TITLE: The role of anaerobic fungi in fundamental biogeochemical cycles in the deep biosphere

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ABSTRACT

A major part of the biologic activity on Earth is hidden underneath our feet in an environment coined the *deep biosphere* which stretches several kilometers down into the bedrock. The knowledge about life in this vast energy-poor deep system is, however, extremely scarce, particularly for micro-eukaryotes such as fungi, as most studies here have focused on prokaryotes. Recent findings suggest that anaerobic fungi indeed thrive at great depth in fractures and cavities of igneous rocks in both the oceanic and the continental crust. Here we discuss the potential importance of fungi in the deep biosphere, in particular their involvement in fundamental biogeochemical processes such as symbiotic relationships with prokaryotes that may have significant importance for the overall energy cycling within this vast subsurface realm. Due to severe oligotrophy, the prokaryotic metabolism at great depth in the crust is very slow and dominantly autotrophic and thus dependent on e.g. hydrogen gas, but the abiotic production of this gas is thought to be insufficient to fuel the deep autotrophic biosphere. Anaerobic fungi are heterotrophs that produce hydrogen gas in their metabolism and have therefore been put forward as a hypothetical provider of this substrate to the prokaryotes. Recent *in situ* findings of fungi and isotopic signatures within co-genetic sulfide minerals formed from bacterial sulfate reduction in the deep continental biosphere indeed seem to confirm the fungi-prokaryote hypothesis. This suggests that fungi play a fundamental biogeochemical role in the deep biosphere.

Keywords: Anaerobic fungi; continental crust; oceanic crust; deep biosphere; geomycology
1. Introduction

The deep biosphere comprises microorganisms several kilometers below the ground surface and ocean floor (Lin et al., 2006). Active deep ecosystems have been reported from such diverse settings as marine sediments (Parkes et al., 2005), deep-sea hydrothermal vents (Jorgensen et al., 1992), subseafloor igneous rocks (Schrenk et al., 2009), and terrestrial sedimentary (Fredrickson et al., 1995) and igneous rocks (Pedersen et al., 2008). The deep biosphere in igneous rocks is among the least understood ecosystems on Earth. Although the microbial processes are relatively slow because of the low energy supply (Wu et al., 2016) the deep ecosystems are proposed to play an important role in the energy cycling of the Earth and comprise a substantial part of the Earth’s biomass (McMahon and Parnell, 2014). Until recently, the majority of the microbiological investigations in the deep biosphere have been focused on prokaryotes, and the potential presence of eukaryotes such as fungi has been largely neglected. In this respect, recent identifications of fungi in a wide variety of deep environments, including various marine settings (Connell et al., 2009; Le Calvez et al., 2009; Nagano and Nagahama, 2012; Orsi et al., 2013), subseafloor basalt (Ivarsson et al., 2012; Ivarsson et al., 2016a), and in the deep continental crust (Sohlberg et al., 2015; Drake et al., 2017a) definitely shed new light on the presence of fungi in the deep biosphere and suggest fungi to play a major role in the energy cycling. Identification of fungi from anoxic deep sea environments (Jebaraj et al., 2010; Raghukumar et al., 2010) and at depths in the crust where strictly anoxic conditions prevail indicates that anaerobic conditions are not a limiting factor for fungal growth, and that the fungi have adapted to anaerobic metabolism.

Anaerobic fungi are so far poorly understood in an environmental context, and the most thorough description is from rumina of ruminating herbivores (Khejornsart et al., 2010; Liggenstoffer et al., 2010), where the anaerobic fungi produce H₂ during their respiration and consort with H₂-dependent methanogenic and acetogenic archaea, which enhances growth of
both organisms. The fungi that form symbiotic relationships with acetogens and methanogens in the rumen have recently been described from marine sediments (Picard, 2017). Potentially any H₂-dependent chemoautotrophic microorganism could be fuelled by H₂ produced by anaerobic fungi in an anoxic environment (Ivarsson et al., 2016b), which is supported by recent findings of fungi-prokaryote consortia in the igneous crust (Drake et al., 2017a). Consequently, anaerobic fungi have been proposed to be a neglected geobiological force in subsurface ecosystems (Ivarsson et al., 2016b; Drake et al., 2017a). Here we discuss this previously unknown biogeochemical agent and its potential implications.

2. **Fungi in oceanic crust**

Deep sea environments host diverse fungal communities, mainly represented by Ascomycota, but also Basidiomycota and Chytridiomycota (Nagano and Nagahama, 2012). A majority of Chytridiomycota in deep sea environments represent novel deep-branching lineages, including a new branch forming an ancient evolutionary lineage (Le Calvez et al., 2009). Ascomycetes and Basidiomycetes have been isolated from seafloor-exposed basalt (Connell et al., 2009), but from the underlying igneous crust, only one fungal isolate (genus *Exophiala* of the order Chaetothyriales) has been reported so far (Hirayama et al., 2015). Apart from this single observation our understanding of the fungal presence in the oceanic crust is based on a fossil record, such as the findings by Ivarsson et al. (2012) describing mycelium-like networks of hyphae with preserved chitin in their cell walls, in vesicular basalts from the Emperor Seamounts in the Pacific Ocean. Bengtson et al. (2014) and Ivarsson et al. (2013a) revealed a close symbiotic-like relationship between fungi and two types of chemoautotrophic prokaryotes in subseafloor basalts, and that the prokaryotes used the structural framework of the mycelia for their growth (Fig. 1). The close relationship with
chemoautotrophic prokaryotes was also described by Ivarsson et al. (2015a) and probably was essential for fungal colonization in the oligotrophic environment. The most likely source of carbohydrates that are essential for the fungi in these oligotrophic environments has been proposed to be living or dead bacterial biofilms (Gadd, 2006). Fungi from the Emperor Seamounts were interpreted as Ascomycetes or stem-group Dikarya and fossilized fungi from the Vesteris Seamount in the Greenland Basin (Ivarsson et al., 2015b) were interpreted as Zygomycetes. Thus, the fungal diversity seems high in both marine sediments and in subseafloor basalts. Fossilized fungi have been found in ophiolites as old as 2.4 Ga (Bengtson et al., 2017). The igneous oceanic crust is a fungal niche that likely is of great importance considering the spatial and temporal distribution of fungi.

3. **Fungi in continental crust**

The deep subsurface of continental igneous rocks has not been as extensively studied as deep sea sediments and subseafloor basalts, and because of this and because the studies have focused on prokaryotes, observations of fungi are very few. Fossilized fungal hyphae have been reported from the bedrock in Sweden and Germany (Reitner et al., 2006; Ivarsson et al., 2013b). Ekendahl et al. (2003) isolated a limited number of yeast fungi strains from waters at Äspö, Sweden and Sohlberg et al. (2015) found a high fungal diversity in deep bedrock aquifers at 300-800 m depth at Olkiluoto, Finland. Most of the observed fungal sequences in the latter study belonged to the phylum Ascomycota, with minor contribution of the Basidiomycota and Chytridiomycota phyla. Anoxic conditions prevail at these depths in the crust and the fungi are thus considered to be anaerobic, but still, the possible role of the detected fungi in this oligotrophic environment is unknown. Recently, we presented *in situ* evidence of fungi from great depth within the continental crust, from a fracture cavity in
Proterozoic igneous rocks at Laxemar, Sweden (Fig. 2), giving a new piece in the deep biosphere puzzle (Drake et al., 2017a) and suggesting that anaerobic fungi are widespread in the continental crust, down to almost 1 km depth.

4. The biogeochemical role of fungi in the deep biosphere

Anaerobic fungi

Facultative anaerobic fungi are known from all major fungal divisions and frequently found in extreme environments including deep subsurface environments (Sohlberg et al., 2015). A correlation between facultative anaerobic fungi and nitrogen cycling seems plausible as fungi use nitrate or nitrite as alternative terminal electron acceptors in the absence of oxygen (Kurakov et al., 2008). Obligate anaerobic fungi, however, are only known from the phylum Neocallimastigomycota, which is best known from rumen of herbivores but have been reported from the gut and coelomic fluid of the coastal sediment-dwelling sea urchin Echinocardium cordatum (Thorsen, 1999) and in the guts of the algae-grazing marine iguana Amblyrhynchus cristatus (Mackie et al., 2004). They have also been reported from landfill soils (Lockhart et al., 2006), as well as lacustrine (Wurzbacher et al., 2016), estuarine (Mohamed and Martiny, 2011), and marine sediments (Picard, 2017). This growing number of observations support that obligate anaerobic fungi not only exist as endosymbionts in rumens but also in marine animals as well as free-living in anoxic sediments and soils.

Considering the vast anoxic environments of the igneous crust, just recently recognized as a fungal niche, it is reasonable to assume that the abundance and diversity of obligate anaerobic fungi are far from understood. The recently reported dominant presence of zoosporic fungi in extreme environments, of which a majority still are unknown, calls for a re-evaluation of fungal tolerance for extreme conditions such as anoxia (Nagano and Nagahama, 2012).
A majority of fungi reported from the subsurface are fungi that are known aerobes in surface environments. Their metabolisms in the anoxic subsurface environments are, however, unknown. Previously there was a clear distinction between aerobic and anaerobic eukaryotes; those who have mitochondria and those who have hydrogenosomes (Muller et al., 2012). Aerobic and anaerobic eukaryotes were believed to be evolutionary divided and anaerobic eukaryotes only to be found in the basal branches of the eukaryotic tree. However, studies have shown that hydrogenosomes are reduced mitochondria and that the reductive evolution of mitochondria to hydrogenosomes or other mitochondrial homologues is common across the eukaryotic tree (Hjort et al., 2010). The transition from mitochondria to hydrogenosomes is, in fact, a simple result of oxygen deficiency in the environment (Embley et al., 1995). Thus, aerobic micro-eukaryotes exposed to permanent anoxia certainly have the capability to adapt and develop anaerobic metabolic pathways over time.

A widespread deep hydrogen-based lithoautotrophic realm

The deep anaerobic biosphere hosts both heterotrophic and autotrophic prokaryotic metabolisms. Studies in deep bedrock ecosystems, such as of aquifers at the Äspö Hard Rock Laboratory in Sweden (Wu et al., 2016) and the Outokumpu deep drill hole in Finland (Nyyssönen et al., 2014) have shown that there is a depth-related transition from dominantly heterotrophic processes in the upper part of the crust to a dominantly autotrophic realm at greater depth, consistent with the current paradigm of a hydrogen-driven deep biosphere at great depth in the crust (Stevens and McKinley, 1995; Pedersen, 1997; Chapelle et al., 2002; Hallbeck and Pedersen, 2008b). The hydrogen gas that is proposed to be an important substrate for these deep subsurface lithoautotrophic ecosystems is also a limiting factor for the persistence of the widespread sulfate reducing bacteria (SRB) communities in this
environment (Pedersen, 2010). The formation and origin of H₂ remain elusive, and several different processes have been proposed, including radiolysis of water from uranium decay (Lin et al., 2005) over long time spans in the subsurface environment. Investigations from deep aquifers in the Precambrian Fennoscandian shield show highly variable H₂ concentrations (Hallbeck and Pedersen, 2008a, b; Kietäväinen et al., 2013). In aquifers studied in Sweden, concentrations of up to 190 µL/L have been reported (Hallbeck and Pedersen, 2008a, b), but these are correlated neither with depth nor with residence times of the waters, which in several cases are in the order of just a couple of thousand years (Laaksoharju et al., 2008). These residence times are certainly too short time periods for build-up of significant H₂ concentrations by radiolysis (cf. Lin et al., 2005) implying that radiolysis is not the sole source of the elevated H₂ concentrations. Other processes that can lead to abiotic H₂ formation are serpentinization of ultramafic rocks (Sleep et al., 2004) and oxidation of graphite rich schists (Kietäväinen et al., 2017), but at least at the Swedish sites referred to above, these rock types are either non-existing or very minor, and there is no spatial relation between rock type and elevated H₂. Instead, based on our findings and ambiguous traces of fungi in the deep aquifer at Olkiluoto, Finland (Sohlberg et al., 2015) where active fungi were detected in most of the investigated fracture zones, we propose that subsurface fungi are neglected and likely significant providers of H₂ for autotrophic microbial processes in the oligotrophic igneous crust.

**Implications of in situ evidences of fungi-prokaryote consortia in the deep biosphere**

Confirmation of fungi providing hydrogen gas to lithoautotrophic prokaryotes in the deep biosphere requires direct evidence. For instance, Bengtson et al. (2014) presented fossilized remnants of a prokaryote-fungi consortium in subseafloor basalt, but the prokaryotic remains could not be morphologically related to any specific kind of prokaryote known to process H₂.
Therefore, it is a major step forward that we recently were able to link *in situ* evidences of fungi in deep granite fractures in Sweden to coeval activity of SRB (Drake et al., 2017a), which can use H$_2$ as an electron donor in lithoautotrophic environments (Hallbeck and Pedersen, 2008a, and references therein). This consortium was interpreted using high spatial resolution stable isotope analysis within the minerals that occur along with the fungi. The light stable sulfur isotope composition ($\delta^{34}$S) within tiny pyrite crystals in spatial relation to the hyphae was shown to be diagnostic for bacterial sulfate reduction (Drake et al., 2017a). In addition, the $^{13}$C-depleted composition of calcite showed that anaerobic oxidation of methane had occurred in the fracture cavity (cf. Drake et al., 2015; 2017b). H$_2$, and potentially some other substrate such as acetate, provided by anaerobic fungi have triggered SRB growth. This is the first *in situ* finding of fungi at great depth in the continental crust and confirms the previously hypothesized coupling between the fungi and SRB in the deep biosphere, a coupling that has yet been unsupported by direct evidence in nature.

**Concluding remarks**

If fungi can survive at extremely oligotrophic conditions deep in the crust they may be significant providers of hydrogen gas that fuels the omnipresent prokaryotic hydrogen-based autotrophic processes in the vast deep biosphere. Although more studies are needed to determine how widespread fungi are in the deep biosphere, the recent findings of anaerobic fungi clearly suggest a re-evaluation of the energy cycling within the igneous crust. Eukaryotes have been neglected in the deep biosphere research, but the recent findings propose that they indeed may be key players in this vast realm. The abundant findings of fungi in subseafloor basalt in the last decade endorsed Ivarsson et al. (2016a) to propose that the igneous oceanic crust might be the largest fungal habitat on Earth. The recently reported
direct evidences for fungal activity in the deep oligotrophic biosphere of igneous continental rocks may indeed suggest that this environment is a neglected vast fungal habitat of almost similar proportions.

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Fig. 1. Fungal–prokaryote community in vesicular basalt, Koko Seamount, 67.5 m.b.s.f.

ESEM micrograph (left) of mycelium and ‘cobwebs’ and Synchrotron Radiation X-Ray Tomographic Microscopy (SRXTM) isosurface rendering (right) of the same region as in the left image, highlighting the fungal cobweb structures (cw) and Frutexites (fx). Images originate from Bengtson et al. (2014)
Fig. 2. ESEM image of biomineralized and organically preserved fungal hypha from a fracture cavity at 740 m depth in the Precambrian bedrock at Laxemar, Sweden. Modified from Drake et al. (2017b).