeric substance (EPS) production is important for the formation of a pellicle in response to a H_2 gradient and may require a time-scale longer than used in the capillary experiment.

The modified Keller-Segel model was used to quantify and compare chemotactic responses. A ligand-receptor dissociation constant (k_d) has not been determined experimentally for H_2 , and the receptors remain unknown. The k_d value was varied across the range of published values for other effectors and receptors, and the chemotactic coefficient (χ) and random cell diffusion coefficient (μ) were kept constant at average literature values (table S1). The best fit for average swimming velocity was obtained with a k_d value of 0.70 mM, with 0.30 and 2.30 mM corresponding to the 95% confidence interval of the data (figure 4A). This range for k_d is similar to that observed for *Escherichia coli* AW405 to α -methyl aspartate (19). It is; however, quite different from values reported for *Bacillus subtilis* receptor affinity to O_2 (0.0015 and 0.075 mM for the high and low affinity of the receptor, respectively), which is the only previously reported k_d for a gas (20).

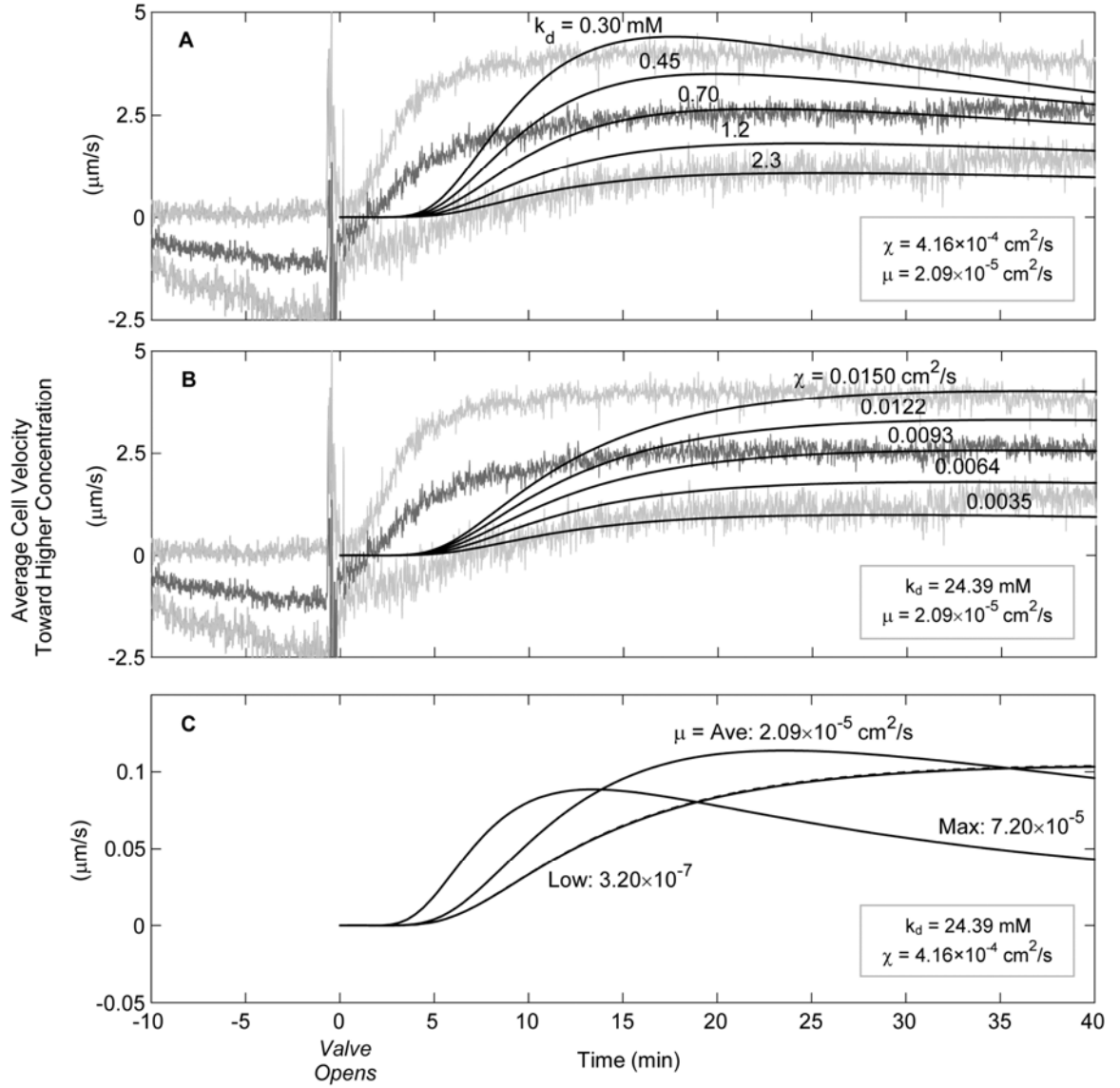


Fig. 4. Model fitting results (smooth black lines) overlaid on experimental results with 95% confidence intervals (grey lines) **(A)** using average literature values for χ and μ and varying k_d over the range shown or **(B)** using average literature values for k_d and μ while varying χ over the range shown **(C)** or μ varied across the range of literature values.

The model could also be fitted to the velocity curve by varying χ and keeping k_d and μ constant at average literature values (figure 4B). The average χ of $9.3 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$ used to fit the experimental results is higher than the literature range 7.20×10^{-5} to $1.24 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$ (table S1). The experimental results were best fitted to the model that assumed the average published value for χ , and then by varying k_d (figure 4A). The model could not be fitted by varying μ according to published values; however, changing μ does change the shape of the velocity curve (figure 4C).

The response times predicted by the model do not fit the rapid response observed in the experiment, and perhaps this is related to the random cell diffusion coefficient, in that response time should be faster for cells with a larger μ (figure 4C). This is based on the proportionality of μ to swimming speed and run time, and inverse proportionality to one minus the cosine of the turn angle θ (21). Although not measured directly here, *M. maripaludis* has been shown to have longer runs and small changes in direction or turn angles (2). Turn angles between 20 and 45° would result in the largest value of μ for a given swimming speed and run time, so it is reasonable to assume μ would be higher for this type of swimming. Another possible explanation for the rapid experimental response not verified with the model, is that more than one type of H_2 receptor, with varying affinities, might be present in *M. maripaludis*, allowing it to respond across varying H_2 concentrations. This has been shown in *B. subtilis*, which has an O_2 receptor with two distinct affinities and binding components (20). Although unlikely, the rapid observed response could also be due to an inaccurate prediction of mass flux of H_2 into the liquid domain. The presented data demonstrate a chemotactic response to H_2 , but model refinement is needed to better represent the phenomenon for H_2 .

The hydrogenotrophic methanogen, *M. maripaludis*, displays chemotactic behavior toward H_2 . The ability to move toward higher concentrations of H_2 could incur an advantage to organisms that are otherwise outcompeted by anaerobes that are able to use H_2 at lower concentrations as well as maintain desired localization with respect to an energy source with low flux. The observed hydrogenotaxis could represent a widespread eco-physiological strategy of methanogens and other hydrogen-utilizing microbes that are important to processes such as bioremediation, and that produce greenhouse gas and biofuels (22, 23). To the best of our knowledge this is the first direct observation of taxis towards hydrogen in any domain of life.

Supplementary Materials:

Supplementary Materials for

Taxis Toward Hydrogen Gas by *Methanococcus maripaludis*

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This PDF file includes:

Materials and Methods

Figs. S1 to S4

Table S1

Captions for Movies S1 to S2

Other Supplementary Materials for this manuscript includes the following:

Movies S1 to S2

Materials and Methods

Culturing Conditions

Methanococcus maripaludis strain S2 was grown in Balch tubes or serum bottles fitted with black butyl stoppers (Geo-Microbial Technologies Inc., Ochelata, OK) and aluminum crimp seals. *Methanococcus* Culture Medium (MCC) was prepared under a stream of anoxic 80% N₂, 20% CO₂ and contains per liter 0.33g KCl, 2.7g MgCl₂•6H₂O, 3.5g MgSO₄•7H₂O, 0.14g CaCl₂•2H₂O, 0.5g NH₄Cl, 5g NaHCO₃, 22g NaCl, 0.14g K₂HPO₄, 5 mL FeSO₄ solution (0.19g FeSO₄•7H₂O/100 mL of 10 mM HCl), 1 mL trace metal solution (per 100 mL; 2.1g Na₃Citrate•2H₂O, adjust pH to 6.5, then 0.45g MnSO₄•H₂O, 0.1g CoCl₂•6H₂O, 0.1g ZnSO₄•7H₂O, 0.01g CuSO₄•5H₂O, 0.01g AlK(SO₄)₂, 0.01g H₃BO₄, 0.1g Na₂MoO₄•2H₂O, 0.025g NiCl₂•6H₂O, 0.2g Na₂SeO₃, 0.01g V(III)Cl, 0.0033g Na₂WO₄•2H₂O) 10 mL of vitamin solution (per liter; 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine HCl, 5 mg thiamine HCl, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg DL-calcium pantothenate, 0.1 mg vitamin B12, 5 mg *p*-aminobenzoic acid, 5 mg lipoic acid), 1 mL of Resazurin solution (1 g/L). This solution is boiled under a stream of gas before adding 0.5 g cysteine•H₂O•HCl, then cooled under gas (24). The solution was dispensed anaerobically and autoclaved. After inoculation, the headspace was displaced and pressurized to 25 PSI with anoxic 80% H₂:20% CO₂ through a sterile filter. Modified MCC medium was used for growth experiments with formate, where NaCl was reduced to 10.5 g/L; 200 mM Na-formate and 200 mM 3-(N-morpholino) propanesulfonic acid (MOPS) were added, and no H₂ was added.

Cultures for taxis experiments were grown at 30°C (with no shaking) from frozen glycerol stocks in 40 mL MCC in 125 mL serum bottles to late exponential phase, then transferred to 5 mL MCC (10% inoculum) in 18 x 150 mm Balch tubes. These working cultures were grown to stationary phase (around 120 h and average cell density 69,420 cells/mL). 1 mL of culture was added to 5 mL fresh MCC in a Balch tube with no H₂ and either incubated for 4-5 h at 30°C under starvation conditions (no electron source), or used immediately for non-starvation conditions. Media salts were separated from cell suspension prior to taxis experiments by centrifuging the inverted Balch tube (100 x g; 30°C for 10 minutes).

Electron Microscopy

Pellicles were removed from tubes with a plastic inoculating loop and placed on a round coverslip freshly treated with poly-L-Lysine (1µg/mL) and fixed in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde and 0.05M Na-cacodylate overnight at room temperature. Coverslips were rinsed and stepwise dehydrated in ethanol, and then critical point dried on a Samdri-795 (tousimis, Rockville, MD). Coverslips were mounted on SEM stubs with double-sided carbon tape and colloidal silver, and then sputter coated with Iridium for 35 s at 35mA. Images were collected on a Zeiss Supra55VP FE-SEM.

Capillary Assay

Square glass capillary tubes (1.0 mm) with the ends fitted with norprene tubing connected to a polypropylene female luer-lock hose barb adapter (Cole-Parmer, Vernon Hills, IL) were partially filled with the cell suspension in an anaerobic chamber that

contained only N₂ and CO₂. A 5 mL glass gas-tight luer lock syringe (SGE, Inc., Austin, TX) was used to transfer the cells to the capillary, and left attached to the capillary with the valve closed. A second 5 mL glass gas-tight syringe with 100% H₂ was attached to the gas side of the capillary (figure S1). The entire syringe/capillary assembly was removed from the anaerobic chamber and placed in a petri dish water bath on the microscope stage and firmly secured with poster putty and tape (figure S3)

Microscopic Observation of Swimming Behavior and Image Analysis

A Leica TCS SP5 II upright confocal microscope was enclosed in an incubation chamber and was equilibrated to 30°C. High-resolution time lapse images were collected every 0.753 s at the center of the capillary, 0.5 cm from the cell suspension/gas phase interface. A 25X water-dipping objective was used and a 3X optical zoom was applied resulting in a final field of view of 206.9 x 206.9 μm^2 and a pixel size of 0.20 $\mu\text{m}/\text{pixel}$. Images were initially acquired for 10 minutes with N₂/CO₂ in the gas phase, then the valve was opened to the H₂ syringe and images were captured for 40 minutes. The control experiments were identical to the above except that 100% Ar was used instead of H₂, and images were acquired for a minimum of 8 minutes before the valve was opened and 38 minutes after.

Images were manually thresholded and binarized using MetaMorph v. 7.6 (Molecular Devices, Sunnyvale, CA). Binary images were analyzed using Imaris v. 7.5.2 (Bitplane, Inc., South Windsor, CT) with a particle-tracking module (Imaris Track). 1 s, 5 s, and 10 s filter durations were tested where a given track was only analyzed if it was as long or longer than the specified duration. The 5 s filter was used for the described analyses, and difference were not observed in overall trends between track lengths.

Chemotaxis Model

A one dimensional finite element model was constructed using Comsol Multiphysics Version 4.3a that solves Equations b and d simultaneously in the liquid domain. Diffusion of hydrogen through the gas domains was modeled by Maxwell-Stefan equations. All model parameters were corrected for temperature (30° C) and salinity (2.65% m/v) of the medium, where possible, and hydrogen consumption rate parameters were estimated from literature values. Table S1 shows all constants used in the model.

The geometry of the model consisted of one liquid domain and two gas domains separated by a valve that opens at $t = 0$ to start the diffusion of hydrogen into the system. The short segment between the valve and the far right boundary is the length of the connection between the valve and the main volume of the gas-tight syringe. The geometry for each experimental replicate was slightly different so average lengths were used. The diffusion of hydrogen through the gas domain of the capillary was expected to be much faster than diffusion through the liquid domain so the precise length of the gas domain was not expected to affect the analysis. The diffusion-limiting segment between the liquid-gas interface and the point of observation 0.5 cm into the liquid remained constant through all replicates and is therefore not an average value. Initial conditions are zero hydrogen inside the capillary, 100% hydrogen behind the valve in the second gas

domain and a constant population density in the liquid domain. Figure S1 shows the geometry, initial conditions and boundary conditions used in the model.

The model geometry was meshed at a maximum element size of 0.1 mm and time step discretization was done with a backward differentiation formula (BDF) method. The time steps taken by the solver were allowed to be free with larger steps being taken at later time points where gradients are shallower. A relative solution tolerance of 10^{-20} was imposed and PARDISO (25) was the general direct solver as implemented in Comsol.

Most parameters in the model are well known or able to be calculated from the literature with the exception of the chemotaxis constants χ , μ and k_d . A literature search was performed for observed chemotaxis values in any organism to establish a range of reasonable values. Twelve applicable values were found for χ , twenty-one for μ and seven for k_d . Maximum, average and minimum values for each are found in Table S1. The model was fitted to the average swimming velocity data by independently varying one chemotaxis parameter by trial and error while keeping the other two constant at the average literature value found in Table S1.

Carbohydrate and Protein Measurements

Protein concentrations were determined with the Lowry assay using bovine serum albumin as the standard (26). Hexose sugars were measured by the colorimetric cysteine-sulfuric acid method with glucose as the standard. Pentose sugars were measured with a colorimetric orcinol- FeCl_3 assay with xylose as the standard. A colorimetric carbazole assay was used to measure uronic acid concentration with D-galacturonic acid as the standard (27).

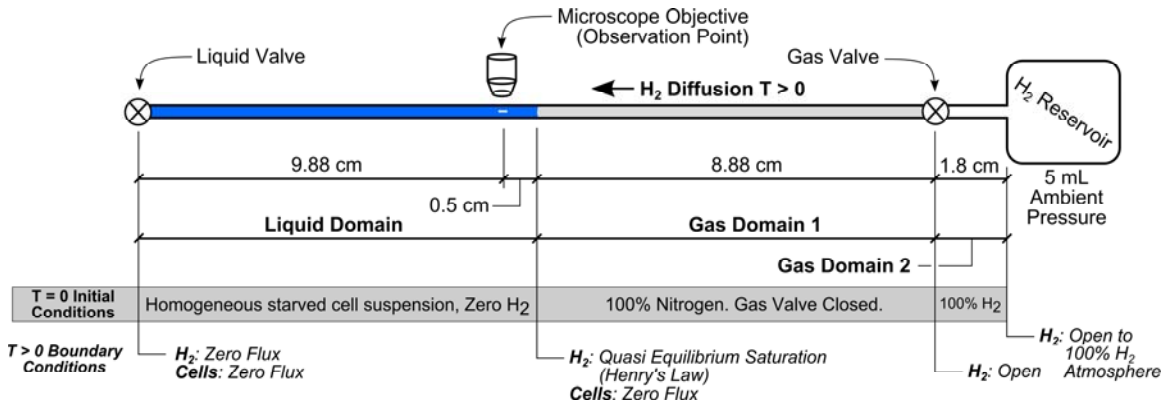


Fig. S1.
Model boundary conditions

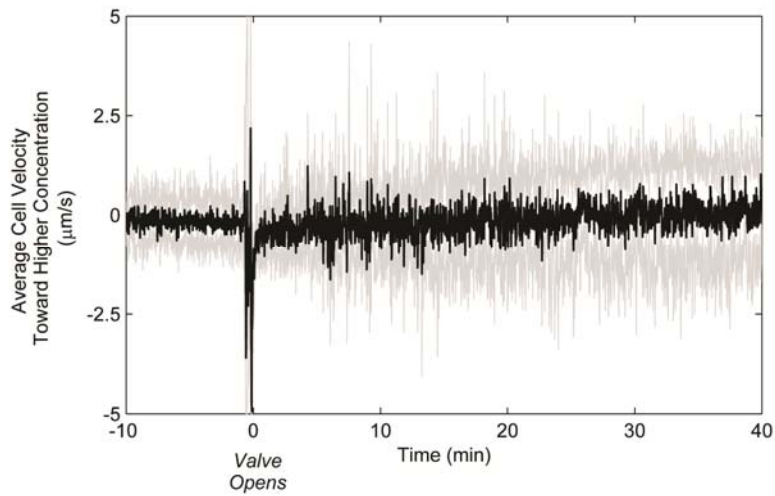


Fig. S2.

Average cell velocity toward hydrogen concentration gradient (positive y-axis) and away (negative y-axis) when *M. maripaludis* cells were not subjected to starvation prior to capillary assay. Black is average with $n=3$ and gray 95% confidence interval.

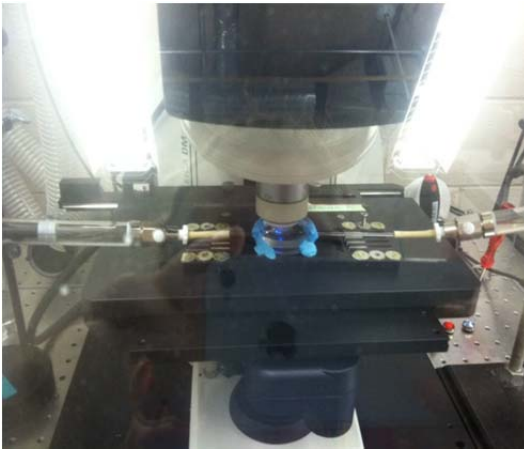


Fig. S3.

Capillary assay setup on microscope stage. The petri dish water bath allowed the capillary to be maintained at a constant temperature and maintained a reliable water column for the water-dipping objective without the need to add water, for the duration of data collection.

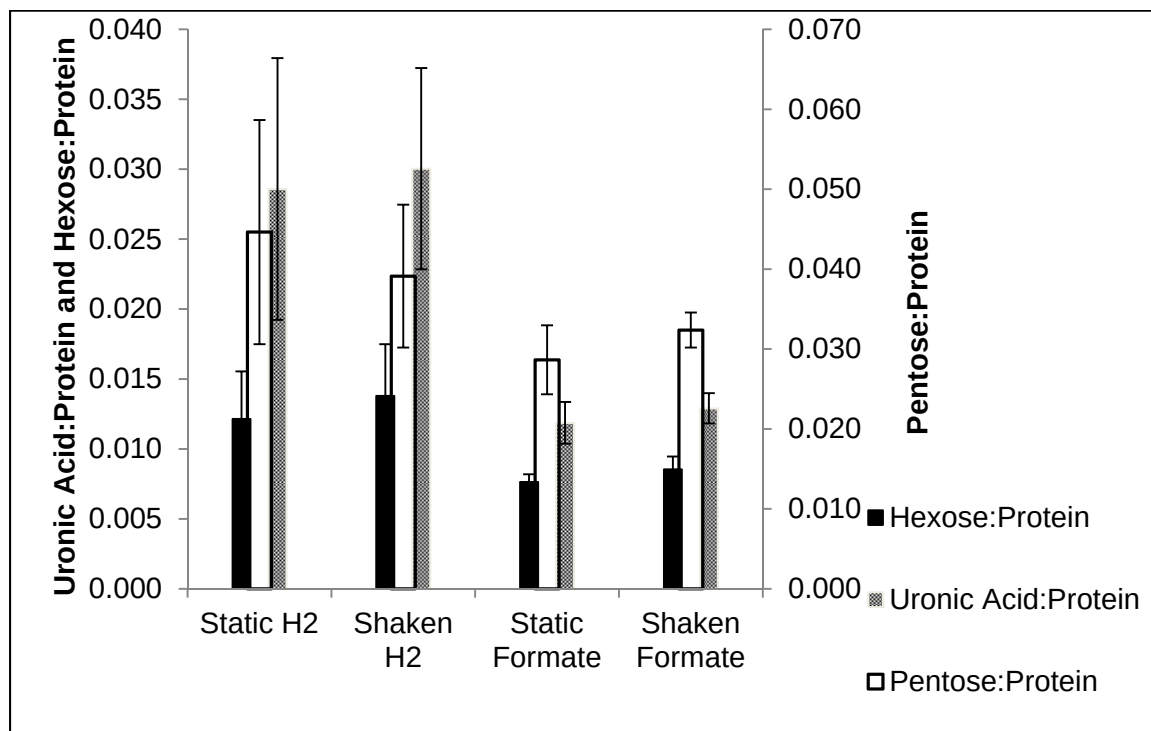


Figure S4.

Comparison of cell-associated carbohydrate concentration including Hexose, Uronic Acid (both primary axis), and Pentose (secondary axis), each normalized to biomass protein. Four culturing conditions include static and shaken for each electron donor, H₂ gas and formate. Error bars represent standard deviation.

Parameter	Value	Unit	Comments	Reference
P	84.3	kPa	Atmospheric pressure at experimental elevation of 1525 m.	Calculated
$D_{\text{H}_2\text{aq}}$	5.32×10^{-5}	$\text{cm}^2 \text{s}^{-1}$	Diffusion coefficient of H_2 in water with a salinity of 2.65% and $T=30^\circ \text{C}$	(28)
$D_{\text{H}_2\text{gas}}$	0.779	$\text{cm}^2 \text{s}^{-1}$	Diffusion coefficient of H_2 in N_2 at $T=297.2 \text{ K}$ and $P=1 \text{ atm}$	(29)
k_{satH}	7.64×10^{-4}	$\text{mol L}^{-1} \text{atm}^{-1}$	Henry's law saturation constant for H_2 in pure water at 30°C	(30)
r_{max}	7.13×10^{-14}	min^{-1}	Michaelis–Menten maximum hydrogen consumption rate.	(31)
k_m	4.1×10^{-6}	M	Michaelis–Menten half saturation constant for hydrogen consumption rate.	(31)
b_0	1.157×10^{10}	cell m^{-3}	Initial cell population density	Measured
μ	7.20×10^{-5} 2.09×10^{-5} 3.20×10^{-7}	$\text{cm}^2 \text{s}^{-1}$	Cell diffusion coefficient	High: (13) Calculated Low: (32)
χ	1.24×10^{-3} 4.16×10^{-4} 7.20×10^{-5}	$\text{cm}^2 \text{s}^{-1}$	Constant chemotactic coefficient	High: (19) Calculated Low: (32)
k_d	170 24.38 1.5×10^{-3}	mM	Receptor-ligand binding dissociation constant	High: (33) Calculated Low: (20)

Table S1.

Physical parameters used in the finite element model. A literature search was performed for μ , χ and k_d and all values found were averaged for this study. The high and low values reported for flagellated, chemotactic bacteria are listed here as well as the calculated averages. No values from any archaeum exist in the literature to the best of the authors' knowledge.

Movie S1

M. maripaludis swimming in a capillary after a five hour starvation period. Gas phase is oriented at the top. There is no hydrogen available initially, then the valve is opened after 10 minutes and hydrogen diffuses into the liquid phase. A fifty-five minute video was minimized by including only every third frame at 10 x speed. The video was compressed by 81% to minimize file size.

Movie S2

M. maripaludis swimming in a capillary after a five hour starvation period. Gas phase is oriented at the top. The valve is opened after 16 minutes and argon diffuses into the liquid phase. A fifty-five minute video was minimized by including only every third frame at 10 x speed. The video was compressed by 81% to minimize file size.

Any Additional Author notes:

K.A.B. developed experimental design, performed experiments, critically evaluated the model, wrote and revised the manuscript. J.M.C. developed experimental design, performed experiments, created the model, wrote and revised the manuscript. C.D. performed experiments and revised the manuscript. R.G. developed experimental design, critically evaluated the model, and revised the manuscript. M.W.F. developed experimental design, critically evaluated the model, and revised the manuscript.

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CHAPTER FOUR

COMPARATIVE PROTEOMICS OF *METHANOCOCCUS MARIPALUDIS* IN A
SYNTROPHIC COCULTURE AND A MONOCULTURE PELLICLEAbstract

The macro-scale structure and physiological responses to growth state have been previously evaluated in a syntrophic coculture between the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough and the methanogenic archaeum *Methanococcus maripaludis*. Molecular level responses have yet to be investigated to determine how the two microorganisms alter resource allocation in a number of environmentally relevant growth states, namely syntrophic biofilm and during a community-level response to hydrogen in *M. maripaludis*. High throughput shotgun proteomics of complex samples allows for identification of changes in relative expression levels of proteins across multiple conditions. Preliminary proteomics experiments were undertaken to determine the difference in protein expression patterns between syntrophic coculture and syntrophic planktonic coculture, monoculture *D. vulgaris* grown with sulfate, and monoculture *M. maripaludis* grown with the soluble electron donor formate, shaken with H₂, and statically with H₂, where a pellicle of chemotactic cells forms a carbohydrate rich community at the gas-liquid interface. The proteomes of both the cellular proteins and the loosely associated extracellular proteins were evaluated separately. Preliminary results indicate that unique proteins are involved in syntrophy versus growth in

monoculture on H₂, and that there is a subset of proteins that may be important for *M. maripaludis* pellicle formation. Sixty-seven ID's with two or more peptides were found only in coculture, and 108 ID's with two or more peptides were only observed in shaken monocultures of *M. maripaludis* grown in H₂ and 122 unique ID's were identified in cultures of *M. maripaludis* growing as a pellicle with H₂.

Introduction

Syntrophic interactions are widespread and especially important in anaerobic environments, where they allow for a coordinated flow and sharing of energy, while mitigating carbon mineralization, and acting as a base and linking point for important biogeochemical cycles (Overmann & Gemerden 2006; Pester et al. 2012; McInerney et al. 2009). While many environments have been surveyed for genomic and metabolic potential, and representatives have been grown in pure culture studies in the lab, little has been done to understand how the mechanism of syntrophic interactions in the context of cellular physiology and metabolism (Walker et al. 2012). A genome may represent all that a species may be capable of now, historically, or in the future, but the proteome represents the direct result of a tightly controlled metabolism. Proteins are expensive to build, and their presence is a very strong indicator that they are serving a purpose, thus revealing valuable information about community processes (Xia et al. 2006).

Mass spectrometry techniques and equipment have been developed to allow for processing complex biological samples quantitatively without the use of a label (Bantscheff et al. 2007; Matzke et al. 2012; Menon et al. 2009; Yates et al. 2009).

Relative abundances of peptides can be determined by spectral counts, peptide peak intensity, or relative numbers of peptides (Bantscheff et al. 2007). For this preliminary discovery study, peptide numbers are counted and compared between sampling conditions as a proxy for relative abundance.

Materials and Methods

Culture Conditions and Biomass Collection

Desulfovibrio vulgaris Hildenborough and *Methanococcus maripaludis* S2 were cultured in biological duplicate for all conditions. For planktonic *D. vulgaris*, *M. maripaludis* shaken, and coculture conditions, cultures were grown in 125 mL serum bottles containing 40 mL of Coculture Medium (CCM) (Walker et al. 2009), a bicarbonate buffered, modified basal salt medium without choline chloride, prepared under 80 % N₂: 20% CO₂. Monoculture *D. vulgaris* medium was supplemented with 25 mM sodium sulfate. Monoculture shaken *M. maripaludis* was grown in CCM, with 30 mM acetate in lieu of lactate, prepared under 80 % N₂: 20% CO₂, then pressurized after autoclaving to 200 kPa with 80% H₂: 20% CO₂. Monoculture static *M. maripaludis* was grown in Balch tubes in 5 mL of CCM with the same modifications as shaken *M. maripaludis*.

Cultures were harvested rapidly on ice by opening crimp seals and pooling 200 mL total culture per replicate (5 bottles or 40 tubes) in sterile 250 mL Nalgene wide-mouth bottles. Cultures were centrifuged at 13,000 g for 10 min at 4°C. Supernatant was reserved and flash-frozen with liquid N₂ and pellets were suspended in 15 mL ice cold

CCM (with no carbon or energy source), and vortexed at maximum speed (VSM-3 Variable Speed Mixer, Pro. Scientific Inc., Oxford, CT) for 30 s to shear extracellular proteins. Following vortexing, tubes were centrifuged again at 13,000 g for 10 min at 4°C. Supernatant presumably containing extracellular, sheared proteins was flash frozen and the pellet containing cell-associated protein was flash frozen.

Coculture biofilms were continuously cultured in modified 1L CDC reactors (BioSurface Technologies Corp., Bozeman, MT) for anaerobic biofilm growth. Biofilm coupon holders were modified to hold glass microscope slides cut to 7.6 cm x 1.8 cm. Headspace (290 mL) was sparged at 20 mL/min with anoxic 80% N₂: 20% CO₂ through a 0-20 SCCM mass controller (Alicat Scientific, Tucson, AZ). Reactors were maintained at 30°C with stirring at 150 rpm. The reactor aqueous phase (375 mL) was inoculated with 20 mL of mid-exponential phase planktonic cultures grown from freezer stocks in 40 mL of CCM in 125 mL serum bottles. Fresh CCM in a 20 L glass carboy was continuously sparged with sterile anoxic 80% N₂: 20% CO₂ and supplied at a dilution rate of 0.01 h⁻¹ starting after 48 hours of batch growth. Biofilms were harvested during steady-state, after 200 hours of continuous culture and rinsed by dipping directly in and out of ice cold deionized water. Both sides of four biofilm slides per reactor were scraped into 15 mL centrifuge tubes and suspended in 10 mL of ice cold CCM (with no carbon or energy source), and vortexed on maximum speed for 30 s. Centrifugation proceeded as planktonic cultures described above.

Lysis Check

To determine the effect of maximum speed vortexing on cell lysis, four vortexing times were tested on planktonic coculture. Initially 1 mL of culture was fixed in 38% formaldehyde for a final concentration of 2% (v/v). The same culture was then centrifuged and the pellet resuspended in ice cold CCM as described above. The resuspended culture was vortexed for 30 s, then 1 mL removed and fixed. The culture was vortexed for an additional 30 s twice, each time removing and fixing 1 mL. Fixed cultures were obtained for 0, 30, 60, and 90 s of vortexing, then stained for 20 min in the dark with an equal volume of filtered 0.3g/L Acridine Orange. Stained samples were collected through a filter chimney on a black polycarbonate track-etched isopore filter (EMD Millipore Corp., Billerica, MA) and imaged on a Nikon Eclipse E800 microscope with a mercury bulb for fluorescence. At least ten random fields of view were analyzed and cells were counted via integrated morphometry analysis in MetaMorph version 7.6 (Molecular Devices, Sunnyvale, CA).

Protein Fractionation

Extracellular and sheared fractions were thawed and cleared by centrifugation at 20,000 g at 4°C for 30 minutes in polycarbonate oak ridge tubes and preserved for analysis. Supernatants were transferred to beakers and ammonium sulfate was added to reach 95% saturation at 4°C with constant stirring with a magnetic stir bar for 10-14 hours. Precipitated proteins were collected by centrifugation at 20,000 g in polycarbonate oak ridge tubes. Protein pellets from both centrifugation steps were resuspended in 50 mM Tris-HCl pH 7 and dialyzed against greater than 2000 volumes of

identical buffer for 10-14 hours at 4°C. Protein concentrations were determined by Lowry assays and equal amounts of protein were loaded onto 10% SDS-PAGE gels and electrophoretically separated at 100V until the dye front reached 1cm from the end of the gel. Gels were stained with coomassie brilliant blue and each lane was excised and cut into 1cm pieces, including the dye front and one control slice beyond the dye front. Gel slices were transferred to 96 well plates and macerated in 50 µl of distilled water, sealed, and stored at 4°C until mass spectrometry was performed.

Protein Digestion

Protein digestion was as described previously (Menon et al. 2009; Cvetkovic et al. 2010) in brief a MassPrep robotic liquid handler (Waters, Milford, MA) was used for automated denaturation, reduction, alkylation, and digestion in 96-well plates. 50 µL of sample was denatured by addition of 50 µL of pure trifluoroethanol and heated at 60°C for 30 min. Reduction of disulfide bonds was achieved with 10 µL of 200 mM dithioreitol in 50 mM ammonium bicarbonate. Samples were alkylated with 14 µL of 200 mM iodoacetamide and incubated at 25°C in the dark for 30 min. Finally the samples were incubated at 40°C for 18 h with 1.5 µg of trypsin (Trypsin Gold Mass Spectrometry, Promega, Madison, WI) to digest proteins, then the pH was lowered to 4 with 5% formic acid to stop digestion.

Separation of Peptides and Mass Spectrometry

Peptides were separated by reverse phase liquid chromatography (LC) (Menon et al. 2009; Cvetkovic et al. 2010) with a gradient of water and acetonitrile with 0.1%

formic acid as the mobile phases, in 96-well plates. 8 µL of the digested sample was injected in an Agilent 1100 microwell autosampler using a quaternary pump (50 mL/min) to a C₁₈ trap cartridge on a six-port switching valve. Flow at 500 nL/min was switched to the nanocapillary pump after 3 min, and peptides were separated on an analytical column/nanoelectrospray tip (75 µm ID, 6 cm long) fabricated with a P-2000 (Sutter Instruments) laser puller and packed with C18 resin (5 µm; Agilent Zorbax SB). A linear ion trap (LTQ) quadrupole mass spectrometer (Thermo Fisher Scientific Inc.) with 2 kV at the tip, was used for MS/MS analysis.

Protein Identification and Annotation

MS/MS data analyses and protein identifications were carried out using Mascot (Matrix Science, Boston, MA) database searching against the complete annotated genome sequences of *D. vulgaris* and *M. maripaludis*. The *M. maripaludis* genome sequence is available at the EMBL/GenBank/DBJ database under accession number BX950229 and the *D. vulgaris* sequences are deposited in GenBank with accession no. AE017285 (chromosome) and AE017286 (plasmid) (Heidelberg et al. 2004; Hendrickson et al. 2004). Protein identifications were limited to cases where 2 or more independent peptides were identified with greater than 98% confidence from the predicted protein sequence. Peptide abundance was used as a first approximation of the respective protein abundance in different samples (Matzke et al. 2012).

Results and Discussion

Lysis Check

After cell collection, cells suspensions for both *D. vulgaris* and *M. maripaludis* were vortexed for increasing time (30, 60, 90 sec) in order to shear loosely attached extracellular proteins. A slight decrease was observed in cell numbers of *D. vulgaris* after 60 seconds of vortexing, and *M. maripaludis* cells did not show significant change in cell numbers after 90 sec. Therefore, 30 seconds was selected to minimize cell lysis (Figure 1).

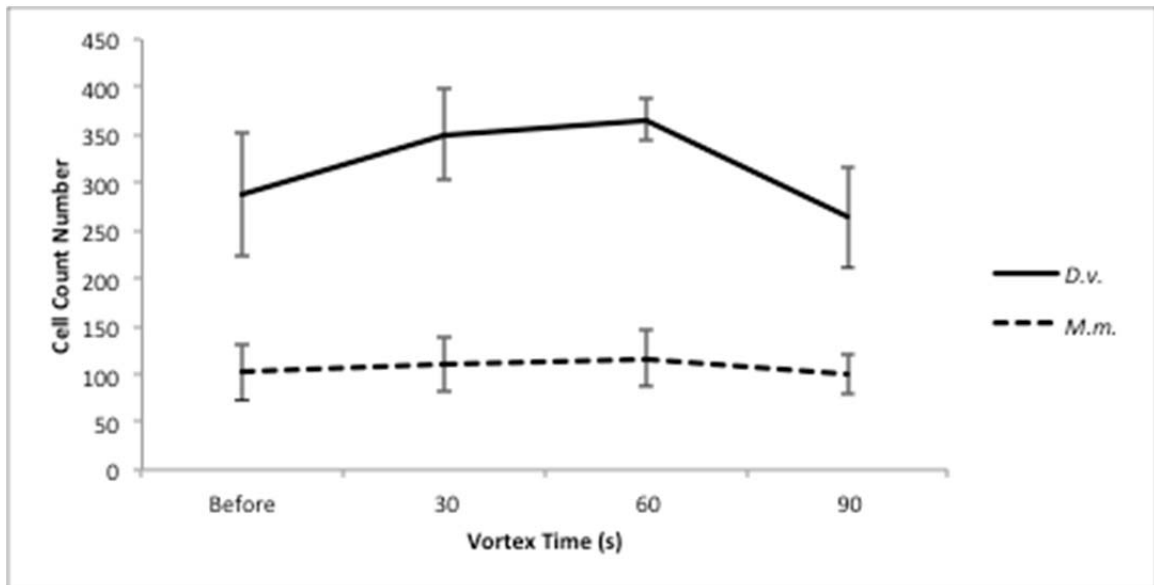


Figure 1. Counted number of *D. vulgaris* (—) and *M. maripaludis* (---) as a function of vortex time. Error bars represent standard deviation.

Protein Fractionation

Polypeptides were fractionated from the extracellular “shear” fraction and examined by staining the SDS-PAGE gels with coomassie brilliant blue from two

separate samples (Figure 2). Panels A and C (Figure 2) represent one sample set while panels B and D represent a second sample set. *D. vulgaris* proteins from both sample sets were not visible in the sheared fraction nor separated by gel electrophoresis. The lack of visible *D. vulgaris* polypeptides was attributed to the presence of sulfide precipitates interacting with proteins and interfering with the separation. The cultures were grown shaken at 150 RPM and upon visual inspection appeared to contain many black sulfide precipitates. When cultures were grown without shaking, the sulfide precipitates were not visible. This issue was only present for monoculture *D. vulgaris*, which grew by the reduction of sulfate to hydrogen sulfide. All the other cultures were grown in the absence of sulfate reduction and sulfide was not produced.

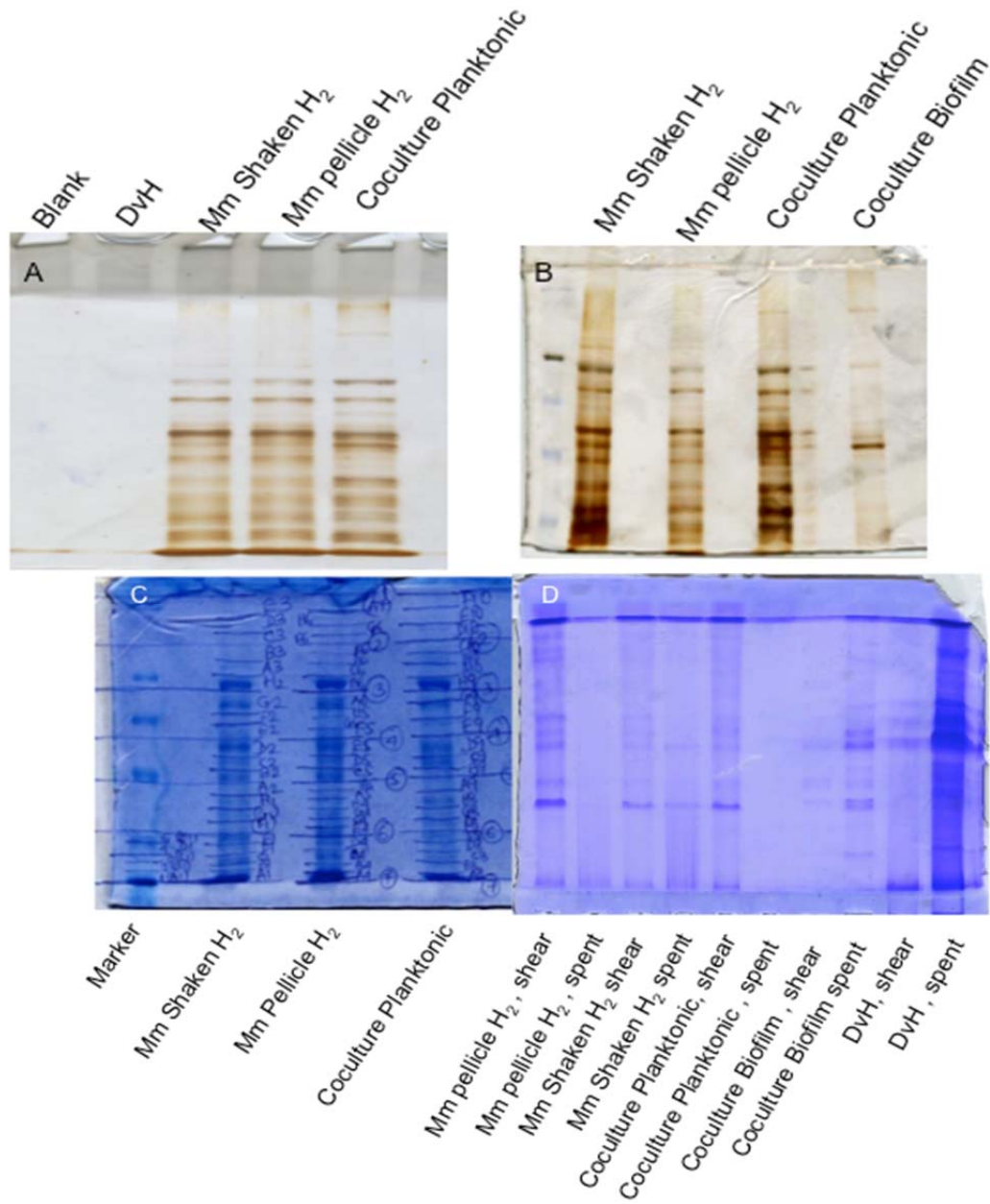


Figure 2. SDS-PAGE gels of proteins fractionated from sheared fraction with (A) and (B) unstained and (C) and (D) stained with Coomassie Blue and (D) including spent media fraction.

A third sample set was collected by growing *D. vulgaris* without shaking, but has yet to be analyzed, so this method modification has not been evaluated. From the first sample

set (Figure 2 A and C), the coculture biofilm samples were used for developing the MS/MS method, and were not analyzed. Additionally all conditions from the second sample set were not analyzed by MS/MS. Preliminary data was obtained for *M. maripaludis* shaken and pellicle, as well as coculture planktonic.

Peptides Unique to Coculture

Sixty-seven polypeptides were identified with 2 or more peptides that were unique to *M. maripaludis* in coculture (Table 1). Sixteen polypeptides, including the most abundant peptide unique to coculture, were annotated as hypothetical, conserved hypothetical, or unknown function. The unknown function of these proteins highlights the need for more work towards a mechanistic understanding of anaerobic syntrophy and methanogenic pellicles. Among the unique coculture polypeptides were several putative helicases and nucleotidyl transferases, possibly indicative of DNA repair mechanisms. Interestingly, proteins predicted to be involved in the methanogenesis pathway were up-expressed in co-culture compared to monoculture. These results suggest that even though all *M. maripaludis* cultures were grown methanogenically, fundamental aspects of carbon and energy flow can be different dependent upon growth modes. A similar result was recently observed for sulfate-reducing *D. vulgaris* biofilms that had up-expressed transcripts and polypeptides unique to the biofilm growth mode (Clark et al. 2012). Sensory proteins were unique in coculture including a receptor tyrosine kinase. A putative type II secretion system protein that had homologs in other methanogens and 60% amino acid similarity to bacterial pili assembly protein was unique to coculture. This is interesting given that parts of the archaeal flagella have homologs to bacterial type

IV pili. Finally two proteins involved in molybdenum biosynthesis and nitrogen-fixation were unique to coculture. Nitrogen was available in the medium as ammonium or N_2 , and nitrogen fixation would not be expected under those conditions. However, it is not known if ammonium utilization is equal between the two populations under the tested growth conditions or if *D. vulgaris* and *M. maripaludis* have equal rates and/or affinities for ammonium utilization.

Unique to Coculture	# peptides
hypothetical protein	37
UvrD/REP helicase	30
tRNA pseudouridine synthase D TruD	26
helicase c2	18
membrane protein	15
Radical SAM domain protein	13
protein of unknown function DUF795	12
TrkA-N domain protein	12
DEAD/DEAH box helicase domain protein	11
Formylmethanofuran dehydrogenase	11
Phosphoglucosamine mutase	10
2, 5-diamino-6-hydroxy-4-(5-phosphoribosylamino)	10
molybdenum cofactor synthesis domain	10
NADPH-dependent FMN reductase	10
alkyl hydroperoxide reductase/ Thiol specific antiox.	9
type II secretion system protein	9
Nitrogenase	8
DEAD/DEAH box helicase domain protein	8
Protein-glutamate O-methyltransferase	8
CO dehydrogenase/acetyl-CoA synthase complex	8
helix-turn-helix domain protein	8
beta-lactamase domain protein	8
hypothetical protein	8
acetolactate synthase, small subunit	8
conserved hypothetical protein	7
Cobaltochelatase	7
CoB--CoM heterodisulfide reductase	6
conserved hypothetical protein	6
TrkA-N domain protein	6
Radical SAM domain protein	6
RNA polymerase Rpb4	6
protein of unknown function DUF1121	6
O-phosphoseryl-tRNA(Cys) synthetase	6
cobalamin biosynthesis protein CbiD	5

Table 1. Peptide ID's unique to coculture listed by annotation and number of peptides identified.

In the context of nitrogen, it would be interesting to determine how the obligate syntrophy would respond to an added mode of interaction (*i.e.*, potential competition).

Unique to Coculture	# peptides
precorrin-6y C5,15-methyltransferase (decarboxylating)	5
FAD-dep. pyridine nucleotide-disulphide oxidoreductase	5
mevalonate kinase	5
4Fe-4S ferredoxin iron-sulfur binding domain protein	5
conserved hypothetical protein	5
isopentenyl-diphosphate delta-isomerase, type 2	5
glycosyl transferase family 2	5
conserved hypothetical protein	5
Protein of unknown function DUF473	4
conserved hypothetical protein	4
conserved hypothetical radical SAM protein	4
extracellular solute-binding protein family 3	4
hypothetical protein	4
metallophosphoesterase	4
2-phosphosulfolactate phosphatase	4
protein of unknown function DUF434	4
conserved hypothetical protein	4
alpha-L-glutamate ligase, RimK family	4
DNA methylase N-4/N-6 domain protein	3
Auxin Efflux Carrier	3
cytidyltransferase-related domain	3
conserved hypothetical protein	3
protein of unknown function DUF75	2
stationary-phase survival protein SurE	2
6-pyruvoyl tetrahydropterin synthase	2
hydrolase, TatD family	2
phosphoesterase RecJ domain protein	2
nitrogen regulatory protein P-II	2
daunorubicin resistance ABC transporter	2
phosphodiesterase, MJ0936 family	2
conserved hypothetical protein	2
intracellular protease, Pfpl family	2
Aminoacyl-tRNA hydrolase	2
UDP-N-acetylglucosamine 2-epimerase	2

Table 1. (continued) ID's unique to coculture listed by annotation and number of peptides identified.

This is especially interesting given that a role was recently given to alanine as a transfer metabolite that can be used by *M. maripaludis* grown syntrophically with *D. vulgaris* (Walker et al. 2012). No unique peptides were observed for alanine transport or utilization.

Polypeptides related to presumptive TrK-like proteins were detected, and these proteins can be associated with redox-linked regulation of potassium channels and conductance (Schlösser et al. 1993). The exact function of K⁺ channels in *Desulfovibrio* or *Methanococcus* is not well understood, but they could play a role in syntrophic interactions related to NAD⁺/NADH balance. The presumptive phosphoglucosamine mutase was observed unique to co-culture, and the enzyme interconverts glucosamine-1-P and glucosamine-6-P. The mutase is typically associated with cell wall synthesis in bacteria, but the exact role in archaea is not known. In addition, previous work has shown that a similar enzyme, phosphoglucose isomerase, can be extracellular and involved as a differentiation/maturation factor in eukaryotes (Fairbank et al. 2009).

Peptides Unique to Planktonic Shaken *M. maripaludis*

108 unique polypeptides with 2 or more peptides were observed from shaken cultures of *M. maripaludis* grown on H₂ and not in static-grown pellicles grown on H₂ or coculture (Table 2). Twenty of these unique ID's were annotated as hypothetical, conserved hypothetical, and unknown function. The most abundant peptide unique to *M. maripaludis* shaken planktonic cultures was an excinuclease, important for DNA repair. Additional DNA repair proteins were unique to monoculture and while these peptides were not seen in coculture, proteins with similar functions were also unique to coculture.

It is possible that a different subset of proteins is used during coculture growth versus monoculture to accomplish similar functions. It was an unexpected result to find 11 peptides unique to monoculture shaken cultures for the F_{420} -dependent hydrogenase (Frh) as this hydrogenase has been previously observed to be more abundant during H_2 -limited growth. The shaking condition should represent the least hydrogen-limiting growth condition examined, but perhaps this method leads to more rapid consumption of hydrogen in the headspace and depletion occurs earlier than expected. This may also be a result of the bias of these samples. Only the crudely sheared fraction of proteins was evaluated by HT-MS/MS, not the cell associated-fraction. While it seems obvious that the shearing method used did also lyse some cells, as evidenced by the peptides identified as intracellular, it is possible that the lysis was biased, and did not occur to the same degree in all samples.

Unique to Planktonic Shaken	# peptides
excinuclease ABC, A subunit	30
nucleotidyl transferase	24
histidinol-phosphate aminotransferase	23
TOPRIM domain protein	22
GTPase-like protein	19
argininosuccinate lyase	19
peptidase U62 modulator of DNA gyrase	17
glutamyl-tRNA(Gln) amidotransferase, A subunit	16
conserved hypothetical protein	15
type II secretion system protein E	15
Phosphopyruvate hydratase	15
aminotransferase class I and II	14
putative DNA helicase	14
precorrin-4 C11-methyltransferase	14
Radical SAM domain protein	13
GTP-binding signal recognition particle SRP54	13
translation initiation factor 2, alpha subunit	13
L-seryl-tRNA selenium transferase	13
protein of unknown function UPF0027	12
histidyl-tRNA synthetase	12
AIR synthase related protein domain protein	12
ferredoxin	11
Orotidine 5'-phosphate decarboxylase	11
small GTP-binding protein	11
Translin	11
carbamoyl-phosphate synthase, small subunit	11
coenzyme F420 hydrogenase/dehydrogenase	11
phosphoribosyltransferase	11
helicase c2	10
glutamyl-tRNA(Gln) amidotransferase, subunit D	10
helix-turn-helix domain protein	10
putative CoA-substrate-specific enzyme activase	10

Table 2. ID's unique to planktonic shaken *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Planktonic Shaken	# peptides
AMP-dependent synthetase and ligase	10
cobyric acid synthase CobQ	9
pyruvate flavodoxin/ferredoxin oxidoreductase	9
Succinate dehydrogenase	9
homoaconitate hydratase family protein	9
aspartate carbamoyltransferase	9
Flagella accessory C family protein	9
Radical SAM domain protein	9
phosphoribosylglycinamide formyltransferase 2	9
aminotransferase class-III	8
DNA ligase I, ATP-dependent Dnl1	8
Transketolase central region	8
Radical SAM domain protein	8
protein of unknown function DUF121	8
conserved hypothetical protein	8
ribonuclease Z	7
CO dehydrogenase/acetyl-CoA synthase complex	7
Methyl-coenzyme M reductase, protein D	7
protein of unknown function RIO1	7
phosphoribosyltransferase	7
Methionine synthase vitamin-B12 independent	7
Ribonuclease P	7
putative hypothetical protein	7
Tetratricopeptide TPR_2 repeat protein	6
peptidase M24	6
thymidylate synthase	6
cobalt ABC transporter, ATPase subunit	6
N2,N2-dimethylguanosine tRNA methyltransferase	6
conserved hypothetical protein	6
nitrogen regulatory protein P-II	5
nicotinate-nucleotide pyrophosphorylase	5
Diaminopimelate epimerase	5

Table 2. (continued) ID's unique to planktonic shaken *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Planktonic Shaken	# peptides
asparagine synthase (glutamine-hydrolyzing)	5
anthranilate phosphoribosyltransferase	5
hypothetical protein	5
ABC transporter related	5
protein of unknown function DUF375	5
protein of unknown function DUF366	5
conserved hypothetical protein	5
helix-turn-helix domain protein	5
exsB protein	5
Na/Pi-cotransporter II-related protein	5
magnesium and cobalt transport protein CorA	5
Phenylacetate--CoA ligase	5
hypothetical protein	4
Rhodanese domain protein	4
MMPL domain protein	4
putative RNA methylase, NOL1/NOP2/sun family	4
Alpha-amylase	4
putative transcriptional regulator, XRE family	4
conserved hypothetical protein	4
hypothetical protein	4
DNA-binding TFAR19-related protein	4
non-canonical purine NTP pyrophosphatase	4
hypothetical protein	4
conserved hypothetical protein	4
phosphoserine phosphatase SerB	4
cytidylate kinase, putative	4
TOBE domain protein	4
CMP/dCMP deaminase zinc-binding	3
Histidinol dehydrogenase	3
Protein of unknown function DUF54	3
methionine aminopeptidase, type II	3
4Fe-4S ferredoxin iron-sulfur binding domain	3

Table 2. (continued) ID's unique to planktonic shaken *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Planktonic Shaken	# peptides
putative translation factor pelota	3
putative signal transduction protein	3
hypothetical protein	3
ABC transporter related	3
conserved hypothetical protein	2
EhbP-like protein	2
Cupin 2 conserved barrel domain protein	2
protein of unknown function DUF39	2
small GTP-binding protein	2
Sjogrens syndrome scleroderma autoantigen 1	2
peptidase M50	2
alpha-NAC related protein	2
pyruvate ferredoxin/flavodoxin oxidoreductase	2

Table 2. (continued) ID's unique to planktonic shaken *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Peptides Unique to *M. maripaludis* Pellicle

122 polypeptides with two or more peptides were identified only in *M. maripaludis* grown on H₂ without shaking, where a pellicle was formed (Table 3). Thirty-three of these unique ID's were annotated as hypothetical, conserved hypothetical, or unknown function. The most abundant peptide ID was a glutamyl tRNA synthetase and several other unique proteins were identified as ribosomal proteins indicating again either a bias in the number of lysis events, or perhaps a real difference in cell activity levels between culture conditions. The two primary objectives for analyzing the sheared fraction and cellular fraction of proteins were to identify possible important extracellular proteins in coculture biofilm and to identify the 25 nm diameter spherical structures observed in dehydrated *M. maripaludis* pellicles. Additionally, the pellicle contained a large

Unique to Pellicle	# peptides
glutamyl-tRNA synthetase	45
type I site-specific deoxyribonuclease, HsdR family	26
Aldehyde ferredoxin oxidoreductase	21
hypothetical protein	20
nucleic acid binding OB-fold tRNA/helicase-type	14
glycosyl transferase family 2	14
phenylalanyl-tRNA synthetase, beta subunit	14
translation initiation factor aIF-2	14
thiamine pyrophosphate protein TPP-binding	14
isopropylmalate/citramalate/homocitrate synthase	13
acetate--CoA ligase	13
2-hydroxyglutaryl-CoA dehydratase D-component	12
ATP dependent DNA ligase	12
Chromatin associated protein KTI12	11
protein of unknown function DUF512	11
glutamate-1-semialdehyde-2,1-aminomutase	11
RimK domain protein ATP-grasp	11
CTP synthase	10
PP-loop domain protein	10
putative DNA primase, small subunit	10
thiamine pyrophosphate protein TPP-binding	10
ABC transporter related	10
uroporphyrin-III C-methyltransferase	9
hydrogenase expression/formation protein HypE	9
ribonuclease H	9
beta-lactamase domain protein	9
transcriptional regulator, MarR family	9
conserved hypothetical protein	8
DEAD/H associated domain protein	8
PUA domain containing protein	8
AAA ATPase central domain protein	8
isopropylmalate/citramalate/homocitrate synthase	8
Phosphosulfolactate synthase	8
conserved hypothetical protein	8

Table 3. ID's unique to pellicle *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Pellicle	# peptides
domain of unknown function DUF1743	8
conserved hypothetical protein	8
modD protein	8
Fructose-bisphosphate aldolase	8
selenide, water dikinase	7
hypothetical protein	7
translation initiation factor SUI1	7
Protein of unknown function DUF555	7
protein of unknown function DUF74	7
hypothetical protein	7
restriction modification system DNA specificity dom.	6
Transcription factor CBF/NF-Y histone	6
3-phosphoshikimate 1-carboxyvinyltransferase	6
Ribosomal protein S6e	6
Transcription factor CBF/NF-Y histone	6
hydrogenase accessory protein HypB	6
Ribosomal protein L25/L23	6
type II secretion system protein	6
conserved hypothetical protein	6
CBS domain containing protein	6
aldo/keto reductase	6
protein of unknown function Met10	6
putative thymidine phosphorylase	6
archaeosine tRNA-ribosyltransferase	6
Carbamoyltransferase	6
Brix	6
D-isomer specific 2-hydroxyacid dehyd. NAD-binding	5
oxidoreductase FAD/NAD(P)-binding domain	5
magnesium and cobalt transport protein CorA	5
ribosomal protein S17	5
protein of unknown function DUF1063	5
4Fe-4S ferredoxin iron-sulfur binding domain protein	5
SSS sodium solute transporter superfamily	5

Table 3. (continued) ID's unique to pellicle *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Pellicle	# peptides
THUMP domain protein	5
purine phosphorylase family 2	5
conserved hypothetical protein	5
conserved hypothetical protein	5
Cobyrinic acid ac-diamide synthase	5
30S ribosomal protein S17E	5
conserved hypothetical protein	5
pseudouridine synthase, RluA family	4
Desulfoferrodoxin ferrous iron-binding region	4
quinolinate synthetase complex, A subunit	4
ribosomal protein L7Ae/L30e/S12e/Gadd45	4
amidohydrolase	4
ribosomal protein L29	4
Protein of unknown function DUF356	4
hypothetical protein	4
hydrolyase, Fe-S type, tartrate/fumarate subfam.	4
LSU ribosomal protein L40E	4
putative translation initiation factor aIF-2	4
protein of unknown function DUF211	4
Dinitrogenase iron-molybdenum cofactor biosyn.	4
protein of unknown function DUF214	4
Ribosomal protein S28e	4
peptidase U32	4
cobalamin 5'-phosphate synthase	3
periplasmic binding protein	3
extracellular solute-binding protein family 3	3
preprotein translocase, SecY subunit	3
DNA-binding protein Alba	3
conserved hypothetical protein	3
Abortive infection protein	3
N-acetyl-gamma-glutamyl-phosphate reductase	3
PHP domain protein	3
Protein of unknown function DUF361	3

Table 3. (continued) ID's unique to pellicle *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

Unique to Pellicle	# peptides
sulfolpyruvate decarboxylase subunit alpha	3
formylmethanofuran dehydrogenase subunit E region	3
hypothetical protein	3
translation initiation factor eIF-1A	3
conserved hypothetical protein	3
Uncharacterised conserved protein UCP006600	3
NADPH-dependent FMN reductase	3
Protein of unknown function DUF1867	2
PRC-barrel domain protein	2
protein of unknown function UPF0118	2
A1A0 ATPase, subunit H	2
Protein of unknown function UPF0147	2
protein of unknown function DUF190	2
conserved hypothetical protein	2
transcription termination factor Tfs	2
pyruvate ferredoxin/flavodoxin oxidoreductase	2
Ribosomal protein L44E	2
hypothetical protein	2
conserved hypothetical protein	2
putative signal transduction protein CBS domains	2
protein of unknown function DUF201	2
hydrogenase assembly chaperone hypC/hupF	2

Table 3. (continued) ID's unique to pellicle *M. maripaludis* cultures grown on H₂, listed by annotation and number of peptides.

number of strands that could be both carbohydrate and proteins. A unique polypeptide to the pellicle was annotated as a hydrogenase formation protein (HypE). The presumptive protein is predicted to interact with other Hyp proteins as well as a putative membrane protein annotated as a SNARE associated Golgi protein (IPR015414). These Golgi membrane proteins are predicted to be involved in vesicle trafficking in yeast and to bind mannosyltransferases (Inadome et al. 2007). Interestingly, an interaction map (STRING v9.0) based on different lines of evidence predicted interactions with HypB, HypC, and a

NAD(P)-oxidoreductase (Figure 3), and polypeptides for putative HypB (MMP1520), HypC (MMP1330), and a NAD(P)-oxidoreductase (vhuG) were also detected.

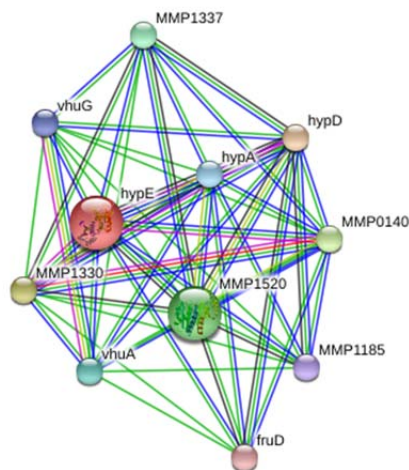


Figure 3. Prediction of interacting functional partners for HypE using STRING v9.0. Prediction of functional links (edges between nodes) is based upon gene loci, gene fusion, cooccurrence, coexpression, experiments, and text mining.

A presumptive glycosyl transferase was observed as unique to the pellicle, and the protein is annotated as a dolichyl-phosphate β -D-mannosyltransferase. Dolichol is a unique lipid carrier observed in eukaryotes and archaea used in the formation of N-linked glycoproteins. The composition of the pellicle EPS and potential vesicles are unknown, but the extracellular localization of these polypeptides suggests a pellicle-dependent response to H_2 .

Most Abundant Peptides

Thirty peptides were counted more than 150 times when all samples were considered together (Table 4). The single most abundant peptide was part of an annotated thermosome protein and was observed in high frequency in shaken and pellicle

monoculture *M. maripaludis* (554 and 517 respectively) but only 22 times in coculture.

The thermosome is part of a type II chaperone system that assists with misfolded proteins (Hendrickson et al. 2004; Furutani et al. 1998). The significant difference in protein expression between monoculture and syntrophic conditions suggests a potential

Most Abundant Peptides-Annotation	Shaken	Pellicle	Cocult.	SUM
thermosome	554	517	22	1093
Coenzyme-B sulfoethylthiotransferase	192	195	126	513
translation elongation factor EF-1, subunit alpha	256	179	60	495
ATP synthase, A subunit	233	171	68	472
glutamine synthetase, type I	192	179	61	432
S-layer protein	128	174	116	418
translation elongation factor aEF-2	221	116	71	408
Coenzyme-B sulfoethylthiotransferase	147	124	120	391
Methyl-coenzyme M reductase, gamma subunit	148	141	80	369
4Fe-4S ferredoxin iron-sulfur binding domain protein	160	111	92	363
alanyl-tRNA synthetase	118	112	94	324
5,10-methylenetetrahydromethanopterin reductase	126	89	99	314
ribosomal protein L3	138	104	58	300
Methylenetetrahydromethanopterin dehydrogenase	132	94	71	297
Alanine--glyoxylate transaminase	122	122	37	281
phosphoenolpyruvate synthase	113	81	81	275
nickel-dependent hydrogenase large subunit	92	89	92	273
methyl-accepting chemotaxis sensory transducer	113	74	64	251
DNA-directed RNA polymerase subunit A'	121	83	32	236
nickel-dependent hydrogenase large subunit	71	35	123	229
H ⁺ -transporting two-sector ATPase alpha/beta subunit	124	62	34	220
anaerobic ribonucleoside-triphosphate reductase	81	76	62	219
carbamoyl-phosphate synthase, large subunit	113	66	32	211
ATP/GTP-binding site motif A (P-loop)	70	55	71	196
methyl-accepting chemotaxis sensory transducer	76	64	54	194
tetrahydromethanopterin S-methyltransferase, MtrH sub.	95	48	35	178
alanine dehydrogenase	48	53	76	177
AAA family ATPase, CDC48 subfamily	49	55	60	164
acetolactate synthase, large subunit, biosynthetic type	103	35	18	156
tetrahydromethanopterin S-methyltransferase, subunit A	56	63	35	154
molybdenum cofactor synthesis domain	0	80	70	150

Table 4. Most abundant total peptides and their distribution across culture conditions including *M. maripaludis* grown on H₂ as a pellicle or shaken, and in coculture. Numbers in the first three columns represent peptide frequency, while the fourth column is a sum of the total occurrence of the peptide across all samples.

protection mechanism incurred from syntrophy resulting in a decrease in protein misfolding and stress response. As expected the most abundant peptides were assigned to IDs involved in core metabolism, including methanogenesis, growth, and energy conservation. Two proteins of the 30 were annotated chemotaxis proteins, which was expected to be important for all of these growth conditions with hydrogen as the energy source.

Conclusion

Differences in protein levels were observed, and each culture condition had a distinct set of 67-122 unique peptides identified, clearly indicating that *M. maripaludis* changes its proteome dependent on environmental conditions. The most abundant peptide identified, a chaperone, was primarily found in monoculture conditions, and less often in coculture. This indicates that some benefit is incurred by syntrophy, although overall slower growth as a syntroph could explain this observation. The pellicle is a unique growth mode that has not been previously reported for *Methanococcus*. Routinely, hydrogenotrophic methanogens are grown as shaken cultures to increase mass transfer of H₂, and we observed pellicles upon static incubation. When static cultures were provided formate (soluble substrate), pellicles were not observed. The comparison of extracellular proteomes between monocultures in the planktonic versus pellicle growth mode has identified potential proteins that contribute to the response of *M. maripaludis* under H₂-limiting conditions.

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CHAPTER FIVE

EPILOGUE

The motivation behind this research was tied to the concept that physiological strategies of microorganisms are not clearly defined by their genome sequence or their behavior in a test tube of rich media. Organisms once deemed “unculturable” are now more commonly described as “not yet cultured”. Phyla once described as obligate parasites have been more recently found to be capable of free-living when cultured appropriately (Haider et al. 2010). The flagellar tip protein of *Pelotomaculum thermopropionicum* was shown to induce up-expression of methanogenesis genes in *Methanothermobacterium thermoautotrophicus* when it touched the cell (Marx 2009). Syntrophic interactions between a dual-culture community were proposed to affect function quantitatively and qualitatively in a biofilm-dependent fashion. We proposed four primary objectives to explore our hypotheses, and this dissertation represents the cumulative results of that work.

System Development

Successful cultivation of anaerobes requires modification of standard equipment to maintain strict oxygen-free conditions. Continuous cultivation of biofilm also requires very specific vessels that can accommodate surfaces for growth. Previous work with anaerobic biofilm in our lab led to modifications of the CDC reactor to accomplish this, namely clamps and gaskets for maintaining a tight seal, and modified biofilm coupon

holders with greatly increased surface area so that sufficient biomass could be produced to complete experiments (Clark et al. 2007; 2012). These modifications worked well in sulfidogenic conditions; however, additional modifications had to be made to maintain anoxia in the absence of sulfide for the cultivation of the SRB-methanogen coculture. In addition, function of community members was quantified based on measurements of carbon mass flux in the aqueous phase and gas phase, which required strict flow control. The lid of the CDC reactor was modified to accommodate additional gas ports with Swagelock fittings, and gas exited to an online gas chromatography station, while sterile sparging gas entered through a mass controller for precise gas flow measurements. This system was adjusted continuously until coculture biofilm and planktonic phases could be grown together reproducibly with the base case parameters described in this dissertation. Once the system was well established, I performed perturbation experiments for comparison.

Characterization of Biofilm Structure and Function

The second objective was to characterize the structure of biofilm formed by a syntrophic coculture of *D. vulgaris* and *M. maripaludis* and describe the associated change in function. Initially I assumed that methanogenesis was the function of interest, as it was the terminal step in the described syntrophy. The base case coculture formed a thick, structured biofilm with deep valleys, tall ridges, and channels throughout. The biofilm at steady-state consisted of approximately 2:1 *D. vulgaris* to *M. maripaludis* and produced methane at a constant rate. Hydrogen produced by *D. vulgaris* was consumed

directly by *M. maripaludis* and H_2 was never detectable in the presence of biofilm. The base case had an associated planktonic phase that was close to 1:1 on average, and frequently *M. maripaludis* would outnumber *D. vulgaris*. This system reached a steady-state when lactate was consumed as fast as it entered the reactor, leaving the cells in a lactate-limited state representative of environmental conditions where low levels of organic acids are produced in anaerobic environments and immediately consumed.

In an attempt to separate the function of biofilm from the function of the planktonic phase, flow rate was increased to exceed the specific growth rates of both organisms, thereby washing planktonic cells out of the chemostat. The increased lactate loading rate quickly changed the chemostat from lactate-limited to lactate-excess, and the lactate was not entirely consumed. The planktonic phase began to slowly wash out of the chemostat, yet despite the increased substrate availability, the biofilm did not increase in biomass. Instead it responded to the increased lactate within one hour by producing significantly more methane. During the four retention times that I continued increased loading rate, the planktonic phase was not successfully washed out. For this reason I cannot attribute increased methane production to one growth phase or the other. I made two conclusions from this perturbation experiment. Since the biomass did not increase and the biofilm structure remained the same as the base case, I determined that the biofilm biomass increased only until it reached a specific concentration. This target cell density can be described as the carrying capacity of the system, or the maximum number of each species that can be maintained given a set amount of nutrient. Biomass retention is not a new idea, and many industrial processes use retention of biofilm biomass as a

strategy to keep desired organisms in a system, such as wastewater treatment (Thiele et al. 1988; Silva et al. 2006; Tappe et al. 1999; Gross et al. 2007; Halan et al. 2012).

Biomass retention as a strategy of microorganisms could represent a group effort to survive stress, where a specific concentration of cells is required to accumulate enough of a signaling molecule to affect the whole community in a positive way, as in quorum sensing (Irie 2008; Goo et al. 2012; Zhang et al. 2012). It could also represent a way to give collective genetic information to other members of the community as biofilms have been shown to facilitate the transfer of DNA (Molin & Tolker-Nielsen 2003).

The second conclusion from the washout experiment was that the whole biofilm community could be metabolically active, based on the immediate response to increased lactate loading rate. To test this theory we incubated the biofilm with CTC, a fluorescent stain that is indicative of respiratory potential. We observed that nearly all of the cells appeared to be actively respiring. This result is in contrast to the normal paradigm for aerobic slab-type biofilms, where the upper layer only remains active as the lower portion of the biofilm becomes oxygen-limited (Xu et al. 2000). The syntrophic coculture biofilm seemed to be structured to alleviate mass transfer limitation and conversely facilitate transfer between the biofilm and the bulk phase. This structure could be a result of *D. vulgaris* structuring itself to prevent hydrogen accumulation or to ensure lactate diffusion is not limited, or it could even be the result of the syntrophic interaction with *M. maripaludis* specifically. This is very interesting considering once again that aerobic slab biofilm that grows over itself, to a thickness that prevents the bottom layer from accessing a terminal electron acceptor. It is tempting to speculate about altruism and

group fitness in light of this observation. Such behavior is attributed, in macroecology, to an innate desire to protect the genes that are most closely related to the individual performing the “selfless” behavior. That is a simple idea for diploid, sexual organisms, but is certainly compounded in the case of social insects, and likely asexual microorganisms (Stilling 2003). Gene transfer facilitated by biofilms and quorum sensing could certainly be described as group fitness behavior in that sense. This notion is supported by the fact that the biofilm had a more evenly distributed carrying-capacity with a ratio of 2:1 compared to previously reported non-biofilm cocultures (4:1 to 3:1).

For an additional test of function compared to the base case (*i.e.* biofilm and planktonic), the biofilm coupons were removed from a reactor at steady-state. The response (*i.e.*, methane production) to increased lactate availability was delayed and transient, and the reactor contained much less biomass without a biofilm phase. Biomass yield per lactate mass flux was therefore much lower than it was in the presence of biofilm. This observation confirmed that biofilm and biomass retention play an important role in community stability and function. In addition, two *D. vulgaris* mutants deficient in biofilm formation ($\Delta flaG$ and $\Delta pilA$) were grown in co-culture with *M. maripaludis*. While the $\Delta pilA$ mutant *D. vulgaris* could grow in closed, batch tubes with *M. maripaludis*, the open, reactor system could initiate growth but become unstable within a week and both organisms washed out. These results suggest that biofilm contributed to the stability of the syntrophic interaction.

Two observations were made during completion of the first objectives where *M. maripaludis* exhibited a previously undescribed behavior in the presence of a syntrophic

hydrogen-producing partner. The methanogen did not form a biofilm under our culture conditions unless it was initiated by the SRB. In addition, when *M. maripaludis* was grown statically in Balch tubes, versus shaken as is traditionally the preferred method for incorporating hydrogen into the aqueous phase, a pellicle formed at the gas-liquid interface. We hypothesized that *M. maripaludis* was chemotactically responding to hydrogen in the form of a H₂ concentration gradient from the gas-phase or from a H₂-producing syntroph as in the case with *D. vulgaris*. Hence, we sought to make a direct observation of the chemotactic behavior of *M. maripaludis*.

“Hydrogenotaxis”

Microorganisms employ a huge range of motility strategies including swimming and twitching by flagella and pili, periplasmic flagella in spirochaetes, secretion of propulsion slime from nozzles in *Myxococcus xanthus* or pores in cyanobacteria, contractile cytoskeletons in *Spiroplasma melliferum*, ratchet gliding in *Flavobacterium johnsoniae*, gas vesicles for floating, and the hami of the *Euryarchaea* SM1 which resembles a grappling hook (Bardy 2003; Wirth 2012). Hydrogen is traded in anaerobic communities as readily as stocks on Wall Street. It seems that taxis toward such a valuable currency would have been one of the first described chemotactic responses. A quote from Steve Zinder sums this up “It is likely that methanogenic substrates, such as H₂ or formate, are chemoattractants, but this has not been tested for methanogens, perhaps due to the formidable technical task of establishing anaerobic gradients of these substrates, which are also energy sources” (Ferry 1993).

We were able to construct an anaerobic capillary system, modified from the Adler version (Adler & Dahl 1967) where swimming behavior could be observed by high-resolution time-lapse microscopy, in response to a gradient of hydrogen or argon control. The result was the first direct observation of swimming toward hydrogen, which we termed - hydrogenotaxis. New words are not always used to describe different types of chemoattractants, for example there is no specific word to describe acetate taxis. There are descriptive words for some of the most important and well-studied tactic responses including aerotaxis, energy taxis, phototaxis, thermotaxis, redox taxis, rheotaxis, and magnetotaxis (Taylor et al. 1999; Harris et al. 2012; Josenhans 2010; Shapiro et al. 2010; Fu et al. 2012; Oprian 2003; Gluch et al. 1995). For this reason we believe the name “hydrogenotaxis” is a good choice for describing this likely common, yet overlooked strategy.

The results of the hydrogenotaxis experiment were fitted to a modified Keller-Segel continuum model albeit with mixed results (Tindall et al. 2008). The average swimming velocity could be obtained by the model; however, the rapid nature of the response could not be fitted. Deviation from the predicted hydrogen diffusion or convection within the capillary could explain this, but it is more likely that a physiological response, left unaddressed by the model is to blame for the poor fit. Despite the lack of agreement between the model and the experimental results, the primary result remains that this represents the first direct observation of taxis toward hydrogen gas in any of the three domains of life.

Comparative Proteomics

The final objective of this research was to incorporate all of the major observations from the first three objectives, to determine appropriate culture conditions for comparative proteomics. High-Throughput Tandem Mass Spectrometry (HT-MS/MS) based proteomics can be used to effectively and comparatively analyze complex biological samples (Yates et al. 2009). We sought to use proteomics to observe the entire range of realized metabolism on a descriptive micro-scale, across these conditions. The culture conditions selected to compare uniquely observed function in these two organisms included coculture biofilm and planktonic proteomes, monoculture *D. vulgaris*, monoculture *M. maripaludis* grown with either formate or hydrogen. For the monoculture *M. maripaludis* hydrogen condition, cultures were grown shaken or statically to allow for the formation of a hydrogenotactic pellicle.

Preliminary results from crudely- sheared extracellular proteins of coculture planktonic and *M. maripaludis* pellicle and shaken on hydrogen indicated that there are many differences in the proteomes under these distinct conditions. The data also indicated that the methods for shearing extracellular proteins will need to be optimized to prevent contamination of that fraction by cellular protein. The samples I prepared have not been fully analyzed, although it is expected that the full proteomics results will eventually be incorporated into a manuscript that encompasses additional high-throughput approaches.

General Conclusion and Future Work

In conclusion, our hypothesis was confirmed that the biofilm growth-mode contributed to a more efficient metabolism between syntrophic populations. However, we anticipated that the more efficient metabolism would result in an increased function in terms of methanogenesis. Instead, the increased function was biomass production, and moreover, the biomass was produced at a more evenly distributed carry-capacity. Secondly, based upon observation from coculture biofilm and methanogenic monocultures, hydrogenotaxis has been definitely demonstrated for the first time.

The future potential for this type of culturing system is immense, now that a baseline has been established for “normal” syntrophic biofilm structure and function. This is especially true in light of the interest in this consortium for their role in numerous environments including, waste sites, wastewater treatment, and carbon cycling. The model syntrophic community could be used to test various stressors and this type of work is already in progress. Future work should include stressing with relevant compounds under both nutrient deprivation and nutrient-excess conditions. In terms of heavy-metal bioremediation, the stresses should include U(VI) and Cr(VI), as well as nitrate, and sulfate. Oxygen stress is of interest too, based on personal observations that the coculture biofilm could handle a considerable amount of oxygen exposure without obvious deleterious effects and the evidence that SRBs can consume oxygen as a group fitness type of protection strategy (Barton & Hamilton 2007). Adding additional community members may be of interest too as would using mutant strains of *D. vulgaris* or *M. maripaludis* that are deficient in motility or attachment to surfaces. Preliminary

characterization of cocultures between WT *M. maripaludis* and mutant strains of *D. vulgaris* including Δp , $\Delta flaG$, $\Delta echA$, $\Delta pilA$, and the complement strain of $\Delta pilA$, was done as part of this dissertation. All these cultures produce methane at similar levels (data not shown) and $\Delta flaG$ and $\Delta pilA$ cocultures were tested in the reactor system and found to be deficient in biofilm production. Interestingly these planktonic cocultures could not reach a stable steady state without the presence of biofilm (data not shown). Established mutant cocultures were stored as glycerol stocks for future work.

The hydrogenotaxis observation leaves the door open to many types of future studies using hydrogen as a chemoattractant. To better characterize this response, multidisciplinary projects could be started with engineering students, to build microfluidic devices better suited for creating stable chemoattractant gradients, and minimizing large magnitude shear forces the swimming cells experience in a large capillary (Ahmed et al. 2010). Multiple hydrogen-utilizing organisms could be tested to determine the extent of the response in nature. Proteins important for chemotaxis sensing and motility in archaea could be characterized and crystal structures obtained with collaborative projects with the chemistry and biochemistry department. All work of this nature could provide more insight to the original question of community function.

“HC SVNT DRACONES”

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APPENDICES

APPENDIX A

IN SILICO APPROACHES TO STUDY MASS AND ENERGY FLOWS IN
MICROBIAL CONSORTIA: A SYNTROPHIC CASE STUDY

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Research article

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In silico approaches to study mass and energy flows in microbial consortia: a syntrophic case study

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Abstract

Background: Three methods were developed for the application of stoichiometry-based network analysis approaches including elementary mode analysis to the study of mass and energy flows in microbial communities. Each has distinct advantages and disadvantages suitable for analyzing systems with different degrees of complexity and *a priori* knowledge. These approaches were tested and compared using data from the thermophilic, phototrophic mat communities from Octopus and Mushroom Springs in Yellowstone National Park (USA). The models were based on three distinct microbial guilds: oxygenic phototrophs, filamentous anoxygenic phototrophs, and sulfate-reducing bacteria. Two phases, day and night, were modeled to account for differences in the sources of mass and energy and the routes available for their exchange.

Results: The *in silico* models were used to explore fundamental questions in ecology including the prediction of and explanation for measured relative abundances of primary producers in the mat, theoretical tradeoffs between overall productivity and the generation of toxic by-products, and the relative robustness of various guild interactions.

Conclusion: The three modeling approaches represent a flexible toolbox for creating cellular metabolic networks to study microbial communities on scales ranging from cells to ecosystems. A comparison of the three methods highlights considerations for selecting the one most appropriate for a given microbial system. For instance, communities represented only by metagenomic data can be modeled using the pooled method which analyzes a community's total metabolic potential without attempting to partition enzymes to different organisms. Systems with extensive *a priori* information on microbial guilds can be represented using the compartmentalized technique, employing distinct

control volumes to separate guild-appropriate enzymes and metabolites. If the complexity of a compartmentalized network creates an unacceptable computational burden, the nested analysis approach permits greater scalability at the cost of more user intervention through multiple rounds of pathway analysis.

Background

Complex microbial communities drive the Earth's biogeochemical cycles [1]. In spite of their importance, the biochemical interactions within these communities are not yet well understood, nor have *in silico* methodologies for studying them matured. An improved understanding of how natural microbial communities mediate biogeochemical cycles will augment predictions of how these processes respond to disturbances from climate change to anthropogenic chemical deposition. Improved understanding may also provide a rational basis for using microbial consortia to produce biofuels and biomaterials from renewable resources [2].

Microbial communities described in terms of their environmental chemistry, ecophysiology, and phylogenetic diversity can be used as a foundation to develop, test, and compare *in silico* tools for analyzing community interactions. The phototrophic microbial mats of Octopus and Mushroom Springs of Yellowstone National Park (Wyoming, USA) represent an ideal test case due to the extensive available data [e.g. [3-9], and numerous other references found throughout this study]. These alkaline siliceous hot spring mats (50-74°C) are inhabited predominantly by unicellular cyanobacteria related to *Synechococcus* spp. and filamentous anoxygenic phototrophs (FAP) related to *Chloroflexus* and *Roseiflexus* spp. The community also contains sulfate-reducing bacteria (SRB) and other prokaryotes sustained by the primary productivity of the photosynthetic bacteria [3].

Previous studies of the mat have revealed diel (day-night) metabolic variation in various community members, driving shifts in the concentration and fate of dissolved metabolites [5,6,10-12]. Organic metabolites including glycolate and other acids, along with hydrogen, form the basis for mass and energy exchanges between community members (Figure 1A). For example, during the day, the photosynthetic cyanobacteria consume CO_2 and produce O_2 as a by-product of photosynthesis. High levels of O_2 relative to CO_2 promote oxygen competition at the ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) active site, leading to the production of glycolate [9,13,14]. Other community members, including the photoheterotrophic FAP, can use glycolate as a carbon and energy source [15]. The cyanobacteria can also store excess photosynthate as polyglucose [16]. This carbon and energy storage material is fermented at night

to organic acids (Figure 1B) [10]. FAP can incorporate fermentation products photoheterotrophically [12,15,17], while SRB appear to oxidize some of these products under anaerobic conditions [6,15,18].

Molecular level metabolic models were constructed to represent the central metabolism of three functional guilds thought to be important to material and energy flows through this community [12]. A guild is a group of species that exploit the same class of environmental resources in a similar manner [19]. Oxygenic photoautotrophs related to *Synechococcus* spp. were selected to represent the mat's primary source of fixed carbon and

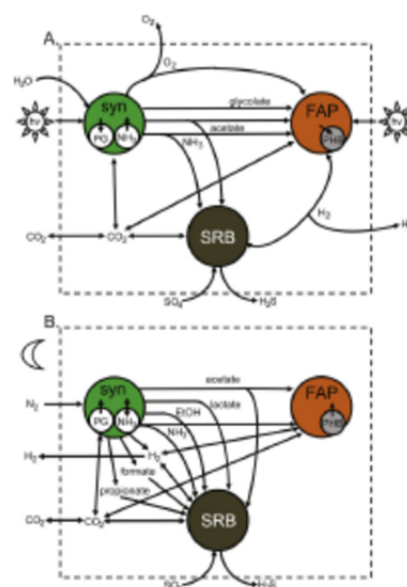


Figure 1
Guild interactions. Modeled consortium interactions are shown for (A) daylight and (B) nighttime simulations. Abbreviations: EtOH, ethanol; hv, photosynthetically-available photons; PG, polyglucose; PHB, polyhydroxybutyrate; syn, cyanobacteria (i.e. *Synechococcus*).

nitrogen. FAP from the family *Chloroflexaceae*, an important structural component of the mat community, were incorporated as metabolically versatile (photo) heterotrophs, capable of capturing light energy as phosphodiester bonds (cyclic photophosphorylation), but requiring externally supplied reducing equivalents. An SRB guild was included as a consumer of the metabolites produced by photosynthetic guilds. Other sub-dominant organisms (e.g. aerobic heterotrophic bacteria and methanogenic archaea) present in the mat [3] were not included in this first *in silico* representation of the system (see methods).

Metabolic networks were studied using the stoichiometry-based network analysis approach known as elementary mode analysis (EMA) [20-22]. EMA evaluates a metabolic network by decomposing the system into a complete set of the simplest biochemical pathways satisfying steady state conservation constraints with enzymatic fluxes occurring in physiologically reasonable directions (i.e. unidirectional enzyme-catalyzed reactions are not permitted to run backwards). These explicitly defined pathways are known as elementary modes and are frequently represented as vectors containing relative rates for every reaction in the metabolic network. The approach permits the description of any sustainable physiological behavior as a linear combination of the simplest biologically meaningful metabolic pathways [23,24].

Metabolic network modeling methodologies have been used extensively to analyze single organisms and monoculture systems [e.g. [25,26]]. An alternative stoichiometry-based technique, flux balance analysis, has been applied to a two-species laboratory microbial system [27]. That study used a compartmentalized approach analogous to strategies for modeling metabolic fluxes between organelles in eukaryotic organisms [28,29]. Topological approaches to metabolic network analysis have also been used at tree of life scales to analyze evolution of metabolism and the interactions of metabolic networks among species and with the environment [30-34]. However, these solely topological studies implicitly assume all genome encoded genes are simultaneously active with no consideration of critical reaction quantitative aspects like carbon and electron conservation relationships.

In the present study, novel methods for handling multispecies EMA are developed and applied to a microbial consortium representing a well-studied thermophilic community from Yellowstone National Park (YNP). To the best knowledge of the authors, this is the first time a stoichiometry-based network analysis approach has been applied to a natural, *in situ* microbial community and the first time these approaches have

been used to analyze microbial biofilms/mats. Mass and energy fluxes were investigated using three different approaches each representing different levels of detail and computational complexity. The three approaches provide a general set of tools for studying microbial communities that can be selected based on the levels of available knowledge and community complexity. Each approach has advantages and disadvantages which are discussed along with examples illustrating how these methods can be used to gain insight into basic community metabolic processes. In particular, our work demonstrates a trade-off between knowledge of the distribution of metabolic capabilities between guilds in a consortium and the ability to conservatively predict and interpret limits for that community's behavior.

Results

Development and implementation of three consortium analysis approaches

The primary aim of this theoretical study was to develop, analyze, and compare different *in silico* approaches for molecular-level analyses of mass and energy flux through a microbial community. The following section details three distinct approaches, using the Yellowstone thermophilic phototrophic mat system as a test case. Advantages and disadvantages of each technique are highlighted and discussions detailing which approach is most appropriate for a given set of microbial data are provided. For each of the three methodologies discussed, both day and night scenarios were considered (Figure 1). All metabolic models, including an explicit listing of the reactions and metabolites, can be found in the additional file 1: Supplement.

Compartmentalized consortium analysis approach

A compartmentalized model was constructed in which each of the three guilds was a distinct compartment and exchangeable metabolites were transferred through a fourth compartment representing the extracellular environment (Figure 2A). This strategy has been used in the past for spatially and chemically segregated eukaryotic and microbial systems [27-29]. The approach was implemented by assigning reactions and metabolites to a network representing each guild (suffixes on metabolite identifiers prevented sharing of compounds common to the metabolism of multiple guilds), while explicit transport reactions accounted for the exchange of metabolites between guild members and the extracellular space. A short illustration of the separation mechanism is provided in additional file 1: Supplement.

The compartmentalized modeling approach has the advantage of conceptual tractability. Dividing the community into guild-level compartments linked by transferred metabolites (e.g. glycolate) is an intuitive way to

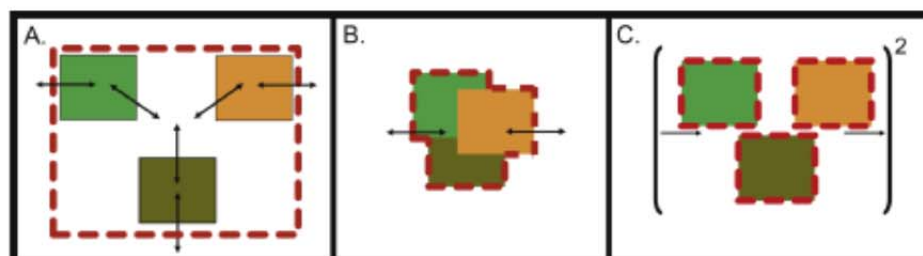


Figure 2
Multi-species modeling approaches. Conceptual depiction of the (A) compartmentalized, (B) pooled, and (C) nested consortium modeling approaches. Dashed red lines indicate system boundaries while thin solid lines represent guild boundaries.

represent interactions within a consortium. It is also an ideal method for understanding which guild performs a particular metabolic transformation. For example, the fraction of total biomass (carbon moles) or total maintenance ATP (used to account for energy-dependent cellular processes other than growth) produced by each guild is clear from the output.

One drawback to the compartmentalized approach is the sheer quantity of unique interactions possible. The size of the resulting network can lead to a 'combinatorial explosion' of elementary modes [35]. To address this limitation, the models for each guild member were constructed to capture the necessary metabolic capabilities while maintaining computational tractability (see methods section). A second drawback of the compartmentalized method is the requirement for significant *a priori* information or assumptions, as enzymes must be assigned to each separate guild. The Octopus and Mushroom Spring phototrophic mats are well-studied, permitting the use of compartmentalized results as a benchmark for the other two methodologies.

Pooled reactions consortium analysis approach

The second approach, referred to as the pooled reactions consortium analysis, treated the consortium as a single entity (Figure 2B). All metabolic reactions and metabolites from the three guilds were combined into a single compartment. Reactions catalyzed by more than one guild were only considered once. The method captures the metabolic constraints of the overall matter and energy transformations without the need for detailed knowledge of every organism in the community.

The pooled reactions approach is ideally suited for investigating the metabolic potential of a community

based solely on metagenomic data because the assignment of each reaction to a constituent guild is unnecessary. The technique is quite flexible and can be scaled to different levels of detail. An additional advantage of the pooled reactions approach is the reduction of computational burden. With these advantages, the method is uniquely suited for initial and exploratory analyses of diverse or poorly understood communities. For scientists accustomed to well-documented laboratory microbes, this approach may seem coarse however, the vast majority of organisms on the planet have not been cultured in the laboratory, much less isolated to purity [36]. This method represents a flexible starting point for analyzing such systems.

A weakness in the pooled reactions approach is that model output does not specify which guilds employ a particular enzyme or produce biomass and maintenance ATP. Instead, the results describe potential performance of the entire consortium. The method also neglects the logistics associated with transferring metabolites between organisms, including conversion of the given metabolite into one for which transporters are available. The approach is presented here using the well characterized cyanobacterial mats to highlight strengths and drawbacks to the method.

Nested pathway consortium analysis approach

The third approach, termed the nested consortium analysis or EMA 'squared', uses successive rounds of EMA to analyze potential interactions within a consortium (Figure 2C). The first round of EMA is applied to each guild model in isolation. The output data are mined for ecologically relevant elementary modes, such as efficient biomass production from sunlight or efficient ATP generation from lactate and sulfate. The selected

elementary modes are then compiled and used as input reactions for a second round of EMA to examine the potential for interactions between guilds. Conceptually, the first round of EMA provides guild-level stoichiometries relating substrates to products. These stoichiometries can be further processed in the same way that traditional EMA uses enzymatic stoichiometries to consider inter- and intraguild interactions. An example of the process is provided in additional file 1: Supplement.

The first round model output was explored for metabolic strategies representing 'selfish' and 'altruistic' operation of each guild. Selfish selection criteria were based on the efficient production of biomass and maintenance ATP. Altruistic selection criteria were based on efficient production of metabolites that could be consumed by other guilds, including for instance H_2 , NH_3 , acetate, and glycolate. Efficiency was evaluated by normalizing the amount of production by the required input (e.g. amount of glycolate or sunlight). A list of the ecological criteria considered can be found in additional file 1: Supplement.

Stable numerical implementation of the nested approach using CellNetAnalyzer v 9.0 required some heuristics. Elementary modes from the first round of analysis were normalized to vectors of unit length prior to the second round of EMA; without this step, errors occurred during calculation of elementary modes; specifically, the attempted inversion of matrices that are very nearly singular. Normalized coefficients were rounded to two decimal places to prevent similar problems. When rounding converted an otherwise non-zero coefficient to zero, the entire elementary mode was scaled so the smallest coefficient was equal to 0.01. The MATLAB script used for normalizing and rounding intermediate results is provided in additional file 1: Supplement. This step was necessary although it introduced a modicum of error. Error analysis is included in the following sections. These steps are not necessary if EMA is performed using the new bit pattern tree algorithm which is numerically more stable [37].

The nested pathway approach allows for the analysis of more complex metabolic networks and larger communities than the compartmentalized approach because each guild is initially considered separately. It also has the advantage of retaining guild-specific output lost in the pooled technique. The manual selection of specific modes permits a guided community analysis based on ecological strategies of interest for each guild; results can then be re-examined in light of the selection criteria used, relating community stoichiometry and guild strategy. The ability to assemble multiple sets of first-

round output and concatenate the results allows the system to expand freely. The nested approach is unrelated to the decomposition approach for the analysis of large cellular networks with EMA [38], but is well-suited for reconnecting the output of the resulting subnetworks tractably.

The nested pathway analysis approach has the disadvantage of requiring two rounds of processing. In addition, manual selection of ecologically interesting modes from individual models requires *a priori* knowledge and can significantly influence the solution space. Finally, intermediate processing introduces some rounding error.

Comparison of consortium analysis approaches

Two test cases were analyzed to compare the three approaches in terms of their ability to describe and explain the flows of carbon, nitrogen, and energy through the mat community. The first case study considered daylight production of biomass fueled by solar energy, while the second case study considered the fermentation of stored polyglucose to drive nitrogen fixation during the night. Table 1 highlights key model outputs associated with the different daytime simulations, including the number of modes identified as well as important parameters like maximum yield of biomass or maintenance ATP per photosynthetically-available photon. Table 2 shows similar results for the nighttime simulations.

Table 1: Elementary mode analysis output summary for comparison of daylight modeling approaches

	compartment	pooled	nested
# of modes	74507	38216	428
$Y_{X/H}$ ^a	0.117	0.128	0.116
$Y_{ATP/H}$ ^b	1	1	1
$Y_{X/psph}$ ^c	0.117	n/a	0.116
$Y_{X/FAP/H}$	0.078	n/a	0.075
$Y_{X/SRB/H}$	0.016	n/a	0.015
$Y_{ATP/psph}$	1	n/a	1
$Y_{ATP/FAP/H}$	0.5	n/a	0.506
$Y_{ATP/SRB/H}$	0.033	n/a	0.031
$Y_{PG/H}$	0.122	0.125	0.121
$Y_{PHB/H}$ ^d	0.089	0.111	0.090

n/a = not applicable.

^a $Y_{X/H}$ is the yield of X on H (i.e. production of X divided by consumption of H). X represents C-moles of biomass and H denotes moles of photosynthetically-available photons.

^bATP represents moles of ATP available for cellular maintenance.

^cpsph designates a parameter associated with the *Synechococcus* guild.

^dFAP indicates a parameter associated with the filamentous anoxygenic phototrophic guild and 'SRB' denotes a parameter associated with sulfate-reducing bacteria guild.

^ePHB represents C-moles of polyhydroxybutyrate (a carbon/electron storage compound associated with the filamentous anoxygenic phototrophic guild).

Table 2: Elementary mode analysis output summary for comparison of nighttime modeling approaches

	compartment	pooled	nested
# of modes	14004	36233	46038
$Y_{\text{cypc}}^{\text{PG}}$ ^a	0.063	0.103	0.063
$Y_{\text{cypc}}^{\text{SO}_4}$ ^b	0.259	0.265	0.203
$Y_{\text{cypc}}^{\text{X}}$ ^c	0.218	0.229	0.209
$Y_{\text{cypc}}^{\text{ATP}}$ ^d	0.737	0.744	0.692
$Y_{\text{cypc}}^{\text{ATP}}^{\text{PG}}$ ^e	0.958	0.958	0.976
$Y_{\text{cypc}}^{\text{ATP}}^{\text{SO}_4}$ ^f	2.25	2.25	2.11
$Y_{\text{cypc}}^{\text{ATP}}^{\text{X}}$ ^g	0.148	n/a	0.150
$Y_{\text{cypc}}^{\text{ATP}}^{\text{SRB}}$ ^h	0.218	n/a	0.184
$Y_{\text{cypc}}^{\text{ATP}}^{\text{FAP}}$ ⁱ	0.5	n/a	0.5
$Y_{\text{cypc}}^{\text{ATP}}^{\text{SRB}}$ ^j	0.625	n/a	0.638

n/a = not applicable.

^a Y_{ij}^k is the yield of i on j (i.e. production of i divided by consumption of j). 'cypc' denotes moles of nitrogen fixed and stored as cyanophycin, while 'PG' represents Cmoles of polyglucose.

^b SO_4 represents moles of sulfate used as an oxidant.

^c'X' represents Cmoles of biomass.

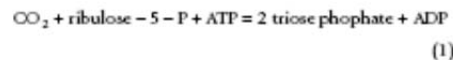
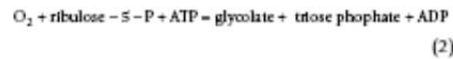
^dATP represents moles of ATP available for cellular maintenance.

^e'cypc' designates a parameter associated with the *Synechococcus* guild.

^fFAP indicates a parameter associated with the filamentous anoxygenic phototroph guild and 'SRB' denotes a parameter associated with sulfate-reducing bacteria guild.

Case one: Consortium photo-efficiency

The relationship between solar energy input and community productivity is a fundamental subject of ecological inquiry, as is the relative abundance of various species. The model community is an example of how metabolic knowledge feeds into these questions, providing a natural point for comparison of the three methods. Oxygenic photosynthesis drives primary productivity in the community by splitting water into reducing equivalents and O_2 . The reducing equivalents can be used to fix CO_2 via the Calvin-Benson-Bassham cycle. The O_2 , however, can compete with CO_2 for the rubisco active site, resulting in the production of glycolate instead of an additional Cmole of reduced carbon (equations 1 and 2). A Cmole is a mole of carbon atoms; one mole of glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) is equivalent to six Cmoles of glucose.



Experimental characterization of these fluxes from the Octopus and Mushroom Spring microbial mats has been reported. The O_2/CO_2 flux ratio at the rubisco active site is estimated to be approximately 0.03-0.07 when dissolved gas concentrations represent mid-afternoon conditions at the springs [9]. The derivation of these estimates from the reported data is demonstrated in additional file 1: Supplement.

The output from the three *in silico* community analysis approaches was plotted using two physiological variables thought to be important to community interactions: 1) O_2 competition at the rubisco enzyme (O_2/CO_2) and 2) the quantity of biomass (Cmole) synthesized per mole of absorbed photons (Figure 3). Relationships between these properties are examined below as a vehicle for comparison of the three modeling approaches.

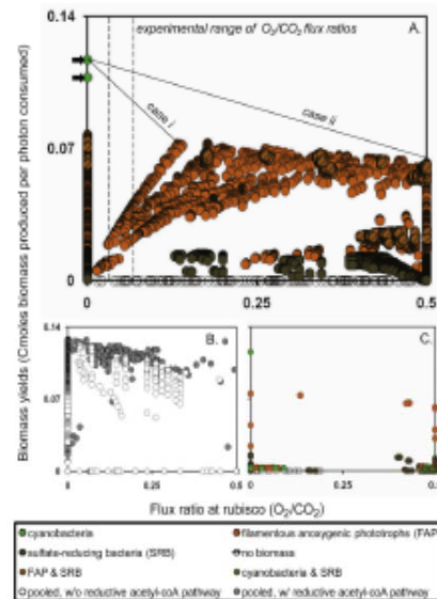


Figure 3
Daytime case study output. Daylight biomass-producing elementary modes, plotted for (A) compartmentalized, (B) pooled, and (C) nested simulations in terms of total consortium biomass yield on photons (Cmole/mole) and oxygen competition at the rubisco enzyme (O_2 flux relative to CO_2 flux). Each point represents a single mode. Solid line segments in (A) connect modes combined to represent ecological scenarios, while dashed lines represent the experimentally-derived limits for oxygen competition at the rubisco enzyme in the mid-afternoon. Arrows denote modes producing cyanobacterial biomass (see text for more details). Points are color-coded by biomass-producing guild (A & C) or SRB-associated reductive acetyl-CoA activity (B).

Compartmentalized consortium analysis

The compartmentalized approach, wherein guilds interact through a mass-balanced external compartment (see section 1a, Figure 2A, and additional file 1: Supplement), served as a basis for comparison of the community modeling techniques. This method revealed only two elementary modes (out of a total of 74,507 distinct elementary modes) producing cyanobacterial biomass. These modes, highlighted with arrows in Figure 3A, differ based on ATP production strategy. The higher-yielding elementary mode generated ATP by oxidizing reducing equivalents from non-cyclic photophosphorylation, while the lower-yielding elementary mode produced ATP through cyclic photophosphorylation.

All other biomass-producing elementary modes generated FAP, SRB, or a combination of FAP and SRB biomass. At low levels of O_2 competition at the rubisco enzyme, Figure 3A illustrates that optimal FAP and SRB biomass synthesis efficiency increases with oxygen competition (positive trend for highest biomass-yielding elementary modes for each increment of oxygen competition). This is due to an increase in glycolate production, shuttling electrons either directly or indirectly to the other guilds. The trend continues until a rubisco O_2/CO_2 flux ratio near 0.13 is reached. At O_2 competition levels greater than about 0.17, O_2 interferes with the efficient photon-based production of cellular carbon (i.e. the reducing equivalents liberated with the oxygen are insufficient to cover the costs of processing the additional glycolate).

Theoretical ecological studies have suggested that organisms adapted to a biofilm or mat lifestyle will draw on available resources in a manner which maximizes yields and thus favors the whole community [39,40]. Combining this theory with the experimentally determined O_2/CO_2 competition at the rubisco enzyme made it possible to predict relative yields of the cyanobacteria and FAP. Two different cases were considered. The first case (i) examined the ramifications of each guild striving to maximize its own biomass yield and the second case (ii) examined different guilds working in concert to maximize biomass for the entire consortium (see Figure 3A).

Cellular metabolism can be expressed accurately as a linear combination of distinct elementary modes [24,41]. It is hypothesized that a consortium's metabolic activity can also be described using linear combinations of the elementary modes (with non-negative coefficients). When exactly two elementary modes are considered, the contribution of each mode to the overall metabolic process (represented by a point on the connecting line segment) is fixed by the ratio of any two fluxes for which the constituent modes have non-

identical ratios. That relationship is demonstrated in additional file 1: Supplement. For each considered case, a combined metabolism was identified using the ratio of fluxes through rubisco (O_2/CO_2) derived from experimental data [9]. The derived values were used to bound the solution space (i.e. set of acceptable flux distributions).

This relationship was applied to the highest-yielding biomass-producing modes from the compartmentalized model to predict the relative abundance of cyanobacteria and FAP for a fixed photon input budget (line segments in Figure 3A connect the elementary modes of interest). Modes producing SRB biomass could easily be combined in the same way, but were not among the highest-yielding strategies. The scenario normalized biomass to a per photon basis. The case i scenario predicts ratios of cyanobacteria to FAP biomass production ranging from 2.5:1 to 6.5:1 (cyanobacteria to FAP) based on the experimentally determined oxygen competition at the rubisco enzyme. The case ii scenario predicts ratios from 8.2:1 to 17.7:1 on the same basis. The numerical procedure is demonstrated in additional file 1: Supplement. The analysis control volume consists of the top 1 mm of mat. The control volume growth is exactly equal to the rate at which biomass leaves the control volume. Physically speaking, this represents the new growth burying the old growth.

The ratio of cyanobacterial to FAP biovolume in a Mushroom Spring mat sample was experimentally measured to be 1.6:1 [16]. It is assumed here that biomass and biovolume are related in the same manner for both species. The relative abundances of metagenomic reads suggest biomass ratios for these guilds between about 1.5:1 and 3.5:1 in the top 1 mm of Octopus and Mushroom Spring mats, assuming no bias in the sampling and sequencing pipeline (Klatt *et al.*, in preparation). The reported ratios are closer to the modeled scenario where each individual guild seeks to maximize its own biomass yields rather than the scenario optimizing biomass yield of the entire consortium. A potential explanation for the modest discrepancy between prediction and experimental data is the experimental underestimation of glycolate production due to consumption of radiolabeled photosynthate during incubation. Increasing the flux ratio (O_2/CO_2) at rubisco (to correspond with higher glycolate production rates) drives the solution space for the case i scenario into stronger agreement with experimental values much more quickly than for the case ii scenario. It should also be noted that *in situ* environmental systems vary considerably in both space and time, and the flux ratios were derived from measurements at a single condition.

Pooled reactions consortium analysis

The same variables were plotted for elementary modes identified with the pooled technique, wherein all reactions and metabolites were combined into a single metabolic unit (Figure 3B). The predicted maximum community biomass efficiency was 9.4% higher for the pooled approach than the compartmentalized approach. The cyanobacteria (responsible for the highest-yielding, compartmentalized biomass mode) do not possess a full citric acid cycle; with the pooled treatment, however, the entire cycle becomes available, efficiently catabolizing photosynthate without the usual logistical costs of metabolite transport between guilds. The genome of RS-1, a FAP isolated from a hot spring microbial mat, appears to code for the enzyme missing from OSA and OS-B', sequenced cyanobacteria from Octopus Spring.

Contrary to the compartmentalized approach, the pooled output suggests that oxygen competition at the rubisco active site does not necessarily affect the biomass yield per photon (note the nearly flat trend of uppermost points in Figure 3B). This represents an uncoupling of heterotrophy and photorespiration, neglecting the role of glycolate as a transfer metabolite. These seemingly contradictory elementary modes all utilized the SRB-associated reductive acetyl-CoA CO₂ fixation pathway, and are striped gray in Figure 3B. This pathway is metabolically inexpensive in the pooled approach due to the direct availability of reducing equivalents derived from oxygenic photosynthesis. These enzymes are generally very sensitive to O₂ [42], calling the feasibility of these particular elementary modes into question. SRB activity has been reported in the upper 0.5 cm of the mat during day [6], but the activity may be limited to small anoxic microenvironments and is unlikely to represent a major daytime CO₂ fixation strategy.

Filtering out elementary modes using the reductive acetyl-CoA pathway leaves a trend of decreasing optimal biomass per photon yields with increasing O₂/CO₂ flux ratios at the rubisco enzyme. The trend appears very similar to case ii from the compartmentalized analysis as a result of the pooled approach's treatment of biomass. A non-guild-specific biomass term means that maximizing the yield optimizes production of biomass for the consortium as a whole, the same criteria used to generate the case ii scenario.

This analysis highlights potential challenges associated with the pooled community approach. First, pooled reactions can result in higher predicted yields than compartmentalized reactions because there are no logistical costs associated with inter-guild metabolite transfer. The second challenge is the combination of enzymatic activities that are not compatible. The use of

oxygenic photosynthesis to drive extremely oxygen-sensitive enzymes, for instance, seems unreasonable. The pooled analysis generates more appropriate trends if *a priori* information regarding the oxygen sensitivity of the reductive acetyl-CoA enzymes is applied as a filter. In addition, because enzymatic activities are shared rather than apportioned between individual guilds, maximizing any yield does so for the entire consortium rather than a single guild. This approach is well suited for initial work on systems lacking sufficient *a priori* data to construct a compartmentalized model. The fact that such data is lacking for the vast majority of all natural ecosystems emphasizes the usefulness of this approach.

Nested consortium analysis

The nested approach produces similar results to the compartmentalized method. While this may not be readily apparent from a cursory comparison of Figures 3A and 3C, connecting the extreme upper-left and upper-right points on each subfigure shows that the limits of the solution space (in terms of biomass yield on photons and oxygen competition at the rubisco enzyme) are quite comparable, although the nested approach identifies fewer suboptimal solutions. The biomass yield on photons decreases with increasing O₂/CO₂ flux ratios at the rubisco enzyme. The initial increasing trend seen in the compartmentalized model consisted of suboptimal solutions not identified as ecologically relevant in the first-round processing. Those metabolic behaviors are all outperformed by linear combinations denoted by the case i line in Figure 1A. The optimal biomass yield on photons is equivalent (within the error introduced by rounding) to the compartmentalized prediction. Combination of modes, as in the case i compartmentalized scenario, identifies very similar ratios of biomass productivity for cyanobacteria to FAP (from 2.0:1 to 5.9:1) at the O₂/CO₂ flux ratios of 0.03-0.07 suggested by experiment (calculations are demonstrated on the compartmentalized output in additional file 1: Supplement). The total number of elementary modes is lower than the compartmentalized approach (428 vs. 74,507) because the initial round of EMA selects only strategies deemed ecologically competitive or relevant. The first round of processing removes many mathematical solutions that are not necessarily of interest for additional analysis. Caution is required; neglect during first-round selection can prevent discovery of interactions or cause underestimations of metabolic potential.

Any system characterized well enough to construct a compartmentalized model can also be analyzed using the nested approach. The approach is best suited for complex systems that prove computationally difficult to analyze using a compartmentalized simulation. In

addition, the nested community analysis approach facilitates interrogation of the competitive strategies employed by individual guilds to produce a compartmentalized modeling result. For instance, the experimentally relevant case i results shown in Figure 3A (biomass production efficiency optimized by guild rather than as a community) require the FAP guild to maximize biomass yield (based on carbon influx) for the strictly heterotrophic co-metabolism of glycolate and acetate. The cyanobacterial guild must concurrently operate a metabolism comprised of three distinct elementary modes: the highest yielding strategies for biomass, glycolate, and acetate production all based on photons. This information is embedded in the criteria used to select the first-round elementary modes.

The nested community analysis approach requires the elementary mode coefficients to be normalized and rounded. Error analysis was performed to gauge the effect of this processing on the results. Each mode was examined for the error in carbon fixed and the amount of absorbed energy. The maximum error introduced was 0.004 Cmoles per mole of photons. The complete error results from both case studies are included in additional file 1: Supplement.

Case two: Nitrogen fixation

Nitrogen fixation fueled by the fermentation of polyglucose is thought to be an important process in the mat during night and early morning [5,11]. The presented models only consider nitrogen fixation at night, since the oxygen sensitivity of nitrogenase should render it inoperable during the day when the mat is superoxic. Future modeling efforts will consider the morning separately, acknowledging evidence that suggests a large fraction of nitrogen fixation *in situ* occurs after sunrise but before O_2 production exceeds consumption [5].

The second case study examined the efficiency of N_2 reduction with polyglucose and the corresponding production of five potentially inhibitory or toxic fermentation by-products: formate, acetate, ethanol, lactate, and propionate. Their toxicities have not been explicitly characterized in this system, but their total molar yield on polyglucose represents a convenient 'toxicity parameter' for comparison of modeling approaches. It should be noted, however, that nitrogen fixation and the incidental production and fate of fermentation products are separately questions of ecological interest.

The FAP guild was included in the night-time simulations. They were inactive however, because unlike the SRB, they are incapable of drawing on sulfate as an

electron acceptor. While the FAP guild can jettison electrons as hydrogen, this requires energy input from light or an alternative oxidant (oxygen was assumed to be unavailable during the night scenario).

Figure 4A depicts the relationship between the secretion of fermentation products and the efficiency of the metabolic strategy according to the compartmentalized results. The high density of points is an explicit illustration of the system robustness. As the yield of NH_3 synthesis increases, more fermentation products are produced. If heterotrophic guilds such as the SRB rapidly metabolize the fermentation products (or those products are removed efficiently through physical transport processes), the high-yielding strategy would likely be the most ecologically competitive strategy for the cyanobacteria. If, alternatively, fermentation products

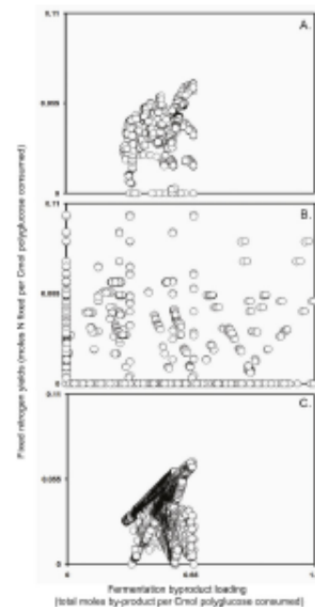


Figure 4
Night case study output. Nighttime nitrogen-fixing elementary modes, plotted for (A) compartmentalized, (B) pooled, and (C) nested consortium simulations in terms of total fixed nitrogen yields on polyglucose (Nmmole/Cmole) and fermentation byproduct loading (total moles of fermentation by-products produced for each Cmole of polyglucose consumed). Each point represents a single mode.

are building up to toxic levels, than the mode associated with lowest by-product loading could be preferred.

The pooled results in Figure 4B demonstrate the effects of neglecting constraints on the exchange of metabolites between guilds. The results suggest, in contrast to predictions by the other two models, that nitrogen fixation at night is possible without associated production of potentially inhibitory or toxic fermentation products (all points in Figure 4B located on the y-axis above the origin). Similar to the photo-efficiency test case, the pooled analysis provides a less conservative solution space than the other approaches. Less information is required to build the pooled model, but predicted yields have to be viewed as theoretical upper limits, with the actual consortium probably operating at lower yields. The approach offers a rational, constraint-based approximation to the metabolic potential of a poorly characterized microbial consortium.

The night nested results have nitrogen-fixing efficiencies similar to the compartmentalized approach. The similarity of the shape and size of the solution spaces between nested and compartmentalized techniques (Figures 4A and 4C) indicates that modes of interest were selected appropriately during first-round processing. Considering the corresponding first-round criteria allows mapping of results into ecological strategies for individual guilds. For example, in the elementary mode resulting in the highest yield of fixed nitrogen on polyglucose, the cyanobacteria guild uses two strategies, maximizing production of free NH_3 and cyanophycin and co-producing acetate, formate, and ethanol. Three distinct SRB physiologies are employed, maximizing biomass production on each fermentation product individually. Error analysis on the night nested model was performed by comparing error in the carbon balance with polyglucose consumption for every mode. The largest error due to rounding was a reasonable 0.033 Cmoles for every Cmole of polyglucose consumed. The complete error results from both case studies are included in additional file 1: Supplement.

Consortium food web robustness analysis

The compartmentalized consortium analysis approach was used to explore additional community properties. Metabolic networks within individual cells are highly branched, forming robust and decidedly redundant systems thought to be resilient to perturbations and disruptions [34,43-45]. Figure 5 details how the 74,507 unique daytime elementary modes can be divided into 16 different inter-guild mass and energy exchanges. Interactions associated with a large number of elementary modes are more likely to remain functional during environmental perturbations [46,47].

Figure 5 indicates that a large number of metabolic strategies (93.9% of elementary modes revealed by the compartmentalized simulation) exist for the daytime transfer of carbon and electrons from the cyanobacterial guild to the FAP guild with associated FAP production of H_2 providing electrons to the SRB guild. In fact, daylight SRB activity *in silico* is overwhelmingly dependent on such hydrogen (97.1% of compartmentalized elementary modes with non-zero SRB fluxes). There are only 58 unique elementary modes involving the transfer of acetate from the cyanobacteria to SRB which do not involve any FAP activity. The interaction analysis also highlights the importance of the well-connected and functionally versatile FAP guild in the mat community metabolism. Only 74 out of the 74,507 unique elementary modes (0.1%) identified by the daytime compartmentalized simulation can function without the FAP guild.

Discussion

Three *in silico* consortium analysis methods were developed and applied to a thermophilic phototrophic mat community from Yellowstone National Park as a test case. The results of this systems ecology study demonstrate the applicability of consortium EMA, explicitly mapping the genes-to-function emergent properties associated with the connectivity of metabolic reactions via the exchange of mass and energy. Since the presented techniques are culture-independent, they are compatible with metagenomic approaches and other studies in which culturing or isolation has proven difficult. This study provides a foundation for further work to refine and test the models using co-culture studies; potential inquiries include the measurement of substrate uptake and growth rates, biomass compositions, mRNA and protein expression, and quantification of internal fluxes. Toxicity profiles of the various fermentation products to representative microbes would also be of interest. Representative organisms or enrichment cultures are available for each guild, and the results of such experimentation will be quite useful for model validation and improvement. That work, however, is beyond the scope of the current study, which seeks only to provide methods for the extension of EMA to microbial consortia.

An alternative approach to the mass-balance-based analysis of large networks, flux balance analysis [48,49], has been applied to genome-scale metabolic networks [50], which is currently not possible with EMA [37]. Flux balance analysis has been used to investigate central carbon and energy metabolism of a two species consortium [27]. Flux balance analysis could likely be applied to the presented model system and could be

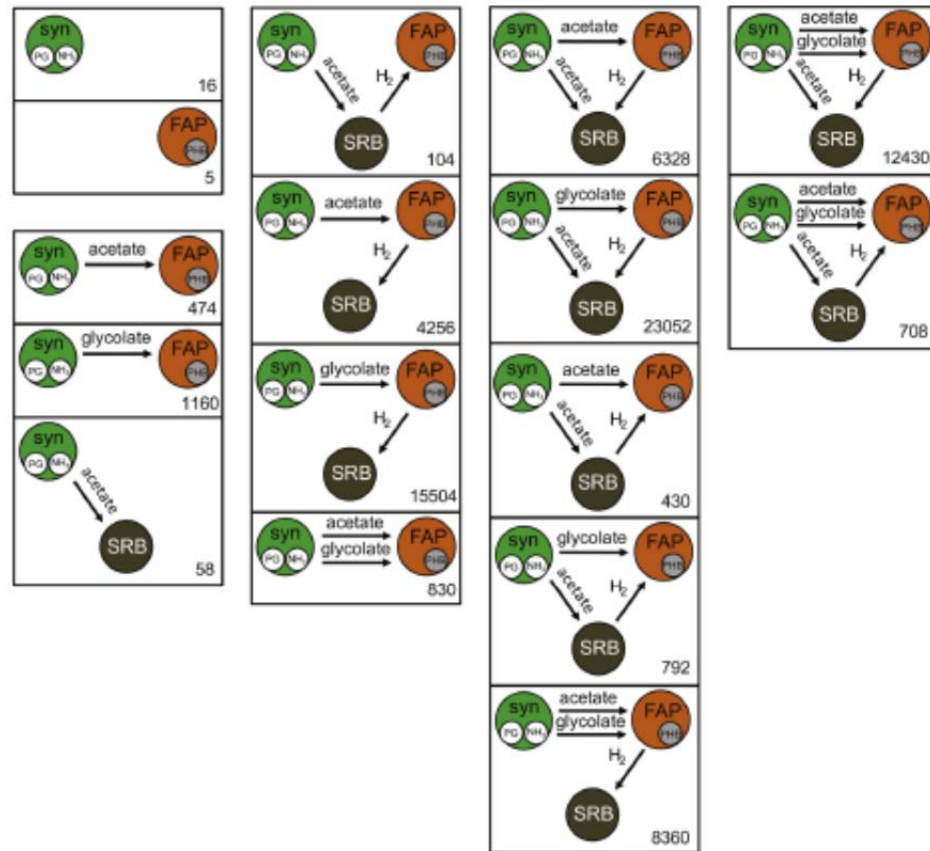


Figure 5

Daytime compartmentalized guild interaction classes. All 74,507 elementary modes identified in the daylight compartmentalized model, binned into 16 classes of guild interactions. Number of modes belonging to each classification is listed in lower right. Columns from left to right contain interaction classes requiring an increasing number of inter-guild metabolite transfers. Abbreviations: PG, polyglucose; PHB, polyhydroxybutyrate; syn, cyanobacteria (i.e. *Synechococcus*).

used to ask similar questions. The output, however, would be a single flux distribution denoting an optimal use of the network as defined by an objective function, rather than a generating set for all flux distributions allowed by the network (although methods have been established to examine the effects of variations in external constraints on the value of the objective function[51]). As the choice of an appropriate objective

function is not always clear prior to extensive experimentation [52,53], a generating set is useful for exploratory modeling. The utility of a generating set for explanatory modeling has also been recently demonstrated [24]. These benefits justify the development of methods for the application of EMA to multispecies systems alongside large-scale network methods. It is worth considering how the methods developed here will

be affected by larger network sizes. This is important because potential applications include more diversified communities and more complex cellular metabolisms. Systems cast as compartmentalized models which prove to be too computationally heavy for current EMA algorithms can be analyzed using the nested approach.

The compartmentalized community analysis method has the advantage of intuitive tractability and separates activity and function by guild, but requires substantially more knowledge of the community than the pooled reactions approach. Tables 1 and 2 illustrate how the energy added to electrons during oxygenic photosynthesis is dissipated with the movement of mass and energy through the consortium. The cyanobacteria exhibit a predicted maximum biomass yield of 0.117 Cmoles for each mole of photons absorbed by the mat. This value is 0.078 for the FAP guild, with the difference providing a rough measure of the cost of transferring reducing equivalents between guilds. The SRB also rely on the by-products of the primary producer guilds for electrons, but are further constrained by an inability to derive energy from light or respiration on O_2 . The highest SRB biomass yield predicted by the daytime compartmentalized model is 0.016 Cmoles per absorbed mole of photons, slightly more than one tenth of the cyanobacterial yield. The ratio between theoretical optima for photosynthetic and SRB biomass yields is similar to observed ratios between primary producers and the secondary productivity of consumers in macroscale ecological systems [19,54].

The compartmentalized method also lends itself uniquely to investigation of the robustness of specific consortium interaction types (see Figure 5). Investigating interactions in the model showed that the FAP are central to the metabolic capabilities of the consortium as a whole; FAP involvement was required for the vast majority (99.9%) of elementary modes. Finally, the highest-yielding biomass-producing modes from the compartmentalized approach were combined to show that experimental data supports strategies optimizing productivity by individual guilds more strongly than strategies optimizing productivity for the entire consortium [19,16; Klatt et al., in preparation].

The pooled reactions consortium analysis method modeled community metabolic potential by treating all enzymatic activities and metabolites as residents of the same physical space, without membrane boundaries. The pooled reactions approach represents the coarsest-scale methodology, requires the least *a priori* information, and is easier to implement than alternative approaches. The pooled approach can often be used when other approaches cannot (due to complexity) or

should not (due to lack of detailed data). These advantages are balanced against a tendency to over-estimate the metabolic potential. This is unsurprising, as real communities are not super-organisms; individuals are membrane-separated and must contend with the logistics associated with matter and energy transport. The pooled technique is best for initial work on 'poorly' characterized systems. The vast majority of ecosystems are not characterized to a level permitting a compartmentalized analysis, suggesting the pooled approach will be relevant for a long time to come.

Finally, the nested community analysis has properties very similar to the compartmentalized approach, but with the important advantage of easy scalability, achieved by concatenating multiple rounds of EMA analysis. The approach also provides additional ecological insight into the competitive strategies underlying each guild's function. This information is contained within the criteria used to select first-round building blocks. The nested method also easily captures interactions between different guilds as well as between members of the same guild expressing different physiologies. For instance, different cyanobacterial elementary modes can combine, representing exchange of metabolites between cyanobacteria expressing different metabolic activity due to differing positions across spatial gradients of light, temperature, and concentration. This type of exchange appears to be very relevant to actual mat function, as other work suggests the presence of functionally distinct *Synechococcus* populations that are adapted to particular microenvironments [55]. As with the compartmentalized approach, the daylight case study found that optimal guild strategies were more strongly supported by experimental data than optimal consortium strategies.

Combinations of the pooled and compartmentalized methods can be used to provide information not easily obtainable with any single approach. As an ecologically relevant example, a cost-benefit relationship was quantified for a complete citric acid cycle in the cyanobacterial guild, as opposed to the incomplete cycle indicated by the genomic sequence of relevant isolates. *Synechococcus* spp. OS-A and OS-B' genomes do not contain the oxaloacetate-producing malate dehydrogenase. The pooled reactions modeling approach identified a cluster of optimal elementary modes using a primarily cyanobacterial metabolism along with the missing citric acid cycle enzyme, which is FAP-derived. The biomass yields in this cluster are a 9.4% improvement relative to purely cyanobacterial metabolic potential, given by the compartmentalized methodology. This is a large difference in yields and laboratory evolution experiments have shown that much smaller differences (0.5% difference in growth

rate) are selectable under competitive conditions [56]. These hot spring mat communities are thought to be modern derivatives of ancient prokaryotic communities, and the long history of cyanobacterial and FAP cohabitation suggests the potential for horizontal gene transfer. Evidence for actual horizontal gene transfer events between cyanobacteria and FAP has been published elsewhere [57]. The cyanobacteria in our study have not acquired and passed on the missing Krebs cycle gene, suggesting that the costs of production, maintenance, and regulation for malate dehydrogenase are unlikely to be offset by the benefit of a 9.4% improvement in biomass yield.

Conclusion

Each of the three *in silico* modeling approaches developed in this study provides a mathematical description of physical constraints on metabolic activity in a consortium. These techniques allow the extension of EMA to ecologically relevant multi-species, biofilm systems. Their contrasting strengths can be combined to arrive at a more holistic description than is possible with any of the methods alone, allowing a broad perspective from which to frame observations and base predictions. These approaches can be adapted to a wide range of microbial communities including both natural and anthropogenic systems. Potential applications include modeling communities involved in wastewater treatment, bioprocess engineering, and environmental remediation, as well as the study of host-pathogen interactions in medicine and symbiotic relationships such as nitrogen-fixing root communities.

Methods

Metabolic models

The metabolic models presented in this study exist in a control volume consisting of the upper 1 mm of the microbial mat. The steady state control volume considers biomass production but does not include an explicit biomass degradation term. To maintain steady state, biomass would leave the control volume at the same rate it is produced. This would correspond with the physical process of new growth burying old growth. Central carbon and energy metabolism network models for each guild considered to be in the control volume were constructed from literature reviews and annotated genomes of representative organisms including *Synechococcus* spp. OSA and OS-B' [58] as well as *Roseiflexus* sp. RS-1 [59]. GenBank accession numbers are CP000686.1, CP000239.1, and CP000240.1 for the RS-1, OSA, and OS-B' genomes, respectively. These genomes are highly representative (>90% nucleotide identity to metagenomic reads) of native dominant FAP and cyanobacterial populations in these mats (Klatt *et al.*, in preparation).

The metabolic potential of the SRB guild was based on several well-studied organisms (*Desulfovibrio vulgaris* Hildenborough, *Desulfotalea psychrophila*, *Desulfovibrio desulfuricans* G20, *Desulfobacterium* sp., and *Archaeoglobus fulgidus*), as well as *Thermodesulfobacterium yellowstonii*, a thermophilic SRB related to isolates from Mushroom Spring [6,60-63]. GenBank accession numbers for these genomes, in the stated order, are AE017285.1, CR522870.1, CP000112.1, CP001087.1, AE000782.1, and CP001147.1.

The use of guilds to minimize community complexity is useful for the compartmentalized approach. For the current study, guilds were defined based on four important parameters: energy sources, carbon sources, electron donors and electron acceptors. Each guild utilized a different combination of metabolites to satisfy these parameters. Subdominant guilds were not included in the model (e.g. methanogenic archaea). Here it is assumed that the methanogens would be responsible for degrading phototroph biomass buried within the mat. Since the control volume only considers the active phototrophic region, the decaying biomass would occur outside this control volume removing any possible role played by this subdominant guild.

The metabolic model input consists of the carbon and electron balanced substrates and products for each considered reaction. It should be noted that these organisms are not characterized at a level which would justify genome-scale reproductions. While such models are admirable for well documented organisms, problems with inaccurate, automated genome annotations call into question the benefit of such attempts with any but the best studied microbes [64]. The biomass compositions of these functional guilds have not been experimentally determined due to tight physical coupling of the cells within the microbial mats. The functional guilds reside within a distance of 100 μ m. Biomass reactions were written to represent bacterial biosynthetic requirements for a composition of 78% proteins, 16% nucleic acids, and 10% other macromolecules. While the biomass compositions of actual mat constituents were not measured, previous published accounts [e.g. [48]] as well as unpublished observations from the authors suggest that overall yields are relatively insensitive to the biomass equation as long as the biomass has a biologically relevant degree of reduction (e.g. approximately 4.8 on an N_2 basis). The supplemental material contains a degree of reduction analysis of the utilized biomass expression. Its degree of reduction is 4.7. The methods developed here could be applied with alternative biomass synthesis reactions based on future experimental knowledge. Carbon storage consisting of polyglucose or polyhydroxybutyrate was considered

separately from biomass production. The metabolic models used in this study are included in additional file 1: Supplement.

Separate models were constructed to represent two distinct phases of a diel cycle, day and night. Differences between the modeled phases were based on the extensive geo- and biochemical data at these sites [5,11,12,55,65] and included sources and shuttles for carbon and energy as well as oxygen and light availability (see Figure 1 for graphical summary). The day and night metabolisms are connected through the fluxes of reduced carbon and fixed nitrogen. The amount of CO_2 fixed during the day determines the mat's overall dark phase reduced carbon budget while the nitrogen fixed during the night using energy stored as polyglucose sets the consortium's fixed nitrogen budget.

Briefly, during the day, the *Synechococcus* spp. convert solar energy, CO_2 , and water into oxygen, ATP, and reduced carbon. Concurrently, the anoxygenic FAP derive energy from sunlight and exploit the reduced carbon (glycolate and acetate) produced by the *Synechococcus* guild. By-products of cyanobacterial activity also serve as carbon and energy sources for SRB. These interactions were used to guide selection of relevant organismal modes for the day nested approach.

At night, the mat was treated as anoxic due to the lack of oxygenic photosynthesis and low oxygen solubility at 60°C. This is a simplification: low levels of oxygen are actually available at the mat surface while most of the mat is anaerobic. The model *Synechococcus* guild ferments stored polyglucose into a variety of organic compounds including lactate, propionate, acetate, and formate as well as ethanol. In addition to being the primary producer of reduced carbon, *Synechococcus* is also thought to be the primary producer of fixed nitrogen for the mat [11]. These were the interactions used to guide first-round selection of modes for the night nested method. The nitrogen fixation reactions are considered solely in the night model because of the sensitivity of nitrogenase to oxygen. This could be a simplification of actual mat behavior: mRNA expression evidence and *in situ* activity assays suggest that a large fraction of nitrogenase synthesis occurs in the morning, when light-derived reducing equivalents are available but before the mat becomes oxic [5].

The Mushroom and Octopus Spring phototrophic mat communities are found within flowing geothermal springs. Consequently, it was assumed that the mass of each metabolite lost from the control volume due to convective transport was exactly equal to that gained by the control volume through convective transport from

upstream microbes. The gases CO_2 , O_2 and H_2 were not constrained by this treatment and could vent to the atmosphere.

The nested models require the defining of 'transfer metabolites', which are permitted to serve as metabolic sinks and sources for the individual guild simulations, but not for the community simulation. They were chosen based on knowledge of the springs and compounds commonly transported across bacterial membranes and are shown in Figure 1, associated with arrows between guilds. Individual guild networks were then evaluated for elementary modes with 'transfer metabolites' allowed to accumulate (or deplete). This was accomplished by defining them as external metabolites in CellNetAnalyzer. The resulting modes were sorted in Excel based on product synthesis yields considered relevant to the system. These criteria are listed in additional file 1: Supplement. The modes with high yields, which represent overall guild stoichiometries, were then used as inputs for the second round of EMA (with 'transfer metabolites' now treated as internal metabolites in CellNetAnalyzer). The first round of EMA used enzyme substrate and product stoichiometries to determine how the numerous enzymes can work together as a system, while the second round of EMA used guild stoichiometries to determine how the guilds could work together as a system.

Elementary mode analysis

Algorithms for calculating elementary modes from metabolic network topology are described elsewhere [46]. These algorithms are based on a field of mathematics known as convex analysis. Calculation of elementary modes in this work was performed using the software CellNetAnalyzer 9.0 and MATLAB v 7.6 [66]. CellNetAnalyzer is freely available to academics at <http://www.mpi-magdeburg.mpg.de/projects/cna/cna.html>, but requires MATLAB version 6.1 or higher. Due to computational burden, the models had to be constructed to avoid missing essential enzymatic activity while still permitting a computationally tractable network. Necessary simplifications included rounding, neglecting the accumulation of fermentation products at night, combining macromolecular syntheses into 'lumped' reactions based on draws from the central metabolism, using a proxy for cellular maintenance energy requirements, and streamlining treatment of nitrogen storage. The models presented in additional file 1: Supplement were used to produce all discussed results. A new algorithm for EMA has been recently described that permits the use of a 64 bit linux based system [37]; this will likely permit analysis of larger networks than was previously possible. Algorithms for parallel processing have been

proposed and will likely reduce the need for simplification, but are not yet publically available [67].

Authors' contributions

DMW, RG, RPC, and WPI were the authors responsible for conception and design of the experiment. Data was acquired, analyzed, and interpreted by CGK, JEA, KB, NM, RPC, RT, SM, SM, and ZJ. The manuscript was drafted by CGK, JEA, KB, NM, RPC, RT, SM, SM, and ZJ. DMW, RG, RPC, and WPI revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

Additional material

Additional file 1

Supplement. This spreadsheet contains all additional information referenced in the text. This material includes the criteria used to select nodes during the first round of pruning for the nested models, a short illustration of the mechanisms used to separate metabolites by guild for the modeling methods, the script used to normalize first-round vector for the nested approach, the full error results from the nested models, a sample calculation illustrating how nodes were combined in the daytime case study, and the final models used to produce all discussed results (along with diagrams of the individual and pooled metabolic networks). Final network models will be submitted in SBML format to the BioModels database <http://www.ebi.ac.uk/biomodels-main/> upon manuscript publication.
Click here for file
<http://www.biomedcentral.com/content/supplementary/1752-0509-3-114-S1.xls>

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APPENDIX B

STRONG INTER-POPULATION COOPERATION LEADS TO PARTNER
INTERMIXING IN MICROBIAL COMMUNITIES

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Strong inter-population cooperation leads to partner intermixing in microbial communities

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Abstract Patterns of spatial positioning of individuals within microbial communities are often critical to community function. However, understanding patterning in natural communities is hampered by the multitude of cell-cell and cell-environment interactions as well as environmental variability. Here, through simulations and experiments on communities in defined environments, we examined how ecological interactions between two distinct partners impacted community patterning. We found that in strong cooperation with spatially localized large fitness benefits to both partners, a unique pattern is generated: partners spatially intermixed by appearing successively on top of each other, insensitive to initial conditions and interaction dynamics. Intermixing was experimentally observed in two obligatory cooperative systems: an engineered yeast community cooperating through metabolite-exchanges and a methane-producing community cooperating through redox-coupling. Even in simulated communities consisting of several species, most of the strongly-cooperating pairs appeared intermixed. Thus, when ecological interactions are the major patterning force, strong cooperation leads to partner intermixing.

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Introduction

Biological interactions drive pattern formation at different levels of organization (Murray, 2003), ranging from developmental patterning within multicellular organisms and biofilms (Shapiro, 1998; Lewis, 2008; Vlamakis et al., 2008; Chuong and Richardson, 2009), to ecological patterning within multi-species communities (Levin, 1992; Rietkerk and van de Koppel, 2008; Momeni et al., 2011). Patterning, reflecting the relative spatial positioning of individuals with respect to each other, can be critical for the proper functioning of a community. Consider microbial communities: in a synthetic community, three bacterial species, each contributing an essential benefit while simultaneously competing for these benefits, can only grow when they are separated by an intermediate distance (Kim et al., 2008); different types of patterning are correlated with different levels of biofilm growth (Christensen et al., 2002; Brenner and Arnold, 2011); branching colony morphology allows more effective spreading across a nutrient-poor surface (Levine and Ben-Jacob, 2004); and in waste treatment granules, the layered pattern of bacteria and archaea is thought to facilitate the sequential degradation of substrates (Sato et al., 2007).

Despite the wide-ranging importance of microbial communities in, for example, human health and the biogeochemical cycling of elements, it is still unclear how cell-cell and cell-environment interactions govern the patterning of communities (Elias and Banin, 2012). Understanding the mechanistic basis of pattern formation from observations of natural communities is stymied by the multitude of cell-cell and cell-environment interactions, as well as environmental variations within and across communities. Thus, it is not uncommon to observe qualitatively different patterns in samples of essentially the same type of community (Christensen et al., 2002; Wilmes et al., 2008; Dekas et al., 2009).

eLife digest Microorganisms such as bacteria, archaea and tiny eukaryotes are found throughout the biosphere. Some of these microorganisms are pathogens that cause diseases in animals, while others provide nutrients, including essential amino acids and vitamins; there are also microorganisms that have critical roles in recycling elements such as carbon, nitrogen and oxygen in the biosphere. In the natural world, microorganisms interact with their environment and with each other, often competing for space, light and nutrients, but sometimes they act cooperatively, which benefits all parties involved.

Microbial communities exhibit spatial patterns that reflect the relative positioning of different microbes in a community. These patterns can be critical for the proper functioning of a microbial community. For example, in the microbial granules that digest organic compounds in waste water, the stratified pattern of different microbial species can be thought of as a sequence of catalysts needed to perform a series of biochemical processing steps. Thus, it is important to understand the mechanisms that drive pattern formation in multispecies communities.

Now, through a combination of simulations and experiments, Momeni et al. have identified two features of spatial patterns in two-population microbial communities when pattern formation is driven by fitness effects related to the ecological interactions between cells. First, interactions that confer significant advantages to at least one of the populations can potentially result in the generation of a stable community; the community is stable in the sense that if it is disturbed, it will return to its stable population composition following the disturbance. Indeed, in engineered *Saccharomyces cerevisiae* communities, very different initial population ratios converged to the same value over time when one strain depended on the other strain, or when the two strains depended on each other, but not when the two strains competed.

The second feature applies to microbial communities composed of two cooperating populations: whereas two populations that compete with each other tend to segregate, cooperation results in the members of the two populations mixing together. Momeni et al. observe the formation of such an “intermixed” community in simulations, and also in two experimental systems that involve cooperation—a community containing two different strains of yeast cooperating through metabolite exchange, and a biofilm in which *Methanococcus maripaludis*, an archaeon that produces methane, cooperates with the bacterium *Desulfovibrio vulgaris*.

These two features of spatial patterning are conceptually similar to the competitive exclusion principle, which states that two species competing for the same resources cannot stably coexist if competition is the sole force at work. This principle has, therefore, encouraged scientists to search for the other forces that must be responsible for the coexistence of different species. Similarly, by predicting the sorts of patterns that will form when the fitness effects of ecological interactions between cells are the only forces at work, Momeni et al. lay the groundwork for investigations into other mechanisms, such as cell–environment interactions and active cell motility, that can govern pattern formation in microbial communities.

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To circumvent the lack of control in natural communities, we employed mathematical and experimental systems to systematically investigate how different types of ecological interactions might lead to distinct community patterning. Interactions can be classified into different ecological types based on their fitness effects on the interacting partners. We focused on the fitness effects rather than the molecular mechanisms of interactions, because diverse molecular mechanisms, ranging from physical associations in cell coaggregates and biofilms (Kolenbrander et al., 2010) to chemical interactions such as quorum sensing (Parsek and Greenberg, 2005), toxin warfare (Vetsigian et al., 2011), and metabolite supply (Christensen et al., 2002), all have fitness consequences which can be positive, neutral, or negative. Among different ecological interactions, we have placed a special emphasis on strong cooperation, interactions with large positive fitness effects on both partners including obligatory cooperation. This is because 1) it is important in a wide variety of microbial communities ranging from syntrophic systems critical for nutrient cycling (Schink, 2002; Falkowski et al., 2008; McInerney et al., 2009) to pathogenic biofilms (Kelly, 1980; Kolenbrander et al., 2010; Elias and Banin, 2012); and 2) the codependence

between cooperative partners poses special challenges for isolating and culturing cells (Schink, 2002; McInerney et al., 2009).

We investigated patterning in three-dimensional communities grown from two fluorescently-marked populations of cells initially randomly distributed on top of a surface (Figure 1A). Starting with a generalized model based on fitness effects of ecological interactions between two populations (A and B) occurring at a local scale ('fitness model'), we predicted: 1) interactions benefiting at least one partner could potentially allow initially disparate partner ratios to converge over time, and 2) unlike other types of ecological interactions that caused partner segregation or layering of one population over the other (A over B or B over A), strongly cooperating partners intermix by forming patches that successively accumulated on top of each other (A over B over A over B, etc). We tested these predictions experimentally in obligatory cooperative systems including engineered yeast communities and syntrophic methanogenic biofilms. Finally, we used the fitness model to show 'strongly cooperating partners intermix' could be generalized to communities consisting of multiple species.

Results

All microbial communities exhibit intra- and inter-population competition as cells compete for shared resources, including space. Therefore, for simplicity, we use [$\sim$] to denote the 'baseline' competition. The fitness effect of baseline competition can start at zero if shared resources are in excess, but will eventually become negative as shared resources become limited. In 'addition' to competition, interactions between two partners can exert positive, negative, or no fitness effects on one or both partners. The 'net' fitness effects of all interactions between two partners, including competition, on the two partners can be represented as two symbols in a square bracket. There are six possibilities: [$\sim$] (no fitness effects other than those from baseline competition), [$\sim$ +] (commensalism, in which one partner experiences nothing more than competition whereas the other enjoys an overall fitness benefit even when competition has been taken into consideration), [$\sim$ -] (amensalism), [- -] (mutual antagonism), [- +] (victim-exploiter), and [+ +] (cooperation). The identities of partners may be added to the notation such that A[- +]B would mean that the overall interaction (including competition) inhibits A and promotes B. Under this notation, inter-population toxin-warfare (Vetsigian et al., 2011) would be [- -] while inter-population cooperation based on the exchange of distinct net benefits would be [+ +]. Some interactions may at the first sight seem cooperative, but the net interaction may turn out not to be cooperative after considering the negative effects of competition (Kim et al., 2008). In the game theory definition, cooperative acts incur fitness costs to the performers. Here we have taken a more liberal definition of cooperation to include mutually-beneficial interactions that may or may not involve fitness costs.

Predicting ecological patterning in simple communities using a generalized fitness model

To search for ecological patterning rules in microbial communities, we built a three-dimensional fitness model that ignored molecular details and instead focused on the fitness effects of interactions ('The fitness model' in 'Materials and methods'). Specifically, two populations of cells, marked as red and green, were initially randomly distributed on a surface (Figure 1A). Cells grew horizontally until sufficiently confined, at which point they grew upward to accommodate the birth of new cells (Figure 1—figure supplement 1, Video 1). Thus, no active cell motility was present during community growth. The growth rate of a focal cell was determined by its basal fitness as a single cell and by its interactions with other cells in a defined interaction neighborhood. To reflect the negative fitness effects of intra- and inter-population competition for shared resources ([$\sim$]), the fitness of the focal cell was decreased as the total population size in the interaction neighborhood increased. In addition, the focal and partner cells affected each other's fitness positively (+), negatively (-), or neutrally ($\sim$). The magnitude of fitness effect is quantitative. Thus, to obtain qualitative ecological patterning rules, we focused on strong interactions in which + and - exert fitness effects large enough to be comparable to the recipient's basal fitness. In most simulations using the fitness model, the basal fitnesses of both partners were non-zero, and therefore + represented strong facultative interactions: a participant could survive on its own at its basal fitness but fared much better in the presence of its partner.

We first analyzed the population composition of communities formed in the fitness model. Simulations (Figure 1—figure supplement 2 and Figure 1—source data 1) and analytical calculations

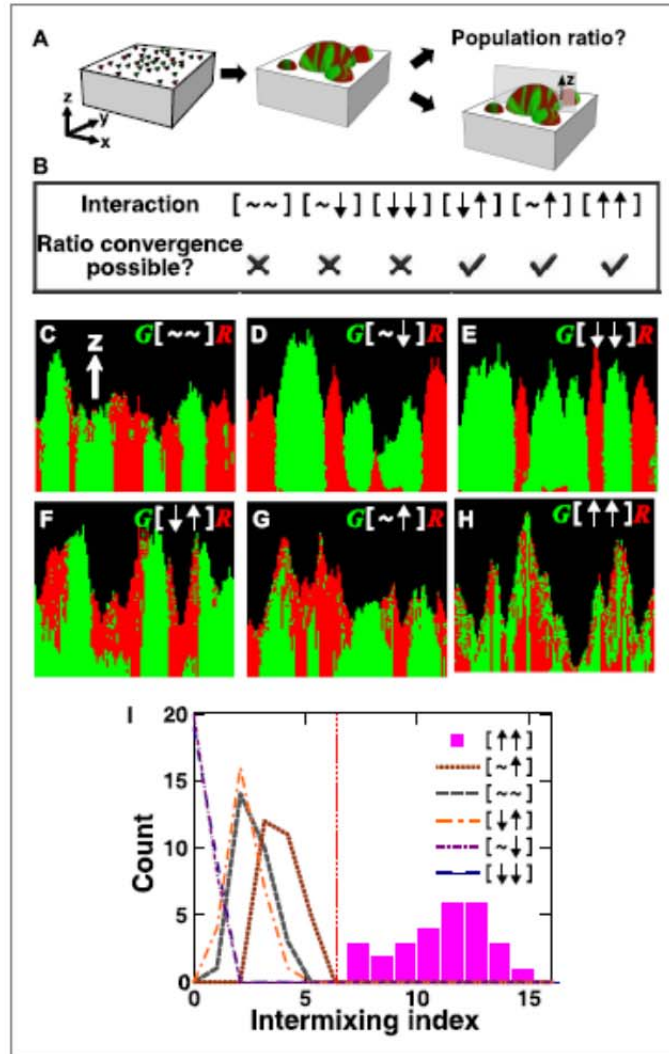


Figure 1. The fitness model generates two ecological patterning predictions. (A) In all simulated and experimental communities (see 'Materials and methods'), two populations of cells, marked in red and green, were initially randomly distributed on a surface unless otherwise stated. The two populations engaged in one of the six ecological interactions. Population ratios for the entire community and patterns of vertical cross sections were examined. (B) The fitness model predicts that strong interactions beneficial to at least one partner can potentially lead to the convergence of initially disparate population ratios (Figure 1—figure supplement 2). (C)–(H) Representative vertical cross-sections of simulated communities, each engaging in one of the six types of Figure 1. Continued on next page

Figure 1. Continued

ecological interactions, are presented. The fitness effects of + and + are large compared to the non-zero basal fitness of the recipient (Figure 1—source data 1), and therefore [+ +] is strong facultative cooperation.

(f) Vertical cross-sections of single-cell thickness from cooperative communities show significantly more intermixing than those from other communities ($n = 28$ sections; $p < 0.01$, Mann-Whitney U test). An intermixing index of 6 (red dotted line) or above separates cooperative from non-cooperative communities in our simulations. To reduce the correlation of sections sampled from the same community, nearest sections were separated by at least seven sections.

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The following source data and figure supplements are available for figure 1:

Source data 1. Parameter values used in the fitness model.

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Figure supplement 1. Cell rearrangement in simulations follows experimental observations on cells that are not actively motile.

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Figure supplement 2. The fitness model predicts that convergence of population ratios is possible when an interaction benefits at least one partner.

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Figure supplement 3. Strong mutual antagonism can lead to rapid divergence of population ratios.

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Video 1. Yeast cells bud to the sides when there is available space and bud upward when sufficiently confined (corresponding to Figure 1—figure supplement 1A). To infer the process of cell rearrangement in three-dimensional communities, we monitored how single YFP-fluorescent yeast cells grew into microcolonies on top of solid agarose. Initially, dividing cells pushed other cells to the side such that all cells remained in the same plane. When a cell was sufficiently confined from the sides by other cells (approximately within a 5-cell radius), it could no longer bud to the side and instead budded upward, as indicated by higher intensities in the fluorescence images. Continued growth of microcolony forced more cells in the middle to send their progeny to upper layers, while cells close to the edge could still push other cells to the side and remain on the agarose surface. All images are taken with the same exposure time. Scale bar is 20 μm .

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(‘Requirements for steady-state ratios in the six types of communities’ in ‘Materials and methods’) show that for interactions benefiting at least one partner, including cooperation ([+ +]), commensalism ([~ +]), and exploitation ([+ -]), different initial partner ratios can potentially converge over time (Figure 1B). Ratio convergence requires ‘balanced’ fitness. For example, for A[~ +]B to achieve ratio convergence, the basal fitness of A must be higher than that of B and after gaining the fitness benefit from commensalism, B must be able to grow at least as fast as A. This way, a situation incompatible to ratio convergence, that is, one partner always fitter than the other, does not occur. Using engineered competitive, obligatory commensal, and obligatory cooperative yeast communities (see below), we confirmed that population ratios converged in the commensal and cooperative but not competitive communities (Figure 2—figure supplement 1). This convergence of population ratios reflects a balance between supply and consumption when growth is limited by supply (Shou et al., 2007): if, for instance, the supplier population suddenly increased in relative abundance, then each individual in the consumer population would receive more benefit and grow faster, which would return the ratio to its original value.

We next examined patterns in vertical cross-sections of communities simulated by the fitness model (Figure 1A, parameters in Figure 1—source data 1), because patterning along the

x and y directions can depend on the initial spatial distribution of cells whereas patterning along the vertical z direction results from growth under the fitness influences of ecological interactions. In [~ ~], [~ +], and [+ +], red and green populations primarily formed columns that are spatially segregated from each other (Figure 1C–E). In [+ -] and [~ -], frequently one of the populations (green) either

formed a column or became covered by the partner population (red) (Figure 1F–G). Only in cooperation ($\uparrow \uparrow$) conferring large fitness benefits to both partners, the two partner populations appeared to be extensively ‘intermixed’, manifested as the two different cell types successively piling on top of each other (Figure 1H).

To compare levels of intermixing in different communities, we defined an ‘intermixing index’ as the average number of cell type transitions spanning community height (‘Spatial analysis’ in ‘Materials and methods’). Since the intermixing index can be a function of community height, we compared intermixing indexes of simulated communities at equivalent heights. Statistically significant differences were observed between strong cooperation versus other types of interactions (Figure 1I). Thus, we predicted that partner intermixing would distinguish strong cooperation from other ecological interactions.

Partner intermixing in engineered obligatory cooperative yeast communities and a corresponding diffusion model

To test the prediction that strong cooperation is the only ecological interaction capable of driving partner intermixing, we engineered yeast communities engaged in competitive ($\sim \sim$), obligatory commensal ($\sim \uparrow$), and obligatory cooperative ($\uparrow \uparrow$) metabolic interactions. Competitive communities represented the baseline intra- and inter-population competition common to ‘all’ communities, while commensalism served as the most stringent control to be discriminated against.

All engineered yeast communities consisted of two non-mating *S. cerevisiae* strains, a G strain expressing GFP or YFP and an R strain expressing DsRed (see ‘Materials and methods’). In competitive communities, prototrophic R and G competed for shared nutrients in agarose and for space (Figure 2—figure supplement 1A). Depending on their genetic backgrounds, the two strains engaged in either equal-fitness or unequal-fitness competition. In obligatory commensal communities, R^{+A} took in lysine from the media and overproduced adenine to feed the adenine-requiring G^{+A} (Figure 2—figure supplement 1B). In obligatory cooperative communities (previously described as Cooperation that is Synthetic and Mutually Obligatory, or ‘CoSMO’), R^{+A} overproduced adenine and required lysine while G^{+A} overproduced lysine and required adenine (Shou et al., 2007) (Figure 2—figure supplement 1C). When mixed, the two cooperative strains could form a viable community, growing from low to high densities in synthetic minimal medium (SD) lacking adenine and lysine (Shou et al., 2007).

To predict and extrapolate experimental results of yeast communities, we developed a three-dimensional model based on the consumption, release, and diffusion of metabolites in the yeast communities (‘the diffusion model’ in ‘Materials and methods’). Specifically, the diffusion model assumed that metabolites diffused in the community and agarose (Figure 2—figure supplement 2) and that cell growth depended on the local concentration of its limiting metabolite according to Monod’s equation (Monod, 1949). Most parameters in the diffusion model were measured experimentally (Figure 2—source data 1). We reasoned that if predictions from the diffusion model were consistent with experimental observations, we could use this model to simulate experiments that would be technically difficult to perform.

To examine vertical patterning in yeast communities, we first used top-view time-lapse images to infer patterns which were subsequently verified by cryosectioning. This is because confocal and two-photon microscopy cannot penetrate deep into yeast communities (Váchová et al., 2009). In competitive communities, whether in the diffusion model or experiments, time-lapse top-views suggested population segregation. For equal-fitness competitive communities (Figure 2A, left; Video 2), individual cells (i) grew into microcolonies (ii) which continued to grow and expand until they reached neighboring microcolonies (iii). After this stage, cells were unable to push other cells to the side, and further cell divisions mainly occurred in the vertical z direction (Figure 1—figure supplement 1). Consequently, columns of primarily a single cell type formed, and top-views of the community remained static (compare iv and v). Competitive communities composed of populations with different fitnesses developed similarly (Figure 2A, right; Video 3), except that the fitter population expanded in the top view during growth (compare the green population in iv and v).

In obligatory commensal communities, time-lapse top-views suggested a ‘layered’ pattern with one population covering the other (Figure 2B; Video 4). We found that in both the diffusion model and experiments, R^{+A} initially grew rapidly by consuming lysine in the agarose (compare i, ii, and iii). In contrast, G^{+A} initially grew slowly, presumably limited by the low level of adenine released by R^{+A} .

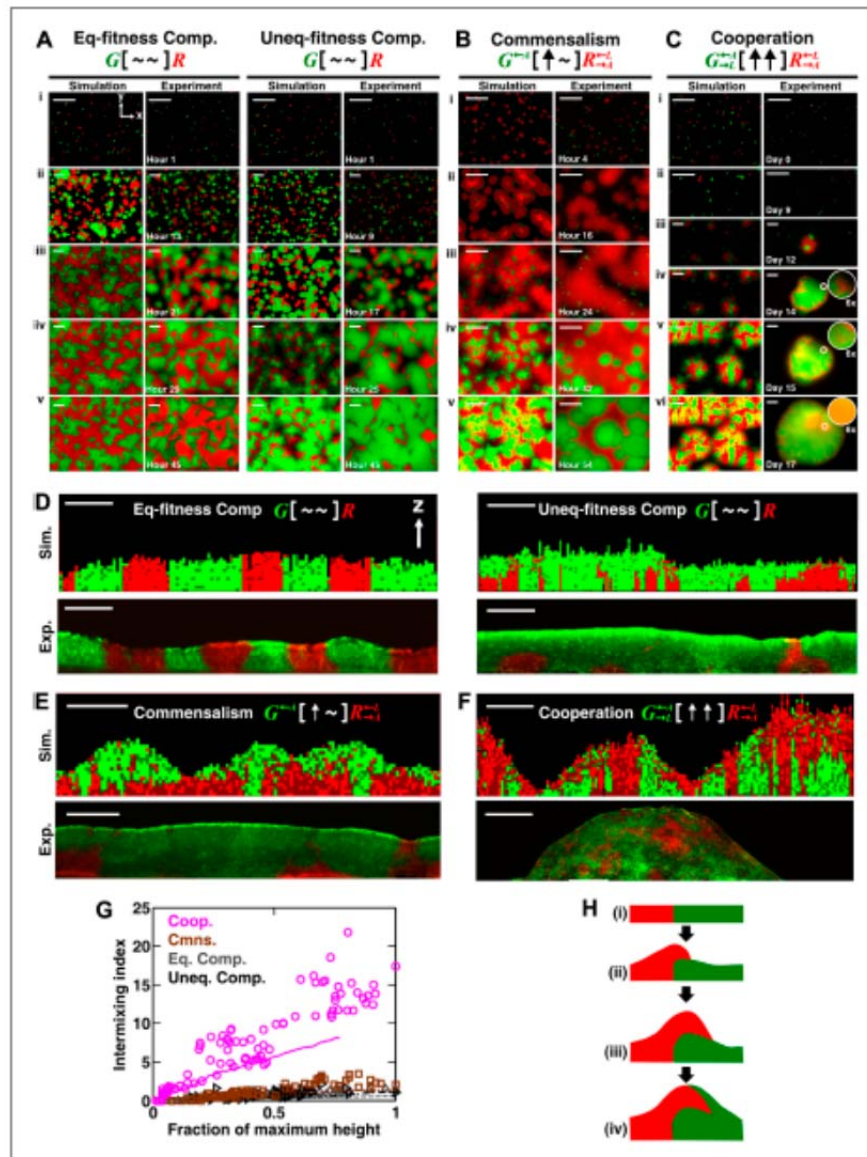


Figure 2. Obligatory cooperation, but not competition or obligatory commensalism, results in substantial partner intermixing in engineered yeast communities and in communities simulated using the diffusion model. Competitive communities of strains with equal fitness (equal-fitness competition, abbreviated as 'Eq-fitness Comp.') showed population segregation as suggested by static late-stage top-views (A, left) and columnar cross-section

Figure 2. Continued on next page

Figure 2. Continued

patterns (D, left). When competing strains had different fitnesses (unequal-fitness competition, abbreviated as 'Uneq-fitness Comp.'), the fitter population G expanded during growth, as evident in top views (A, right) and vertical cross-sections (D, right). In obligatory commensal communities, since one supplier could support the birth of multiple consumers, consumers eventually overgrew and covered suppliers (top-views in B and vertical cross-sections in E). Obligatory cooperative communities showed substantial population intermixing as suggested by alternating cell types in top-views (C, 6x magnification insets in experiments) and patchy cross-section patterns (F). Top views of communities from the diffusion model integrate intensity and color over height such that brighter colors represent higher cell numbers and yellowness indicates the simultaneous presence of green and red. Scale bar: 100 μm . All communities started from total 500 cells/ mm^2 and $R:G = 1:1$. (G) Quantification of intermixing in experimental (symbols) and diffusion-model (lines) communities showed that while the intermixing index remained low for commensal (brown) and competitive (grey and black) communities, it increased linearly with community height in obligatory cooperative (magenta) communities. Results from the diffusion model underestimated intermixing indexes because a confined cell was modeled to divide strictly vertically upward (Figure 1—figure supplement 1) without allowing cell movements in horizontal directions (Figure 3—figure supplement 1F). (H) A conceptual model illustrates the development of intermixing over time in a strongly cooperative community with 1:1 steady-state population ratio. Local deviations from the steady-state ratio result from asymmetric partner properties and/or stochastic fluctuations in cell numbers (i). The under-abundant population (red) grows faster than its neighboring over-abundant partner (green). Due to the spatial localization of large cooperative benefits, cells near population borders grow faster than those farther away. Consequently, cells from the initially under-abundant red population at the border divide the fastest. Progeny that pile on the green partner have more access to cooperative benefits than those who do not (ii), which favors intermixing. When the previously over-abundant partner becomes under-abundant (iii), piling-up in the opposite direction occurs (iv, green on red).

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The following source data and figure supplements are available for figure 2:

Source data 1. Definitions and values of parameters used in the diffusion model.

DOI: 10.7554/eLife.00230.010

Figure supplement 1. In engineered yeast communities, obligatory cooperation and obligatory commensalism allow initially different partner ratios to converge over time.

DOI: 10.7554/eLife.00230.011

Figure supplement 2. Basic assumptions in the diffusion model.

DOI: 10.7554/eLife.00230.012

Figure supplement 3. Cooperative communities exhibit a characteristic patch size associated with the spatial localization of benefits.

DOI: 10.7554/eLife.00230.013

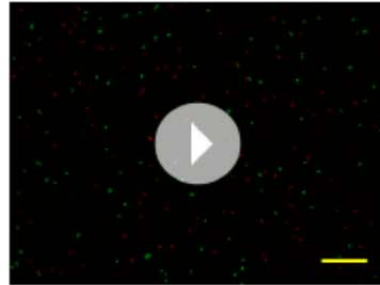
Figure supplement 4. Obligatory cooperative yeast partners intermix.

DOI: 10.7554/eLife.00230.014

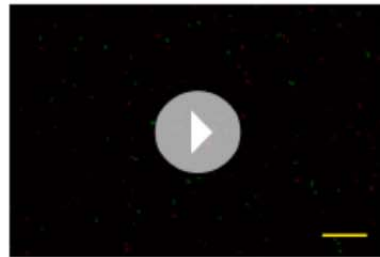
before $R_{\rightarrow A}^{+}$ became abundant (compare i, ii, and iii). During the growth of $R_{\rightarrow A}^{+}$, some G^{+A} cells had been pushed to the top layer of the community (iii). As the $R_{\rightarrow A}^{+}$ population continued to expand and release adenine, G^{+A} started to grow rapidly (compare iii and iv). Eventually, $R_{\rightarrow A}^{+}$ stopped growing after lysine in the agarose had been depleted. Since the amount of adenine released during the lifetime of every $R_{\rightarrow A}^{+}$ cell could support the birth of multiple G^{+A} cells (Shou et al., 2007), G^{+A} population outnumbered and covered $R_{\rightarrow A}^{+}$ (v).

In contrast, time-lapse top-views of obligatory cooperative communities suggested population intermixing (Figure 2C; Video 5). After plating on agarose lacking adenine and lysine (i), cells in both populations divided once or twice by utilizing metabolites stored in their vacuoles (Shou et al., 2007). $R_{\rightarrow A}^{+}$ released adenine and entered the death phase while $G_{\rightarrow A}^{+}$ continued to grow by utilizing the released adenine (ii) (Shou et al., 2007). $G_{\rightarrow A}^{+}$ entered the death phase and released lysine after a significant delay due to their better starvation tolerance compared to $R_{\rightarrow A}^{+}$ (Shou et al., 2007). Released lysine supported the growth of surviving $R_{\rightarrow A}^{+}$ cells into microcolonies (iii). $R_{\rightarrow A}^{+}$ in turn released adenine which promoted growth of nearby $G_{\rightarrow A}^{+}$ cells and led to partial covering up of $R_{\rightarrow A}^{+}$ microcolonies by rapidly growing $G_{\rightarrow A}^{+}$ (iv and v, insets). Abundant $G_{\rightarrow A}^{+}$ cells provided enough lysine for rapid growth of local $R_{\rightarrow A}^{+}$ cells, which gave rise to patches of $R_{\rightarrow A}^{+}$ cells on top of the community (vi, inset). These community growth kinetics were consistent with previous measurements in liquid cultures (Shou et al., 2007).

To confirm that partners intermixed in cooperative but not non-cooperative communities, we obtained vertical cross-sections of competitive, commensal, and cooperative communities at their maximal community heights ('Cryosectioning' for experimental communities in 'Materials and methods'). We found that in both the diffusion model and experiments, equal-fitness competitive communities formed segregated columns (Figure 2D, left). Unequal-fitness (Figure 2D, right) and obligatory commensal communities (Figure 2E) displayed a layered pattern in which the top



Video 2. Top views of an equal-fitness competitive community suggest population segregation (corresponding to Figure 2A, left). Competitive communities of strains with equal fitness showed population segregation as suggested by static late-stage top-views. The community started from a uniform distribution of total 500 cells/mm² and R:G = 1:1. Intensities of both fluorescent channels in images at different times were normalized to the same maximum value for better representation of patterns throughout growth. Blanks in the video were due to removal of the dish to sample other replicate communities for flow-cytometry or sectioning. Scale bar is 100 μ m.
DOI: 10.7554/eLife.00230.015



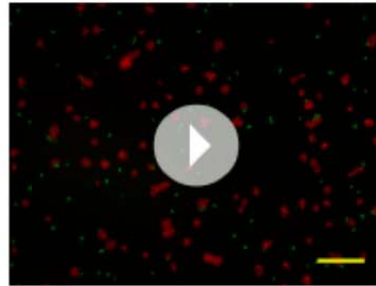
Video 3. In unequal-fitness competition, the fitter population gradually covers the less fit population (corresponding to Figure 2A, right). Here, G is fitter than R. The community started from a uniform distribution of total 500 cells/mm² and R:G = 1:1. Intensities of both fluorescent channels in images at different times were normalized to the same maximum value for better representation of patterns throughout growth. Scale bar is 100 μ m.
DOI: 10.7554/eLife.00230.016

large cooperative benefits cause partners to 'take turns' to grow, which leads to population intermixing (Figure 2H). In summary, in communities engaging in strong cooperation, but not in communities governed by other types of ecological interactions, the intermixing index increases proportionally as a function of community height. This proportionality is due to a fixed patch size determined by localized nutrient supply and consumption.

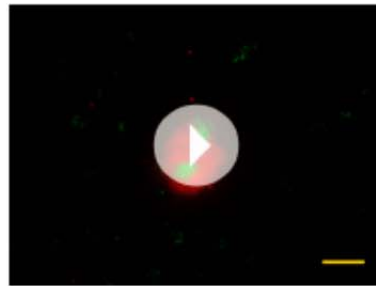
portion of a community was dominated by one partner. In contrast to competitive and obligatory commensal communities, obligatory cooperative communities appeared to show population intermixing with patches of red and green cells emerging on top of each other (Figure 2F and Figure 2—figure supplement 4A and B). Cooperative intermixing appeared to be less in the diffusion model than in yeast communities. This is presumably because the diffusion model assumed that a confined cell would bud strictly upward whereas in yeast communities, cell divisions could stray to the side (Figure 3—figure supplement 1F).

To compare levels of intermixing in different communities, we quantified the intermixing index of each community (Figure 2G, symbols for experiments and lines for results from the diffusion model). As expected, equal-fitness competition resulted in small intermixing indexes at all community heights (Figure 2G, grey). In unequal-fitness competition (Figure 2G, black) and obligatory commensalism (Figure 2G, brown), the formation of a layered pattern caused a small increase in the intermixing index that subsequently leveled off. In contrast, the intermixing index in obligatory cooperative communities increased proportionally to community height in both experiments and the diffusion model (Figure 2G, magenta). This proportionality suggested the existence of a characteristic patch size ('Spatial analysis' in 'Materials and methods'), denoted λ_c , of 10–20 μ m. The characteristic patch size was independent of initial conditions (Figure 2—figure supplement 3A). Indeed, a calculation of the patch size based on experimentally-determined release, diffusion, and consumption of exchanged metabolites yielded comparable results ('Calculating the characteristic patch size in cooperative yeast communities' in 'Materials and methods').

What caused partner intermixing during cooperation? Using the diffusion model, we found that if cooperative benefits were not spatially localized because of instant distribution of benefits throughout the community or because of excessive supply levels, intermixing was diminished (Figure 2—figure supplement 3B). Based on this result and based on patterns observed in top-views and vertical cross-sections of cooperative communities, we propose that local deviations from the steady-state ratio coupled with localized



Video 4. Top views of an obligatory commensal community suggest population layering (corresponding to Figure 2B). For a detailed explanation of the growth kinetics of the community ($R_{\text{co}}^{+A} [\sim +] G_{\text{co}}^{+A}$), please refer to the main text. The community started from a uniform distribution of total 500 cells/mm² and $R_{\text{co}}^{+A} : G_{\text{co}}^{+A} = 1:1$. Intensities of both fluorescent channels in images at different times were normalized to the same maximum value for better representation of patterns throughout growth. Blanks in the video were due to removal of the dish to sample other replicate communities for flow-cytometry or sectioning. Scale bar is 100 μm . DOI: 10.7554/eLife.00230.017



Video 5. Top views of an obligatory cooperative community suggest populations intermixing (corresponding to Figure 2C). For a detailed explanation of the growth kinetics of the community ($R_{\text{co}}^{+A} [+] G_{\text{co}}^{+A}$), please refer to the main text. The community started from a uniform distribution of total 500 cells/mm² and $R_{\text{co}}^{+A} : G_{\text{co}}^{+A} = 1:1$. Intensities of both fluorescent channels in images at different times were normalized to the same maximum value for better representation of patterns throughout growth. Blanks in the video were due to removal of the dish to sample other replicate communities for flow-cytometry or sectioning. The video started at ~300 hr, after the formation of R_{co}^{+A} microcolonies. Scale bar is 100 μm . DOI: 10.7554/eLife.00230.018

cooperative benefits is not growth-limiting. However, cooperative benefits are unlikely to be available in large excess because of the potential fitness cost of generating benefits and because of competition

Strongly cooperative partners intermix under a wide range of conditions

Under what conditions can we observe partner intermixing in cooperation? First, we experimentally tested partner intermixing in obligatory yeast cooperative communities initiated under different population ratios and densities. Next, we used the diffusion model to examine communities in which obligatory cooperative partners interacted with different dynamics. Finally, using the fitness model, we tested intermixing in facultative cooperation by varying the relative magnitude of cooperative benefits compared to the basal fitness of the two interacting populations. We utilized yeast communities when experimentally possible and otherwise took advantage of the diffusion and the fitness models.

We found that intermixing was insensitive to initial conditions in the yeast obligatory cooperative communities. The initial partner ratio did not significantly affect the level of intermixing (Figure 3—figure supplement 1D). At very high initial cell densities, we observed significant intermixing in communities directly above the inoculation area even in the absence of cooperation (Figure 3—figure supplement 1E, yellow shading). This is because high cell densities put different cell types in close proximity to one another, and cell divisions that were not perfectly straight upward (Figure 3—figure supplement 1F) caused intermixing. However, we reasoned that communities beyond the inoculation area might reveal patterns indicative of the underlying interactions, because these regions are formed by cell growth under the fitness influences of interactions. To test this possibility, we spotted cell mixtures at high densities on agarose and allowed the community to expand to new territories beyond the inoculation area. Even though communities directly above the inoculum always appeared highly intermixed (Figure 3A, 'Center' and Figure 2—figure supplement 4C), in edge sections, significant intermixing was only observed in cooperative communities (Figure 3A, 'Edge' and Figure 2—figure supplement 4D).

Intermixing is insensitive to interaction dynamics, so long as large cooperative benefits remain sufficiently localized for both partners. As described above, in the diffusion model, excessive supply amounts or instant distribution of benefits throughout the community diminished intermixing (Figure 2—figure supplement 3B). The former can occur if the local availability of

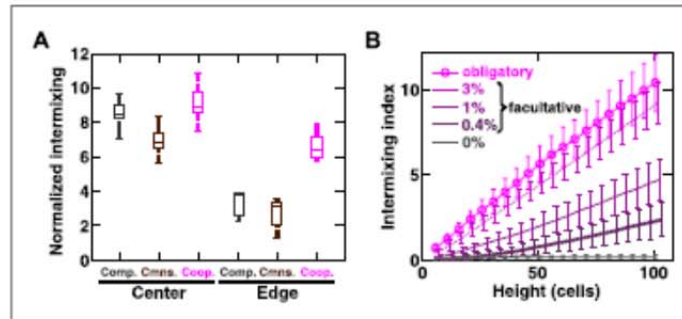


Figure 3. Strongly cooperating populations intermix under a wide range of conditions. (A) In engineered yeast communities, even though both obligatory cooperative and non-cooperative communities directly above the high-density inoculation spot showed high population intermixing ('Center'), edge sections ('Edge') of obligatory cooperative communities showed significantly more intermixing than those from non-cooperative communities (Mann-Whitney U test, $p < 5 \times 10^{-3}$). A total of 10^6 R and G cells at a 1:1 ratio were deposited in an inoculation spot of ~ 2 mm², corresponding to 10 cell layers. Communities were allowed to grow and expand beyond the inoculation spot on a 4-mm-tall agarose pad of 500 mm² area. Vertical sections from the edges were taken at a height approximately half of the maximum community height at the center. Box plots show the 25th to 75th percentile range, with the median marked with a line and whiskers extending to the 95% confidence interval. (B) In simulations using the fitness model, facultative cooperation conferring smaller fitness benefits required greater community heights to exhibit a significant level of intermixing. The strength of facultative cooperation is shown as the ratio of fitness benefit received from each cooperative partner cell in the interaction neighborhood to the basal fitness of the focal cell. Simulation parameters can be found in [Figure 3—source data 1](#). Error bars indicate 95% confidence interval.

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The following source data and figure supplements are available for figure 3:

Source data 1. Parameter values used in the fitness model in [Figure 3B](#).

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Figure supplement 1. Intermixing is observed in obligatory cooperative communities over a wide range of conditions.

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for these benefits in the partner population. As far as diffusion is considered, the diffusion model showed significant population intermixing in obligatory cooperative communities with diffusion constants varying more than 10-fold ([Figure 3—figure supplement 1A](#)). Additional calculations showed that intermixing was largely insensitive to diffusion kinetics, because the characteristic patch size was related to the diffusion constant by 1/5 power ('Calculating the characteristic patch size in cooperative yeast communities' in 'Materials and methods'). The diffusion model also showed that intermixing was present with or without a delay in G_{-L}^A supplying lysine benefits to the partner ([Figure 3—figure supplement 1B](#)). Furthermore, even though asymmetry between properties of partners ([Figure 2C](#)) would seem to facilitate intermixing ([Figure 2H](#)), asymmetry in partner properties was not required to generate intermixed patterns: partners that grew, died, and released and consumed metabolites at identical rates intermixed ([Figure 3—figure supplement 1C](#)).

Using the fitness model, we found that in facultative cooperation, small fitness benefits generated less intermixing than large fitness benefits ([Figure 3B](#)). This result is intuitive: facultative cooperation should resemble obligatory cooperation if fitness benefits are large for both partners. When only one partner receives a large fitness benefit, facultative cooperation should resemble obligatory commensalism. Finally, small fitness benefits for both partners will make facultative cooperation resemble competition. In facultative cooperation with smaller fitness benefits, intermixing would be apparent if communities could grow to greater heights. Further experiments are required to test these predictions. Together, these results suggest that intermixing relies on spatial localization of cooperative benefits that are sufficiently large to both partners, and is otherwise insensitive to initial conditions or the detailed kinetics of interactions.

Communities of naturally mutualistic microbes exhibit intermixing

To test whether cooperative patterning applies to other biological systems, we examined redox-coupling in a two-species methane-producing biofilm consisting of the bacterium *Desulfovibrio vulgaris* and the archaeon *Methanococcus maripaludis*. In the absence of sulfate and hydrogen, the two species engage in obligatory mutualism: *D. vulgaris* ferments lactate and promotes the growth of *M. maripaludis* by supplying the electron donor H_2 , while *M. maripaludis* promotes the growth of *D. vulgaris* by consuming the H_2 gas which can be inhibitory to *D. vulgaris* under these conditions (Figure 4A). Similar types of syntrophic interactions leading to methane production typically occur in microbial consortia that digest organic compounds in freshwater sediments, sewage treatment plants, and the guts of ruminants (Conrad et al., 1985; Schink, 1997).

Vertical cross-sections of independent *D. vulgaris*-*M. maripaludis* biofilms indeed exhibited increasing intermixing as a function of community height (Figure 4B). Thus, naturally mutualistic microbes cooperating through a coupling mechanism different from metabolite exchange also exhibited a significant level of intermixing. Other known cooperative communities, including those degrading herbicide pollutants (Breugelmans et al., 2008) and those colonizing teeth (Palmer et al., 2001) also seemed to display intermixed patterns, although we do not know whether the intermixing indexes of these communities increased linearly with height.

Most of the strongly-cooperative pairs intermix in simulated multi-species communities

In communities with more than two species, indirect interactions can obscure direct interactions (Wootton, 2002). For example, if A promotes B which inhibits C, it will appear that A inhibits C. This is akin to indirect interactions between gene products in a cell. To test whether intermixing between

cooperators was affected by other members of a community, we used the fitness model to simulate communities composed of five interacting species (Figure 5 and Figure 5—figure supplement 1). We randomly assigned one of the six possible ecological interactions between each pair of populations, and consequently, each network had 10 pair-wise interactions. The fitness effects of $+$ and $-$ were sufficiently large to be comparable to the recipient's basal fitness. A total of 240 pair-wise interactions in 24 independent communities were examined in the fitness model. In most cases (26 out of 31), cooperative pairs intermixed (Figure 5A,D). Occasionally, commensal pairs (Figure 5B, ② [$\sim +$] ③) showed substantial intermixing (3 out of 58 commensal pairs, Figure 5D), or cooperative pairs (e.g., Figure 5C, ① [$+$] ③) showed little intermixing (5 out of 31, Figure 5D), presumably due to interactions through other community members (Figure 5B, ③ indirectly promoted ② through ①; Figure 5C, ③ promoted ② which inhibited ①). These deviations are consistent with the notion that the intermixing index reflects the 'overall' interaction between two partners, integrating any additional fitness effects of indirect interactions through other community members. Thus, strongly cooperating partners intermix while deviations from this expectation reflect the presence of strong indirect interactions.

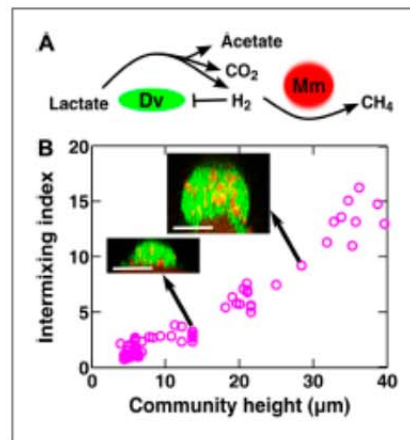


Figure 4. Obligatory cooperation through redox-coupling leads to partner intermixing. (A) In the absence of sulfate and hydrogen, the bacterium *Desulfovibrio vulgaris* (Dv) and the archaeon *Methanococcus maripaludis* (Mm) cooperate through redox coupling. Dv ferments lactate and produces mainly acetate, CO_2 , and H_2 . However, this reaction is not thermodynamically favorable unless H_2 is kept at very low concentrations. H_2 is used by Mm to reduce CO_2 to methane. (B) In cooperative biofilms of Dv (green) and Mm (red), the intermixing index increased with height. Cell identification relied on FISH (see 'Materials and methods'). Scale bar: 20 μm .

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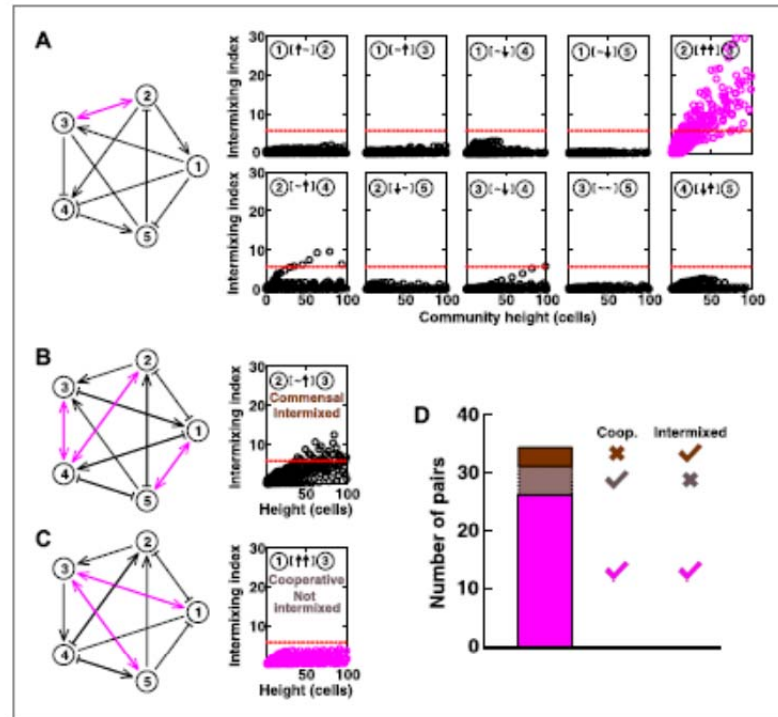


Figure 5. Most of the strongly-cooperative pairs intermixed in simulated five-species communities. (A–C) Examples of networks in which cooperative pairs intermixed (A), non-cooperative pairs intermixed (B), or cooperative pairs did not intermix (C) are shown. In the schematic network diagrams, line termini of $\rightarrow$, $\leftarrow$, and $\leftrightarrow$ represent $\rightarrow$, $\leftarrow$, and $\leftrightarrow$, respectively; cooperative pairs are highlighted in magenta. Simulations were performed using the fitness model. The basal fitness for each population was chosen randomly from a range spanning 0.03–0.05/hr. The fitness effect from each partner cell in the interaction neighborhood was either 0 for $\rightarrow$, or otherwise randomly chosen to be approximately 2–3% of basal fitness to achieve strong interaction. Initial population ratios were randomly assigned such that no population was initially lower than 5% of the total population. Simulations were run for 10 generations, and vertical cross-sections of the final communities were examined for intermixing. We considered intermixing index exceeding a threshold of 6 (Figure 1f) as significant (red dotted lines). The remaining nine panels in Figure 5B and C are provided in Figure 5-figure supplement 1. (D) Quantifying intermixing in a total of 240 interactions from 24 independent communities showed that most of the cooperative pairs intermixed (magenta). Indirect interactions through other community members could make cooperative pairs not intermixed (gray) or non-cooperative (commensal) pairs intermixed (brown). DOI: 10.7554/eLife.00230.023

The following figure supplements are available for figure 5:

Figure supplement 1. The complete results of Figure 5B and C (panel A and B, respectively).

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Discussion

Using controlled systems to identify patterns driven by ecological interactions

Patterning is driven by cell–cell and cell–environment interactions. Here, we examined how the net fitness effects of cell–cell interactions could influence patterning. Using defined mathematical and

biological systems under controlled spatial environments, we have established two expectations for ecological patterning between two interacting partners.

The first expectation is that interactions conferring large fitness benefits to at least one partner ($[+ +]$, $[+ -]$, and $[- +]$) can potentially result in ratio convergence (Figure 1—figure supplement 2 and Figure 2—figure supplement 1). Ratio convergence confers stability to community composition. The composition of a community is defined to be locally (or globally) stable if after small (or any-size) deviations from it, either due to initial conditions different from it or perturbations, the community eventually returns to this composition. An analytical calculation based on the fitness model showed that in $[+ +]$ and $[+ -]$, global stability can be achieved whereas in $[- +]$, local stability can be achieved (Figure 1—figure supplement 2; ‘Requirements for steady-state ratios in the six types of communities’ in ‘Materials and methods’). Experimentally, obligatory cooperation and obligatory commensalism led disparate initial ratios to converge (Figure 2—figure supplement 1). In contrast, in competition between two populations of equal fitness, population ratio is determined by the initial value until a perturbation resets the value which will remain unchanged until the next perturbation. In competition between two populations of unequal fitness, the fitter population should increase in frequency and therefore, population ratio is unstable. In amensalism and mutual antagonism, population ratios at best have an unstable fixed point (Figure 1—figure supplement 2B). This means that even if the population ratio may seem ‘fixed’, slight deviations will send it farther and farther away from the original fixed value. The reason for this instability, using mutual antagonism as an example, is because if A inhibits B and B inhibits A ($A \downarrow B$), then an increase in population A will facilitate inhibition of B and therefore make B less able to inhibit A, further increasing the relative abundance of A.

The second expectation is that strong cooperation results in partner intermixing in the direction vertical to the surface of initial colonization. Cooperative intermixing requires spatially localized benefits that are sufficiently large to both partners (Figure 2—figure supplement 3 and Figure 3B), and otherwise appears to be robust against variations in initial conditions (Figure 2—figure supplement 3A, Figure 3A, and Figure 3—figure supplement 1D) or reaction dynamics (Figure 3—figure supplement 1A–C). We have observed cooperative intermixing in the fitness model simulating strong facultative cooperation (Figure 1) and in obligatory experimental systems including engineered yeast communities (Figure 2) and syntrophic biofilms (Figure 4). The intermixing indexes in these communities increased linearly as a function of community height, because these communities exhibited fixed-size patches associated with the spatial localization of benefits (Figure 2—figure supplement 3; ‘Calculating the characteristic patch size in cooperative yeast communities’ in ‘Materials and methods’). This is in stark contrast with other ecological interactions which lead to segregated or layered patterns (Figures 1 and 2) in which the intermixing index remained low or increased transiently before leveling off. In simulated multi-species communities, strongly cooperative pairs intermixed in most cases; cases where cooperative pairs did not intermix or non-cooperative pairs intermixed were likely caused by strong indirect interactions through other partners. In a theoretical study using a one-dimensional stepping-stone model, strongly cooperating partners also appeared much more mixed than competing partners (Korolev and Nelson, 2011).

Our work defines the expected pattern created by pair-wise interactions if the fitness effects of interactions are the main driving force of patterning. The generality of these ecological patterning conclusions awaits further experimental validations. Many simple communities grown in laboratory environments conform to these findings. For instance, competing bacterial species form a columnar or layered pattern (Palmer et al., 2001; Kreth et al., 2005; An et al., 2006; Hallatschek et al., 2007; Bernstein et al., 2012). *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13(FR1) show spatial segregation as competitors when grown on citrate, but when grown on chlorobiphenyl they engage in metabolic commensalism and exhibit a layered pattern (Nielsen et al., 2000). Similarly, when grown on a non-selective carbon source, *Comamonas testosteroni* WDL7 outcompeted and covered *Variovorax* sp. WDL1, but when fed with an aromatic compound as the sole carbon source, competition switched to cooperation and the two populations intermixed (Breugelmans et al., 2008). Cooperative oral bacteria species intermixed when grown on saliva (Palmer et al., 2001; Periasamy and Kolenbrander, 2009). If cooperative intermixing is suspected, then examining whether the intermixing index increases linearly as a function of community height will be informative to exclude transient increases.

Deviations from the expected ecological patterns suggest other major patterning forces

We have described the expected patterning between two cell populations if the fitness effects of interactions are the major driving force. These expectations are abstract in the sense that in reality, no interactions can exist in the absence of molecular mechanisms or an abiotic environment. Assigning expected patterns to different types of ecological interactions will allow us to identify deviations from expectations. Determining the causes of such deviations will help us better understand the biology of a community.

Fitness effects of ecological interactions rely on molecular mechanisms to manifest themselves. Different types of molecular mechanisms can conceivably alter patterning. For instance, in the fitness and the diffusion models and in the *S. cerevisiae* communities, cells divide upward when sufficiently confined horizontally. This type of cell rearrangement has also been observed in bacterial colonies (Kreft *et al.*, 1998) and was adopted to model biofilm growth (Xavier and Foster, 2007). However, it is conceivable that if at least one population actively moves (Houry *et al.*, 2012) or grows hyphae to penetrate the entire community, two populations might appear intermixed even if they do not cooperate. In biofilms of *Pseudomonas aeruginosa*, two populations differing only in fluorescent colors (Klausen *et al.*, 2003) showed modest intermixing even though the expectation for equal-fitness competition is a columnar pattern with an intermixing index close to zero (Figure 2D, left). This modest intermixing was caused by *P. aeruginosa* differentiating into non-motile 'stalk' cells that anchored to the surface and motile cells that climbed up to form the mushroom-like caps (Klausen *et al.*, 2003).

Environmental influences can also alter ecological patterning. For instance, if two cooperating populations have very different preferences for oxygen, then the two populations are likely not to intermix and instead form layers in which the aerobic population is exposed to oxygen while the anaerobic population is protected from oxygen.

What can cause variability in patterning?

Variability in patterning has been observed within and between communities (Christensen *et al.*, 2002; Wilmes *et al.*, 2008; Dekas *et al.*, 2009), even if they were grown in laboratory-controlled environments (Christensen *et al.*, 2002). Stochastic events such as environmental variability, mutations, or fluctuations in initial conditions can all lead to variable patterns. For instance, if the straight-columnar pattern expected for equal-fitness competition is observed for the majority of community but a layered pattern is observed in occasional locations, then fitter mutants may have arisen in these locations, initiating unequal-fitness competition (Hallatschek *et al.*, 2007; Korolev *et al.*, 2012). For mutually antagonistic interactions, the fitness model showed that population ratios can quickly diverge. Thus, which population eventually dominates depends on the initial population ratio (Figure 1—figure supplement 3). In this case, stochastic variations in initial conditions can result in dramatically different patterns, giving rise to a phenomenon equivalent to 'survival of the fittest'. Indeed, different patterns have been observed for communities formed by two antagonistic bacteria species (Kuramitsu *et al.*, 2007).

In summary, our work is conceptually analogous to that of the competitive exclusion principle (Gause, 1934). The competitive exclusion principle, also known as Gause's law, states that two species competing for the exact same resources cannot stably coexist. Analogous to how the competitive exclusion principle has created a framework to examine forces that cause species coexistence, our work on ecological patterning will hopefully lay the ground for examining mechanisms that shape patterning in microbial communities. We encourage comments, especially those pertaining to the generality of our conclusions, to be posted to the eLife website.

Materials and methods

Engineering yeast strains

In competitive communities, equal-fitness G and R strains were respectively WS931 (MATa ste3::kanMX4 trp1-289::pRS404(TRP)-ADHp-venus-YFP) and WS937 (MATa ste3::kanMX4 trp1-289::pRS404(TRP)-ADHp-DsRed.T4), both from the S288C background. For unequal-fitness competitive communities, G was replaced by WS1246 (MATa ho::loxP AMN1-BY Supercontig17(27163-27164)::TDH3p-yEGFP-loxP-kanMX-loxP) from the RM11 background with a 20% fitness advantage over the S288C background. In commensal communities, R₁ and G⁺ strains were respectively WS950 (MATa ste3::kanMX4

lys2Δ0 ade4::ADE4(PUR6) trp1-289::pRS404(TRP)-ADHp-DsRed.T4) and WS932 (*MATa ste3::kanMX4 ade8Δ0 trp1-289::pRS404(TRP)-ADHp-venus-YFP*), both from the S288C background. In cooperative communities, R_{max}^+ and G_{max}^+ strains were respectively WS950 and WS954 (*MATa ste3::kanMX4 ade8Δ0 lys21::LYS21(fbr) trp1-289::pRS404(TRP)-ADHp-venus-YFP*) from the S288C background.

Culturing communities

For yeast communities, agarose columns were prepared by pouring SD minimal medium (Sherman, 2002) with 2% low melting temperature agarose in 1.3-ml deep-well plates (U96 DeepWell from Nunc, Penfield, NY), and after agarose had solidified, adding drops of melted SD agarose to make the surface flat. For commensal yeast communities, SD was supplemented with lysine (80 μM final concentration). Batch cultures of yeast strains were grown to exponential phase at 30°C in SD with supplements when necessary. Cells were washed free of supplements if any, mixed, and filtered on top of MF membrane (HAWP04700 from Millipore, Billerica, MA). Disks were cut from the membrane using a 6-mm-diameter Harris Uni-Core puncher and transferred to the top of agarose columns, unless otherwise stated.

For the coculture of *D. vulgaris* and *M. maripaludis*, a CDC reactor (BioSurface Technologies Corp., Bozeman, MT) was used for anaerobic biofilm growth. Biofilm coupon holders were modified to hold glass microscope slides (Fisher Scientific #12-544-1, Fisher Scientific, Pittsburgh, PA) cut to 7.6 × 1.8 cm. Cocultures were grown in CCM (Walker et al., 2009), a modified basal salt medium without choline chloride. Headspace was sparged with anoxic 80% N₂:20% CO₂, and the reactor was maintained at 30°C with stirring (150 rpm). The reactors were inoculated with planktonic coculture, and after cell attachment to the glass slides (48 hr), biofilms were allowed to develop and grow over time in the presence of planktonic cells, or initially drained of planktonic cells. Patterns in the two experimental regimes were similar.

Flow cytometry

Flow cytometry was performed in a modified 4-laser FACS Calibur machine (DxP; CyTek Development, Fremont, CA). Cells in communities were diluted to ~10⁶ cells/ml in H₂O. Each sample (90 μl) was supplemented with 10 μl of fluorescent bead stock (Thermo Scientific Fluoro-Max Cat# R0300 at ~8 × 10⁹/ml, counted using a Z2 Coulter counter and a hemacytometer) as a reference to determine total cell density and 3 μl of 1 μM ToPro3 (Invitrogen, Grand Island, NY) to mark dead cells. The laser and filter configurations for different fluorophores were 50 mW 488 nm laser with 530/30 filter for YFP, 75 mW 561 nm laser with 575/26 filter for DsRed, and 25 mW 639 nm laser with 660/20 filter for ToPro3. Using an automatic micro-sampling system (DxP; CyTek Development, Fremont, CA) samples in 96-well plates were processed for 60 s at a flow rate of 0.5–1 μl/s, recording 10⁴–10⁵ events. FlowJo software (Tree Star, Ashland, OR) was used to measure the ratio of different populations of fluorescent cell against the bead standard in order to calculate cell densities.

Fluorescence imaging

A Nikon TE2000 inverted microscope equipped with a Prior stage controller, a Sutter Lambda XL fluorescent lamp, and a Photometric SnapHQ CCD camera was controlled by custom LabVIEW software to auto-focus and acquire images. All images were taken at 10× magnification using a Nikon long working distance CFI Plan FI objective (NA 0.30, WD 16). For imaging YFP- and DsRed-tagged strains, ET500/20×-ET535/30m-T515LP and ET545/30×-ET620/60m-T570LP filter cubes were used, respectively. Timelapse imaging took place in a 30°C chamber (In Vivo Scientific microscope incubator).

Cryosectioning

To obtain vertical cross-sections of communities through cryosectioning, we froze communities in liquid nitrogen for 15 s and fixed them in methanol at −20°C. After 20 min, the communities were transferred to a pre-cooled empty plate at −20°C to allow methanol to evaporate, which typically took 4 hr. The communities were embedded in optimal cutting temperature (OCT) compound for 10 min at room temperature and subsequently frozen over dry ice and kept at −20°C for sectioning. We also froze down communities without fixing by directly embedding them in OCT (Piccirillo et al., 2010) and immediately freezing them on dry ice. Results obtained from the two procedures yielded similar conclusions.

For sectioning, communities embedded in OCT blocks were mounted on a cryotome. The blade was adjusted to ensure cross-sections of the community were vertical. For each community, typically

thirty to fifty 14- μm (3-cell-thick) sections were cut and transferred to glass slides. Cross-sections were imaged using the fluorescence microscope as described above. Only images of cross-sections that were minimally perturbed by the fixing and sectioning processes were included in the analysis. More details of the cryosectioning method can be found in (Momeni and Shou, 2012).

Fluorescence in situ hybridization (FISH)

Biofilms of *D. vulgaris* and *M. maripaludis* were fixed in 4% paraformaldehyde for 4 hr, and then embedded in polyacrylamide (Dajms *et al.*, 2006). The embedded biofilms were dehydrated and hybridized in 1 ml buffer solution (0.9 M NaCl, 20 mM Tris-HCl (pH 8), 0.01% SDS, and 35% deionized formamide) with 3 ng each of probes EUB338 (GCT GCC TCC CGT AGG AGT) 5' and 3'-labeled with Cy3 and ARCH915 (GTG CTC CCC CGC CAA TTC CT) 5' and 3'-labeled with Cy5 (Stoecker *et al.*, 2010) for 5 or 8 hr at 46°C in a humid chamber. Next, samples were washed in 50 ml prewarmed washing buffer (70 mM NaCl, 20 mM Tris-HCl pH 8, and 5 mM EDTA) at 47°C for 20 min, then dipped in ice cold ddH₂O and quickly dried with compressed air (Amann *et al.*, 1995). Finally, each sample was mounted with Citifluor AF1 antifadeant (Citifluor Ltd., Leicester, United Kingdom) and viewed using a Leica TCS SP5 II inverted confocal laser scanning microscope with 488, 561 and 633 nm lasers and appropriate filter sets for Cy3 and Cy5. Confocal voxel size was typically 0.24 $\times$ 0.24 $\times$ 0.49 μm .

Spatial analysis

For both simulated and experimental sections, our unit of analysis was the size of one CCD camera field of view under a 10 $\times$ objective which has a width of 0.7 mm. In any unit of analysis, community height is the value such that 90% of height values are below it. This choice is made to exclude artifacts such as height spikes in simulations. Images of experimental community sections were rotated in ImageJ such that the x - z axes of the frame matched those of the community. We further digitized these images into $f(x,z)$ with assigned values of +1, -1, and 0 for pixels identified as population 1, population 2, and no-signal, respectively. No-signal pixels were defined as having a fluorescence intensity per unit exposure time less than 10–20% above the background in fluorescence channels. For the remaining pixels, the intensity values of 90th percentiles were found in each fluorescence channel. These values were used to normalize the corresponding green- or red-fluorescence intensity for each pixel whose identity was then assigned to be the color with the higher normalized value. In simulated communities with more than two populations, analysis was performed on two focal colors at a time, and in each analysis, pixels identified as other colors were treated as no-signal.

To compare levels of intermixing in different communities, we estimated the number of cell type transitions spanning community height. We define the intermixing index as the average number of color changes along community height: let $h(x_i)$ and $c(x_i)$ respectively be the local height and the number of color changes along z at the lateral position x_i . The intermixing index IM can be calculated as

$$IM = \left(\sum_i c(x_i) h(x_i) \right) / \left(\sum_i h(x_i) \right). \quad (1)$$

Intermixing is small for segregated patterns with few color changes along height, and increases when patches of different cell types successively appear on top of each other. Note that this choice of intermixing index yields small values when one population is very rare compared to the other. We weighted $c(x_i)$ by the local community height $h(x_i)$, thus giving more emphasis to taller regions. This is because taller regions have gone through more growth regulated by the fitness effects of interactions.

To estimate the vertical patch size λ_z^* , the ratio of height $h(x_i)$ to number of cell-type layers, $1 + c(x_i)$, was averaged across the community cross-section

$$\lambda_z^* = \left(\sum_i [h(x_i)]^2 / [1 + c(x_i)] \right) / \left(\sum_i h(x_i) \right). \quad (2)$$

For engineered yeast communities, typically 10–20 frames from different locations of each community were included in the analysis to ensure an unbiased representation of community patterns. For biofilms of *D. vulgaris* and *M. maripaludis*, vertical cross-sections $\sim 2.4 \mu\text{m}$ apart were sampled from confocal z -stack images of biofilms.

Calculating the characteristic patch size in cooperative yeast communities

The characteristic patch size can also be calculated as the effective length of interaction between two partners. We calculate how far inside the community released lysine can diffuse before being consumed; similar discussion applies to adenine. Assume one cell has released β_l fmole of lysine that diffuses at most a distance l before being consumed. We define the sphere of radius l as the diffusion domain of the release event. The number of consuming cells within the diffusion domain can be estimated as $N_c = (2l/c)^3$, where c is the diameter of a cell. The average time nutrient can diffuse in the diffusion domain before being consumed is $t_c = R/2D_l$, where $D_l = 360 \mu\text{m}^2/\text{s}$ is the diffusion constant within the community. Assuming that each consuming cell takes up lysine with a rate $v \leq \alpha_l/T$, with α_l being the amount of lysine required for a cell division and T being the minimum doubling time, we have

$$\beta_l = N_c v t_c \leq \left(\frac{2l}{c}\right)^3 \frac{\alpha_l}{T} \frac{l^2}{2D_l};$$

thus,

$$l \geq \left[\frac{\beta_l D_l T c^3}{\alpha_l} \right]^{1/5} \approx 50 \mu\text{m}.$$

Within radius l , the average number of release events within time t_c is $(2l/c)^2 t_c d_a \approx 0.2$. Thus, the probability of a second release event occurring in the same diffusion domain before nutrient from the first release event has been consumed is low. Therefore, l defines the interaction length scale. Note that diffusion constant contributes by fifth root to l , and therefore, different diffusion constants in the community should not considerably alter patterns, as observed in simulations (Figure 3—figure supplement 1A). The calculated l of $50 \mu\text{m}$ is larger than the experimental patch size λ_c^* of ~ 10 to $20 \mu\text{m}$. What could account for this discrepancy? Experimentally measured diffusion constant of Sulforhodamine 101 in community ($20 \mu\text{m}^2/\text{s}$) reduces l to $\sim 30 \mu\text{m}$. In addition, cells may take up more nutrients than what is required for producing one daughter and may store extra nutrients in vacuoles (Shou *et al.*, 2007), further reducing the estimated interaction length scale.

The fitness model

The individual-based fitness model followed cell growth in a three-dimensional simulation grid of $100 \times 100 \times 300$ cells with periodic boundary conditions along the x and y directions. Consider population i ($i = R$ or G) interacting with population j ($j = G$ or R). Without loss of generality, consider a focal cell from population i . The growth of the focal cell is influenced by cells in its cubic three-axial interaction neighborhood (Deutsch and Dormann, 2004) defined by i -cell-width to the left, right, front, back, above, and below. Let ϕ_l and ϕ_j be the fraction occupancy of i and j in the interaction neighborhood, respectively. The growth rate of the focal cell is $r_i = [r_{i0} + r_i \phi_j (1 - \phi_i)] [1 - \chi(\phi_i + \phi_j)]$. r_{i0} is the basal fitness (growth rate of i without any interactions); $r_i \phi_j (1 - \phi_i)$ represents the fitness effect on i by j , which increases with partner abundance and decreases with recipient abundance due to intra-population competition for partner; $[1 - \chi(\phi_i + \phi_j)]$ reflects intra- and inter-population competition for shared resources with fitness decreasing as the neighborhood becomes more occupied. Cells were inoculated in the bottom surface of the simulation grid. In each simulation time step Δt , the probability of cell division is $r_i \Delta t$. A cell would divide either to the side if there was space within its (x, y) planar confinement neighborhood of n -cell radius or upward otherwise (Figure 1—figure supplement 1). Parameters used are listed in Figure 1—source data 1. $\chi = 0.8$, $i = 3$, and $n = 2$ in all cases. See Source code 1 for an example (and Source code 4 for the MATLAB function).

The diffusion model

The individual-based diffusion model followed actions of cells (nutrient uptake, cell division, cell death, and possibly release of nutrients) and the distribution of nutrients in a three-dimensional simulation grid. Since cell division and death occur at a time-scale much longer than diffusion and nutrient uptake, we used a multi-grid scheme in both space and time. In this model, a three-dimensional simulation domain consisted of cell grids representing individual cells and nutrient grids representing nutrient

concentrations (Figure 2—figure supplement 2). Cell actions and nutrient distributions were updated at discrete time steps over the simulation domain. Simulations were typically performed over an agarose domain of 0.75-mm length $\times$ 0.75-mm width $\times$ 24-mm depth and a community domain of 0.75-mm length $\times$ 0.75-mm width $\times$ 0.3-mm height with parameters listed in Figure 2—source data 1.

Nutrient concentrations as a function of space and time are based on the diffusion equation

$$\frac{\partial S}{\partial t} = \nabla \times (D \nabla S) - U + Q, \quad (3)$$

with

$$U = v_m \frac{S}{S + K_{MM}} n_u \quad (4)$$

$$Q = \rho n_q. \quad (5)$$

Equation (3) states that S , the amount of limiting nutrient in a diffusion grid, depends on three processes: i) diffusion of nutrient with diffusion constant D , ii) uptake of nutrients (Walther *et al.*, 2005) by cells (U), and iii) in cooperative communities, release of nutrients by the partner population (Q). In equations (4) and (5), n_u and n_q are the number of consuming and releasing cells within the diffusion grid, respectively, K_{MM} is the Michaelis-Menten constant for uptake, v_m is the maximum uptake rate per cell, and ρ is the release rate per cell. To solve this diffusion equation, we used a finite difference time-domain method (Crank, 1980) with no-flow ($\partial S / \partial z = 0$) boundary conditions applied to the top and bottom surfaces of the simulation domain and periodic boundary conditions applied to the four vertical sides of the domain.

Cell growth rate r in the model is dictated by Monod's equation

$$r(S) = r_m \frac{S}{S + K_M}, \quad (6)$$

in which r_m is the maximum growth rate when nutrients are abundant, S is the concentration of the limiting nutrient, and K_M is the S at which half maximal growth rate is achieved. We assume that individual cells take up nutrients with $K_{MM} = K_M$ and once they have accumulated the required amount of the limiting nutrient, cell division occurs.

To incorporate realistic assumptions about cell rearrangement upon division in the community, we monitored single yeast cells growing into microcolonies on solid media (Figure 1—figure supplement 1A). Initially, each cell budded in the same plane and pushed others in its immediate neighborhood to the side. Once a cell was completely surrounded on each side by roughly five cells, it budded upward. The same process was implemented in the diffusion model (Figure 2—figure supplement 2). It should be noted that by forcing the confined cells to bud strictly upward, the model underestimates intermixing. As a result, communities show more vertical features in simulations than experiments (Figure 2F).

Temporally, the update time-step for nutrient diffusion and uptake (~ 1 s) was smaller than that for cell division and death (~ 360 s). Spatially, cell actions took place on a cell grid with single-cell resolution (5 μ m), while nutrient distributions were followed on a diffusion grid at lower spatial resolution (~ 15 to 60 μ m). These values were selected considering the trade-off between simulation time and accuracy, while ensuring the stability of simulations. For instance, at each time-step within a diffusion grid, the total amount of nutrients consumed should be considerably smaller than available nutrients. In other words, assuming that the length of a diffusion grid is nc where c is the length of a cell grid which is equivalent to the size of a cell, there are at most n^3 cells in each diffusion grid and

$$S(nc)^3 > n^3 v_m \frac{S}{S + K_{MM}} dt_u, \quad (7)$$

where S is the concentration of the limiting nutrient in the spatial grid, K_{MM} is the Michaelis-Menten coefficient for nutrient uptake, v_m is the maximum uptake rate, and dt_u is the uptake time-step. After simplifying equation (7), we obtain

$$dt_u < \frac{S + K_{MM}}{v_m} c^3. \quad (8)$$

Table 1. Summary of steady-state occupancy, conditions to achieve steady-state, and the stability of steady-state for six types of ecological interactions.

Interaction	Steady-state occupancy	Steady-state condition	Stability
$G[\sim\sim]R$	Any ϕ_G	Only when $r_{G0} = r_{R0}$	Unstable
$G[+]R$	$\phi_G = \frac{r_{G0} - r_{R0} + r_{R1}}{2r_{R1}}$	$r_{R1} > r_{G0} - r_{R0} $	Globally stable
$G[\sim+]R$	$\phi_G = \sqrt{\frac{r_{R1} - r_{G0}}{r_{R1}}}$	$r_{R1} > r_{R0} - r_{G0} > 0$	Unstable
$G[\sim-]R$	$\phi_G = \sqrt{\frac{r_{G0} - r_{R1}}{r_{R1}}}$	$r_{R1} > r_{G0} - r_{R0} > 0$	Globally stable
$G[+]R$	$\phi_G = \frac{r_{R1} - r_{G0} + r_{R0}}{2r_{R1}}$	$r_{R1} > r_{G0} - r_{R0} $	Unstable
$G[+]R$	$\phi_G = \frac{1}{2} + \sqrt{\frac{r_{G0} - r_{R0}}{2r_{R1}} - \frac{1}{4}}$	$r_{R1} > r_{G0} - r_{R0} > 0$ $r_{R1} < 2(r_{G0} - r_{R0})$	Locally stable (when $\phi_G > \phi_{G,c}$; see Figure 1—figure supplement 2)

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In the worst case scenario of S being much smaller than K_{MM} , using parameters of our engineered yeast strains, we find $dt_u \sim 0.5$ s. Thus, we chose $dt_u = 0.5$ – 1 s as the time-step for updating the nutrient uptake and diffusion equations. To ensure stability of the finite-difference equations for diffusion, the diffusion grid-size (nc) and the time-step (dt_u) for the diffusion equation have to satisfy (Iserles, 2009)

$$dt_u < \frac{1}{2} \frac{(nc)^2}{D}, \quad (9)$$

where D is the diffusion constant in the region of interest. From this relation, we chose diffusion grid size $nc = 50$ μm , and consequently, each diffusion grid contains $10 \times 10 \times 10$ cells (Figure 2—figure supplement 2A). Since diffusion is fast within each grid (~ 1 s), this choice of grid size is unlikely to introduce a notable error in our calculations. See Source codes 2 and 3 for examples (and Source code 4 for the MATLAB function).

Requirements for steady-state ratios in the six types of communities

Let ϕ_R and ϕ_G be the fraction occupancy of R and G in an interaction neighborhood, respectively. ϕ_G is the fraction occupancy of G that leads to equal fitness of the two populations and thereby result in a steady state ratio within the interaction neighborhood. Following the assumptions of the fitness model, the growth rate of each population is $r_i = [r_{i0} + r_i \phi_j (1 - \phi_i)] [1 - \chi(\phi_i + \phi_j)]$ where $i = R$ or G and $j = G$ or R . For simplicity, we assume that $\phi_R + \phi_G = 1$, which leads to $r_i = r_i / (1 - \chi) = r_{i0} + r_i \phi_j^2$.

In two-population cooperative communities, using the simplifying assumption of $r_{R0} = r_{G0} = r_{R1} > 0$, we have $\hat{r}_G - \hat{r}_R = (r_{G0} - r_{R0} + r_{R1}) - 2r_{R1}\phi_G$ (Figure 1—figure supplement 2B). Setting $\hat{r}_G - \hat{r}_R$ to 0, the community can achieve a steady-state value of $\phi_G = (r_{G0} - r_{R0} + r_{R1}) / 2r_{R1}$. To satisfy $0 < \phi_G < 1$, $r_{R1} > |r_{G0} - r_{R0}|$ which means that the interaction term has to be strong enough to overcome the difference in the basal fitness of the two populations. Here, ϕ_G is stable, since $\hat{r}_G - \hat{r}_R$ is positive (favoring G) when $\phi_G < \phi_G$, and negative (favoring R) when $\phi_G > \phi_G$. At ϕ_G , two populations grow at the same rate and population ratio is fixed at $R:G = (1 - \phi_G) : \phi_G$.

Similar analysis shows the existence of a stable partner ratio under commensalism $G[\sim+]R$. A steady-state ratio ϕ_G can exist under exploitation. However, initial ratios below the critical value $\phi_{G,c}$ (Figure 1—figure supplement 2B) will not converge to ϕ_G . Other interactions $[\sim -]$, $[\sim +]$, and $[+ -]$ do not converge to a stable ratio. Similar conclusions on ratio convergence hold if we assume $\hat{r}_i = r_{i0} + r_i f(\phi_j)$ for any continuous function f that monotonically increases with ϕ_j (proof not shown). Results are summarized in Table 1.

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Additional files

Source code 1.

• Source code 1. MATLAB code for simulating cooperative communities in the fitness model.

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Source code 2.

• Source code 2. MATLAB code for simulating cooperative communities in the diffusion model.

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Source code 3.

• Source code 3. MATLAB code for simulating competitive communities in the diffusion model.

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Source code 4.

• Source code 4. MATLAB function for in-plane rearrangement of cells in both the fitness and the diffusion models.

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