New Frontiers: The Evaluation of Eukaryotic Populations in the Deep Marine Subsurface: Scientific Goals and Approaches for Future Ocean Drilling Projects

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Abstract.
The composition and activities of microbial communities from diverse and often 'extreme' habitats have been the focus of intense research during the past decade, spurred on largely by advances in molecular biology. These studies have placed microbes (used in the largest sense to include viruses, archaea, bacteria and protists) as essential participants in virtually all biogeochemical processes on our planet. Most molecular-based research to date has focused on bacteria, archaea and viruses, while studies of the diversity and activities of protistan assemblages has lagged behind. Recently there has been a concerted effort to study life in the deepest parts of the oceans and even below the seafloor in the deep subsurface. Bacteria and archaea have been shown to comprise a major portion of Earth's total living biomass in the deep subsurface and their activities catalyze the recycling of buried organic matter. The contribution of microbial eukaryotes (protists and fungi) in this ecosystem is unknown, as these assemblages have been studied in deep-sea sediments only to a few centimeters into the seafloor. Protists and fungi may impact carbon cycling in the marine subsurface through consumption of dissolved organic matter (fungi) and through bacterial/archaeal grazing. Protists may control bacterial and archaeal abundances and community composition, and thereby impact microbial production and nutrient cycling. Despite the pivotal roles played by these organisms, little is known regarding the presence, abundance, diversity and activities of these species in deep biosphere environments. This white paper proposes goals and approaches for initiating research on the deep biosphere with a focus on eukaryotic microbes to complement existing microbial community research in this ecosystem.

Research Focus

Our current view of microbial diversity in the deep ocean is largely shaped by microscopy and molecular information available for bacteria and archaea. The evaluation of diversity and ecology of the single-celled eukaryotic microbes (the protists and fungi) has been much slower despite their important role in marine microbial communities and in expanding our understanding of the evolution of multicellular taxa (Caron et al. 2009a,b). Protistan lineages represent one and a half billion years of evolution and comprise the bulk of eukaryotic phylogenetic diversity and an astounding array of morphologies, physiologies and ecological activities. In the upper water column of the World's oceans protists play pivotal roles in global food webs as primary producers and consumers. Much less is known about the presence and activities of microbial eukaryotes in the deep ocean, although recent studies have provided preliminary insights into microbial eukaryote diversity in the bathypelagic (Countway et al. 2007; Not et al. 2007) and deep-sea sediments (Arndt et al. 2003; Edgcomb et al. 2002). Bacterial and/or archaeal grazing by protozoa in subsurface horizons may significantly impact carbon cycling in the deep oceans and deep-subsurface environments by limiting bacterial and archaeal abundances, altering community composition and controlling net production and

nutrient cycling (Sherr and Sherr 2002). The composition, temporal and spatial dynamics, and possible biogeochemical activities of marine subsurface eukaryotic communities are now emerging as an important, and presently uncharacterized, topic in marine sciences and biogeochemistry (Edgcomb et al. 2007, Teske 2007).

Given the logistical challenges of accessing the deep ocean and deep subsurface biosphere, the history and present character of 'deep' ecosystems is largely an unwritten book. The most pressing need is for a coordinated approach to study the diversity, function, and activity of deep biosphere microbial eukaryotes.

IODP-Aligned Scientific Goals for Studies of Microbial Eukaryotes

1. **A global census of subsurface marine microbial eukaryotes.**

The breadth of microbial eukaryote diversity (and consequently eukaryote activities) present in the deep subsurface is virtually unknown at this time. It is known that bacterial and archaeal abundances are broadly correlated to sediment depth (Parkes et al. 2000) and to organic carbon content (Lipp et al. 2008). If heterotrophic protists are important consumers of these microbes in the subsurface biosphere, their abundances will depend on prey availability, which will, in turn be controlled by organic carbon content of the sediment and environmental conditions (pressure, temperature, oxygen, etc.). A similar correlation to these environmental variables might apply to fungi, which extract carbon directly from organic sources. Evaluation of the diversity and abundances of protists and fungi (in conjunction with bacterial/archaeal assemblages) in subsurface sediments will enable the construction of testable hypotheses on the environmental and geochemical factors dictating their distributions and defining their activities. Such observations may yield unprecedented discoveries, and change our perception of community structure and function in the subsurface biosphere.

Our present state of knowledge is based on a few preliminary 18S rRNA surveys of subsurface sediment and seafloor surface diversity (Peru Margin, Edgcomb e al., *in prep*; 9^{o}N EPR and Guaymas Basin, Countway et al. *in prep*).

The Peru Margin is currently one of the best-characterized deep subsurface microbial ecosystem with extensive background data on bacteria and archaea, and organic carbon content (D'Hondt et al. 2004; Schippers et al. 2005, Parkes et al. 2005; Meister et al. 2005; Webster et al., 2006, Biddle et al. 2006; Sørensen and Teske, 2006; Teske and Sørensen 2008). 18S rRNA primer sets to evaluate eukaryotic diversity in this ecosystem have revealed that fungal sequences affiliated with Basidiomycetes and Ascomycetes dominated the samples (Fig. 1)(Biddle 2005a,b). The remaining sequences were most similar to uncultured fungal sequences from Guaymas Basin hydrothermal sediments (Edgcomb et al. 2002), the Lucky Strike vents at the Mid-Atlantic Ridge (Le Calvez, 2008), Cariaco Basin anoxic water column (Stoeck et al. 2003), non-hydrothermal deep-sea sediments (Bass et al. 2007), and Lassen Volcanic National Park (Genbank EF682454). These initial results confirm that eukaryotes are present in the subsurface, and indicate that fungi may be the important single-celled eukaryotes in the deeply buried biosphere (see also review by Xu, 2006).

Hydrothermal sites are well-studied systems and have been extensively characterized with respect to the prokaryotic assemblages whereas eukaryotic work is largely just beginning (one study excepted, (Edgcomb et al. 2002)). A recent high-throughput molecular survey of microbial eukaryote diversity at Guaymas Basin in cold sediment (1-2 cm depth horizon) yielded ~7,500 eukaryote sequence tags (~130 bp in length) from a diverse array of protistan taxa plus an additional ~14,000 tags that could

not be readily identified due to low homology with known eukaryotic taxa (Countway and Caron, unpublished data). The previous data were generated using a 454-based sequencing method recently developed specifically for assessing the diversity of microbial eukaryotes (Amaral-Zettler et al. 2009). Increased 'read lengths' for the '454' sequencing method (up to ~400 bp) have already been achieved in many labs and may be invaluable for probing life in the deep subsurface biosphere.

Preliminary analyses suggest a diverse array of flagellates in addition to the fungal taxa. Small heterotrophic protists typically prey on bacteria and archaea, and have been detected in hydrothermal (e.g. Lopez-Garcia et al., 2003; Edgcomb et al., 2002) and non-hydrothermal (Scheckenbach et al., 2005) surface sediments from the deep-sea, and in terrestrial subsurface aquifers (reviewed in Novarino et al. 1997). Their small size and ability to subsist at relatively low prey abundances should enable them to persist at low bacterial/archaeal abundances and production rates that might characterize subsurface sediments.

2. **Community structure among *in situ* eukaryotes in the deep biosphere.**

Diversity assessments of the deep biosphere, as with other environments, must consider all components of the microbial community (bacteria, archaea, protists and viruses) in order to adequately characterize the structure and function of these assemblages (reviewed by Heidelberg et al. 2008). This approach will provide the best opportunity to understand the complex interplay between microorganisms, identify potential trophic relationships among them, and infer the influence of the unique deep biosphere environment on shaping and controlling these interactions. This work is necessary for understanding the ecology of microbial eukaryotes, many of which are dependent on other microbes for sustenance, and is ultimately essential for understanding the resultant outcomes that affect global biogeochemical cycles.

Gene assessments will also contribute towards this goal. Genes that occur more frequently in a particular community may be conferring attributes beneficial for maintenance of the function of that particular ecological niche. Novel strategies of adaptation for different environments in Eukaryotic microbes have recently been demonstrated (Allen et al. 2008). Together these findings suggest that marine microbial genomes both free-living and symbiotic, are complex, highly dynamic, and adaptive. Studies could also target mRNA usually expressed only by protists, such as tubulin (Edgcomb et al., 2001). Metabolic potential or evidence for mRNA production *in situ* could be used to design targeted lab based experiments to further evaluate physiological responses with cultured organisms.

3. **Colonization, trophic activities and growth of cultured subsurface isolates.**

Studies of diversity and community structure must be coupled with research to isolate and culture the dominant microbial eukaryote taxa present in the deep biosphere in order to gain a richer understanding of their ecological roles and activities. Metagenomic and other -omic approaches to evaluate microbial communities may eventually generate hypothesis-driven research, but these approaches are presently thwarted by the genome sizes of most eukaryotes. Thus, most information on function must be obtained using laboratory cultures of eukaryotes that have been demonstrated using culture independent methods to be important players in the deep biosphere, and then subsequently isolated and brought into culture. There are extremely few representative microbial eukaryotes in culture from the deep ocean, and therefore this is an area of study ripe for advancement.

Recent advances in culturing techniques and the insights provided by genetic analyses

of natural communities (Caron and Gast, 2008) hold tremendous promise for increasing our abilities to culture and study the ecology and physiology of microbial eukaryotes from targeted environments. For example, microscopical or genetic approaches that indicate that some deep-sea protists participate in obligatory symbioses with other microbes will greatly improve attempts to culture these protists and characterize their ecological roles (Buck and Bernhard, 2001).

Expected new outcomes and capabilities

A wide (global) survey of different sampling sites and sediment types, good sample preservation, and good negative controls, such as bottom water samples, to distinguish authentic subsurface signatures from deep-water communities, would have great scientific rewards. 1) Knowledge of the eukaryotic subsurface biosphere would clarify controls on bacterial/archaeal cell density through predation (protozoa), and on assimilation of buried organic matter (fungi). 2) Novel anaerobic types of eukaryotes are likely to be discovered that can survive at relatively low standing stocks of prey. 3) New symbiotic associations involving protists are also likely to emerge, expanding our knowledge of cell-cell interactions in this unique biosphere. 4) As seen with the bacteria and archaea (Fry et al. 2008, Teske and Sørensen 2008), novel and deeply-branching phylogenetic lineages of subsurface eukaryotes will likely be discovered that are relevant for improving our understanding of the eukaryotic tree of life.

Technological Requirements

Eukaryotic microbes may play significant roles in the biogeochemistry and food webs of the deep subsurface biosphere. A primary goal, then, should be to ensure that sampling is performed that is appropriate for evaluating these assemblages along side analyses of 'more traditional' microbial components. Collaborative approaches promoting the use of established, tested protocols and interdisciplinary approaches will be needed. The scientific community involved with the study of deep-sea and global microorganisms is now beginning to assemble to undertake deep biosphere research (e.g. Edwards et al. deep biosphere coordination proposals/IODP/etc). The incorporation of studies of microbial eukaryotes should be a significant component of this work.

The microbiological laboratory facilities on the drill ship *R/V JOIDES Resolution* and existing protocols allow for acquiring core samples free from surficial or water column contamination. The development of a research coordination network that promotes integrated approaches using established ODP/IODP protocols (Smith et al., 2000; House et al. 2003; Lever et al. 2006) and cooperation among a community of scientists interested in studies of deep sea protists is essential. A framework for this network has already been initiated with an upcoming meeting planned for October, 2009. Networking ensures that directed studies are undertaken at well-characterized and otherwise studied sites, building upon results from preliminary studies and leveraging interdisciplinary expertise in a coordinated approach. Once collected, a collaborative network also promotes building of capacity and interpretation of limited samples through sharing of material for different types of analyses among a broad protistan scientific community.

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Figure 1. Phylogenetic tree (unrooted, under maximum likelihood, GTR+gamma+invar, using RAxML) of representative fungal 18S rRNA phylotypes from Peru Margin sediments (Edgcomb et al., *in prep.*)

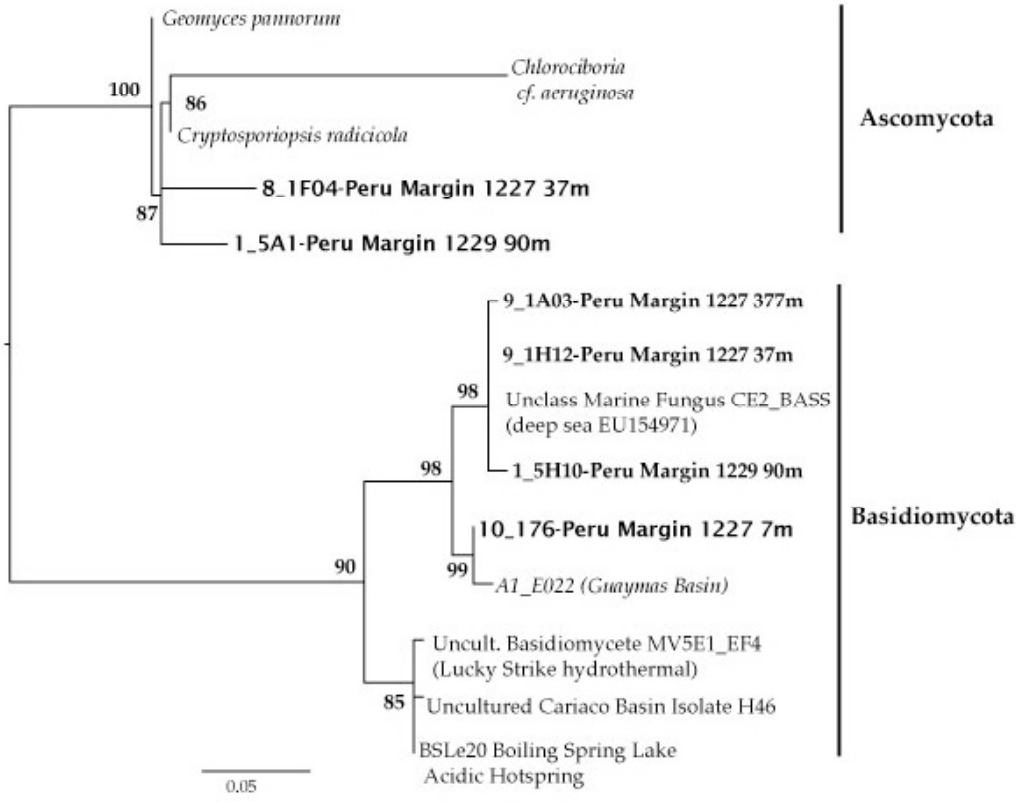

Figure 2. A) Internal Transcribed Spacer (ITS) analysis of DNA extracted from ODP 1229 sediments (depths 4-158 mbsf). Products were amplified by standard fungal ITS primers (ITS1F, ITS4R). The black arrow indicates the product usually associated with sequences of the Ascomycota (Biddle 2006 PhD thesis). B) Number of sequence reads related to fungal clades from 1, 16, 32 and 50 mbsf at Site 1229, from a pyrosequencing survey of sediment DNA without prior PCR amplification (Biddle et al. 2008). C) Cultivation and enumeration of fungal colonies growing on heterotrophic medium, reanalyzed (Biddle et al. 2005a).