Deep-biosphere consortium of fungi and prokaryotes in Eocene subseafloor basalts

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ABSTRACT

The deep biosphere of the subseafloor crust is believed to contain a significant part of Earth’s biomass, but because of the difficulties of directly observing the living organisms, its composition and ecology are poorly known. We report here a consortium of fossilized prokaryotic and eukaryotic micro-organisms, occupying cavities in deep-drilled vesicular basalt from the Emperor Seamounts, Pacific Ocean, 67.5 m below seafloor (mbsf). Fungal hyphae provide the framework on which prokaryote-like organisms are suspended like cobwebs and iron-oxidizing bacteria form microstromatolites (Frutexites). The spatial inter-relationships show that the organisms were living at the same time in an integrated fashion, suggesting symbiotic interdependence. The community is contemporaneous with secondary mineralizations of calcite partly filling the cavities. The fungal hyphae frequently extend into the calcite, indicating that they were able to bore into the substrate through mineral dissolution. A symbiotic relationship with chemoautotrophs, as inferred for the observed consortium, may be a pre-requisite for the eukaryotic colonization of crustal rocks. Fossils thus open a window to the extant as well as the ancient deep biosphere.

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INTRODUCTION

The oceanic crust makes up the largest potential habitat for life on Earth (Schrenk et al., 2009). Yet little is known about the abundance, diversity, and ecology of its biota. Considering that deep-sea sediments are estimated to contain the Earth’s largest proportion of micro-organisms (Parkes et al., 1994; Whitman et al., 1998), we should expect a significant biosphere to be hosted in deep crustal environments as well. Textural alterations of glassy basaltic rocks and cryptoendoliths in basalts (Schumann et al., 2004; Furnes et al., 2008; Ivarsson et al., 2008; Peckmann et al., 2008; Eickmann et al., 2009), and genomic investigations of drill cores in basalts (Lever et al., 2013) suggest ongoing microbial interactions, but direct observations of the living micro-organisms are mostly beyond our reach, and the biochemical pathways enabling life in these extreme environments are largely unknown. Neither is it known to what degree the subsurface environments are sufficiently stable to allow the establishment of mutualistic symbiotic relationships, important to sustain an efficient circulation of nutrients. We report here the discovery of a fossil microbial community consisting of eukaryotic heterotrophs (fungi) and prokaryotic putative chemoautotrophs living in a spatially organized association deep in submarine vesicular basalts.

Fossilized mycelial micro-organisms in subseafloor basalts have been interpreted as remnants of fungi (Schumann et al., 2004; Reitner et al., 2006; Peckmann et al., 2008; Eickmann et al., 2009; Ivarsson, 2012; Ivarsson et al., 2012, 2013). The ability of fungi to live in such extreme environments is corroborated by discoveries of viable yeast
cells deep in continental crust (Ekendahl et al., 2003) and of active fungal genes in deep-sea sediments at various depths below the seafloor (Orsi et al., 2013a,b). These occurrences raise several questions regarding metabolism and ecology of fungal communities in subsurface basaltic environments.

All known fungal species are heterotrophic, and it is questionable whether abiotic sources or flow through basalt are able to ensure a steady supply of accessible organic compounds over geological time. The alternative is a biological source of organic compounds provided by chemoautotrophic communities. At hydrothermal vents eukaryotes are known to live in symbiotic relationships with chemoautotrophs (Cavanaugh et al., 2006), and fungi are known from such environments (Connell et al., 2009). Fungi are notable for entering into symbiotic relationships, although such a relationship with chemosynthetic bacteria has not yet been demonstrated in living forms. Symbiosis with chemoautotrophs would enable fungi to populate and thrive in subsurface settings and expand their ecological niches.

MATERIAL AND METHODS

The Emperor Seamounts in the Pacific Ocean is a submarine chain of seamounts formed by hotspot volcanism between ~81 and ~43 Ma. The seamounts stretch for about 5000 km in a north–south trend. At ~43 Ma, the direction changes to a northwest–southeast trend, and the Hawaiian Islands continue to the Recent. The ages increase, in a classical hotspot fashion, away from the active hotspot in the southeast called Loihi Seamount.

During Ocean Drilling Program (ODP) Leg 197, three seamounts belonging to the Emperor Seamounts were drilled: Detroit Seamount (~81 Ma), Nintoku Seamount (~66 Ma), and Koko Seamount (~48 Ma). Sites 1203 and 1204 were drilled at Detroit Seamount, Site 1205 at Nintoku Seamount, and Site 1206 at Koko Seamount (Tarduno et al., 2002). The material described in this study is from sample ODP 197-1206A-4R-2, 0, Koko Seamount, from a depth of 67.5 m below seafloor (Ivarsson et al., 2013).

Areas with fossils were localized with light microscopy in drill core pieces. Selected areas were reduced to cubes of ~1 × 1 cm in diameter by sawing. The reduced pieces were further investigated with optical microscopy, environmental scanning electron microscopy (ESEM), energy dispersive spectrometry (EDS), synchrotron radiation X-ray tomographic microscopy (SRXTM), Raman spectroscopy, and stable isotope mass spectrometry. The investigated material is deposited in the Palaeobiology collections of the Swedish Museum of Natural History (X5311).

Optical microscopy

Light microscopy and imaging were performed with a Nikon SMZ1500 microscope fitted with a Nikon D80 camera.

Environmental scanning electron microscopy

A Philips XL30 environmental scanning electron microscope (ESEM) with a field emission gun (XL30 ESEM-FEG) was used to analyze the minerals and fossils. The ESEM was equipped with an Oxford X-ray dispersive spectrometer (EDS), backscatter electron detector (BSE), and a secondary electron detector (SE). The acceleration voltage was 20 or 15 kV depending on the nature of the sample, and the instrument was calibrated with a cobalt standard. Peak and element analyses were made using INCA Suite 4.11 software. The samples were analyzed in low vacuum to avoid charging effects at the surface and thus were not coated, with neither C nor Au, to enable EDS analyses of C.

Synchrotron radiation X-ray tomographic microscopy (SRXTM)

The samples were mounted on a 3 mm-wide brass peg. SRXTM was performed at the TOMCAT beamline at the Swiss Light Source at the Paul Scherrer Institute. The X-ray energy was set to 41 keV for maximum penetration. First, for each sample, a fast overview scan (not shown) at low magnification but capturing the entire object was acquired to identify the coordinates of the regions of interest. With this information, selected 1.6 × 1.6 × 1.6 mm³ volumes within the larger 1 × 1 × 1 cm³ specimens were then imaged at higher resolution (local tomography). For the results presented here, 1501 projections were acquired equiangularly over 180°, online post-processed and rearranged into flat- and darkfield-corrected sinograms. Reconstruction was performed on a Linux PC farm using highly optimized routines based on the Fourier Transform method (Marone & Stampanoni, 2012). To mitigate image artifacts occurring as a consequence of the strong incompleteness of these datasets captured in local tomography geometry and strongly affecting the quality of the results, corrected sinograms were constant padded prior to reconstruction (Marone et al., 2010). Slice data derived from the scans were then analyzed and rendered using Avizo software. With the 10× lens used, the resulting voxel size was 0.65 μm.

Raman spectroscopy

The instrument used was a confocal laser Raman spectrometer (LabRAM HR 800; Horiba Jobin Yvon, Villeneuve d’Ascq, France), equipped with a multichannel air-cooled charge-coupled device detector. Excitation was provided by an Ar-ion laser (λ = 514 nm) source. The laser power at the sample surface was 8 mW. The instrument was incorporated with an Olympus BX51 microscope. The laser beam was focused through an 80× objective with a...
working distance of 8 mm; the resulting analyzed spot size was about 1 µm. The spectral resolution was ~0.3 cm⁻¹. The accuracy of the instrument was controlled by repeated use of a silicon wafer with a Raman line at 520.7 cm⁻¹. The Raman spectra were obtained with LabSpec 5 software.

**Stable isotopes**

For stable isotope analysis, calcite crystals were sampled from six different vesicles with varying amounts of fossils present. 0.2 mg carbonate was flushed with helium gas in a septum-seal glass vial. 100 µl of 99% H₃PO₄ was added to each sample for reacting to CO₂. For the analyses of stable isotope signatures, a GasbenchII coupled to a Finnigan MAT 252 mass spectrometer was used. Based on these measurements, the reproducibility was calculated to be better than 0.07‰ for δ¹³C and 0.15‰ for δ¹⁸O.

**RESULTS**

The microbial assemblage investigated here occurs in open or partly open vugs in the vesicular basalt (Fig. 1A). In some of the vugs, the basaltic walls are entirely covered by fossil films, whereas in some the fossils form isolated patches. The assemblage consists of a basal film from which filaments arise to form mycelium-like networks, which in turn are overgrown by cobweb-like structures and microstromatolite. The assemblage co-occurs with secondary calcite in the voids. Stable isotope measurements of the calcite, with δ¹³C ranging between 1.0 and 1.5‰ and δ¹⁸O ranging between 2.7 and 3.6‰, give a seawater signal without evidence of influence from biological processes, which would be expected to produce more negative δ¹³C values (Table S1). There is no indication of recrystallization that might have obliterated the original isotopic composition (Walter *et al.*, 2007). The fossils are mineralized by iron oxides and montmorillonite (Figs S1–S4). The montmorillonite is not visible in the SRXTM images, resulting in apparent morphological differences between SEM (Fig. 1C) and SRXTM (Fig. 1D) renderings.

We will refer to the filaments as hyphae and to the networks as mycelia. These terms are typically applied to fungi and actinobacteria and may be understood in a non-systematic, descriptive sense. However, Ivarsson *et al.* (2012) demonstrated the fungal nature of similar thin hyphae in

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**Fig. 1** Fungal–prokaryote colony in vesicular basalt, Koko Seamount, 67.5 m.b.s.f. (A) Light micrograph of connected vesicles. (B) Close-up of mycelium in A. Broken hyphae show brown hematite core inside light crust of montmorillonite. (C) ESEM micrograph of mycelium and ‘cobwebs’. (D) SRXTM isosurface rendering of the same region as in C, bringing out the thin ferruginous cores rather than the thick montmorillonite crust. (E) SRXTM combined volume and surface rendering of mycelium and ‘cobwebs’ at calcite–void interface. (F) SRXTM isosurface rendering of dog-tooth calcite enveloped, and partly penetrated, by hyphae. (G, H) SRXTM volume renderings of hyphae and ‘cobwebs’. Arrows point to examples of alignment of the minute bodies. (I) ESEM micrograph of hyphae, ‘cobwebs’, and *Frutexites*. Hyphae and ‘cobwebs’ are encrusted with montmorillonite, *Frutexites* is not (cf. B). Legend (for all figures): ba, basalt; bf, basal film; ca, calcite; chy, coarse hypha; cw, ‘cobweb’; Fx, *Frutexites*; gl, globular feature; hy, hypha; my, mycelium; vo, void.

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equivalent rocks, and the new data reported in the present paper are consistent with a fungal affinity of the structures.

**Basal film**

The basal film covers the basalt interfaces with the voids and with the secondary calcite but is nowhere seen to extend over the surfaces of the calcite (Fig. 2A,E, Movie S1). Frequently, it is cracked up and partly flaked off from the basaltic substrate, leaving a space beneath (Figs 1A, 2A–C, 3A). Such detachment occurs both in the voids and in the calcite (Fig. 2A, upper and lower part, respectively). The film consists of a basal hematite layer with carbon, an upper hematite layer, and a montmorillonite crust (Fig. S2). The montmorillonite and upper hematite layers peter out where the film faces the calcite, leaving only the hematite–carbon layer. Morphologically, there is also a distinct difference between the parts of the films that are covered by calcite and those that are exposed to the voids. In the exposed parts, thin hyphae 2–10 µm in diameter emerge from the film to form mycelia filling up part of the voids and entering the calcite through its interface with the voids. These thin hyphae are missing where the basal film is covered by calcite (Movie S1). In both the exposed and calcite-covered parts, the surface of the film is smooth or forms hemispherical to cylindrical protrusions (Fig. 2C) that may extend into a mycelium-like network with coarse hyphae (Fig. 2E).

**Hyphae**

The thin hyphae emerging from the exposed basal film are 2–10 µm in diameter and up to several millimeters in length (Figs 1, 2, 3A,D). They consist of a dense X-ray-attenuating central part of hematite with small amounts of carbon, encrusted by X-ray-transparent montmorillonite (Figs 1B–D,E, S3). Thicker portions are expressed as hematite cylinders with a less-dense (less X-ray attenuating) core (Fig. 3A), whereas thinner ones appear as single hematite strands with no resolvable core (Movie S1). Near the basal film, the hyphae are mostly slender; further from the film they thicken, branch, and anastomose to form a mycelium (Fig. 1B–F) and frequently penetrate the secondary calcite (Figs 1E, 3D, Movie S1). Where they meet calcite surfaces they either (i) creep along the surface of the mineral, (ii) enter crevices in the mineral, or (iii) penetrate the mineral, forming deep galleries (Fig. 1E,F, Movie S1).

**Coarse hyphae**

The protrusions emanating from the basal film are about 20–40 µm in width. Mostly they are hemispherical in shape and form a botryoidal texture on the surface of the film (Fig. 2C, lower part), but occasionally they protrude as coarse hyphae (‘chy’ in Fig. 2). Where the film is exposed to the void these are usually short, up to about 100 µm, and sometimes have a globular termination (Fig. 2A–C), about twice the diameter of the supporting hypha. Where the calcite abuts the film, the coarse hyphae may grow to produce a mycelial network within the calcite (Fig. 2E, 3B). These coarse hyphae have a dense core, similar to the hyphae of the main mycelial network, but the main volume consists of a thick outer sheath, slightly less X-ray attenuating than the surrounding calcite. They frequently form globular bodies, up to 60 µm in diameter, within the calcite (Fig. 2D, Movie S1).

‘Cobwebs’

Sheets of montmorillonite between hyphae become transparent in SRXTM and are seen to contain minute bodies,
1–5 µm in size, commonly aligned in strings (Figs 1G,H, 3D; S1). These strings are suspended within planes determined by the supporting hyphae, forming cobweb-like structures (Figs 1B–I, 2A–C, 3A,D). The ‘cobwebs’ only occur where the supporting hyphae grow in the voids and in crevices in the invaded crystals, not where the hyphae form galleries in the crystals (Fig. 1E). Element analyses by EDS of less-heavily encrusted strings indicate that the bodies are preserved in iron oxides (Fig. S1).

Frutexites

Closely associated with the hyphae and ‘cobwebs’ is another type of structure: fractally branching, cauliflower-like bodies with a distinct direction of growth (Figs 1B,I, 3). The material is strongly X-ray-attenuating owing to a mainly goethite composition with local transitions to poorly crystallized hematite (Fig. S4). The major branches may reach 100 µm in width and more than a millimeter in length. The internal structure shows evidence of lamination suggesting successive layers of growth (Fig. 3C). In some instances, the initial part is attached to a hypha from the void-filling mycelial network (Fig. 3D, coarse arrows), and occasionally hyphae are encrusted by later growth stages (Fig. 3A).

The cauliflower-like bodies frequently penetrate the secondary calcite (Fig. 3B), and in almost all of these instances the apparent direction of growth is into the mineral (Fig. 3B; Movie S1), indicating that, like the void-filling hyphal networks, these structures grew into the mineral, dissolving it along the way, rather than being diagenetically embedded by the mineral. The growth direction into the calcite may be explained by the fact that growth starts on a substrate in the void, such as mycelial hyphae.

These fractally branching bodies are morphologically identical to the microstromatolitic structures known as Frutexites (Maslov, 1960), characteristic of cryptic habitats and hydrothermal vents through the Proterozoic and Phanerozoic (Walter & Awramik, 1979; Myrow & Congiolo, 1991; Böhm & Brachert, 1993; Rodríguez-Martínez et al., 2011). We will refer to them under this name.

DISCUSSION

Temporal relationships between the fossil structures

To understand the assemblage of fossils in the vesicles, it is first necessary to establish their temporal relationships between each other and to the secondary calcite in the voids. The following observations are significant in this regard:

1. The basal film covers the basalt interfaces with both voids and calcite, but it never covers the calcite interface with the voids.
2. The thin hyphae emanate from the basal film, but only in the voids, not in the calcite.
There are further morphological differences in the film at the two types of interface: shorter coarse hyphae with globular terminations occur in the voids, networks of coarse hyphae with large globules characterize the calcite.

The thin hyphae adapt themselves to the calcite surfaces but can also bore into the calcite.

The ‘cobwebs’ are suspended on the thin hyphae in the voids and where the hyphae rest in crevices in the calcite, but they never occur on the hyphae making boring galleries into the calcite.

*Frutexites* is attached to hyphae in the voids and often penetrates the calcite but is not seen to displace hyphae when growing into voids.

Even when forming a dense mycelium, hyphae do not crowd around *Frutexites* or become engulfed by it.

These observations suggest that the basal film became established on the basalt surfaces before the growth of the calcite. It may have become partly smothered by the calcite, but continued to grow and form a coarse hyphal network. Where not covered by calcite it produced short protrusions ending in bulbous terminations as well as thinner hyphae forming a mycelium. These hyphae mainly grew in the voids, but could also penetrate the calcite and form a network within it. The ‘cobwebs’ suspended themselves on the hyphae in the voids and followed the live hyphae as far as they could into crevices in the calcite but not where the hyphae bored into the calcite. *Frutexites* attached itself on the hyphae in the voids and grew to considerable volume but without disturbing or engulfing the hyphal network.

Thus, we propose that the organisms lived at the same time, during the precipitation of the calcite. The hyphae grew and penetrated the mineral after it was formed, rather than having become embedded in growing crystals. These observations provide a time constraint for the calcite, showing that it was formed after the film was laid down in the voids but prior to the development of complex, void-filling, calcite-boring mycelia. Together with the montmorillonite preservation, this indicates that the colonization and subsequent rapid fossilization occurred in an aquatic environment of ambient or higher temperatures, which coincides with the late stages of seawater–rock interactions. The basalt was subjected to seawater alteration during 1–2 million years while the sediment pile built up and finally isolated the volcanic section (Révillon et al., 2007; Ivarsson et al., 2013), which provides the time window for the colonization to occur.

**Nature of the organisms**

Previous work has established thin mycelial structures from these rocks as fungal hyphae based on characteristic fungal morphologies like repetitive septa, anastomoses between branches, a central strand, and the presence of chitin, which together suggest affinity to the Dikarya (Ivarsson et al., 2012, 2013). Because the hyphae seem to emanate from the basal film and become wider with increasing distance from the film, we tentatively interpret the film and its coarser protrusions to belong to the same organism as the hyphae. The short coarse hyphae with globular terminations (Fig. 2A–C) may represent sporophores, but the lack of internal structures does not allow determination of which, if any, dikaryon sporophore type is represented. The larger globular features connected to coarse hyphae within the calcite may also be related to reproduction, but as these hyphae appear to have been actively boring into the calcite (see below), a function to propagate spores into the surrounding medium seems less likely.

The regular alignment in strings of the minute bodies in the ‘cobwebs’ indicates that the bodies are not accidentally trapped particles but belong to an organized structure. The size, morphology, and organization of the ‘cobwebs’ are concordant with prokaryotic cells, and the organized association between the ‘cobwebs’ and the fungal hyphae suggests a symbiotic relationship between the two. The organization and placement of the ‘cobwebs’ would be consistent with micro-organisms that utilize elements or compounds dissolved or in suspension. The ‘cobweb’ structure is similar to that of the sulfur-oxidizing archaea *Pyrodictium* (Rieger et al., 1995) and *Euryarchaeon* SM1 (Moissl et al., 2003), frequent in hydrothermal environments, as well as sulfur-oxidizing bacteria living on sulfidic sediments (Fenchel & Glud, 1998; Thar & Kühl, 2001).

Lack of S in EDS analysis speaks against the presence of sulfur oxidizers, however. As the putative cells in the ‘cobwebs’ consist of iron oxides, there is a possibility that the organisms mediated iron oxidation metabolically, but the composition might also be due to diagenesis, as suggested by the similar composition of the fungal hyphae. The lack of sulfides and the presence of iron oxides in the samples exclude strictly anoxic conditions.

The nature of *Frutexites* has been the subject of discussion in the literature (reviewed by Rodríguez-Martínez et al., 2011). Most interpretations have landed in a bacterial origin, but non-biogenic chemical precipitation has also been entertained as an alternative. This question reflects the well-known problem of how to distinguish biogenic stromatolites from non-biogenic precipitates (Grotzinger & Rothman, 1996). We interpret the Koko Seamount *Frutexites* to be bacterial in origin, for three main reasons:

1. Recent formation of *Frutexites*-like structures in subterranean rocks has been shown to be associated with the chemolithotrophic iron-oxidizing bacteria *Galvo-nella ferruginea* (Rodríguez-Martínez et al., 2011).
2. Chafetz & Guidry (1999), studying precipitates in hydrothermal environments, found a morphological and structural continuum from bacterially mediated ‘shrubs’ to abiotically precipitated dendrites. The

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structures at the biological end of the spectrum are morphologically identical to *Frutexites* and include goethite-rich structures with well-preserved bacterial fossils. ‘Shrubs’ at the non-biogenic end are dominated by crystal shapes and differ substantially from the bacterially induced ones.

3 The Koko Seamount *Frutexites* are dominated by goethite, whereas the basal films and hyphae attributed to fungi mainly consist of hematite of likely diagenetic origin.

We therefore interpret the Koko Seamount *Frutexites* as ferruginous microstromatolites formed by iron-oxidizing bacteria.

**Symbiotic relationships**

We propose that three separate organisms lived simultaneously in physical contact in the voids in the Eocene basalt: the fungi (basal film with protuberances and mycelial network), the prokaryotic cells suspended like cobwebs between the fungal hyphae, and the iron-oxidizing bacteria forming the microstromatolites. From a metabolic point of view, we suggest a symbiosis between chemosynthetic prokaryotes and heterotrophic fungi in the deep crustal environment would be functional. The role of the chemosynthetic symbionts in such an arrangement could be to utilize inorganic carbon such as CO₂ and through oxidation of reduced compounds transform it into organic molecules accessible to the eukaryotic host. The fungal production of CO₂ could be utilized by the chemosynthetic community, although it is not clear whether CO₂ would have been limiting in this environment even without the presence of fungi.

Symbiosis between prokaryotic and eukaryotic cells is a globally important phenomenon that influences the physiology, ecology, and evolution of virtually every organism on Earth. Eukaryotic hosts expand their ecological niches through symbiosis with metabolically diverse bacteria and archaea (Stewart et al., 2005). Establishment of sustainable eukaryotic colonies in a challenging environment such as that of subseafloor basalts would probably be impossible without chemosynthetic symbionts serving as sources of accessible metabolites like carbohydrates.

When the basaltic vesicles were formed in molten magma, they were sterile. A pre-requisite for colonization by prokaryotes and fungi and for preservation of the habitat is that the vesicles are connected to the overlying seawater column and circulating brines by fractures, veins, pores, or chains of adjacent vesicles. The club-shaped sporophore-like structures of the fungi (Fig. 2) probably represent a means of ensuring that propagules are released into the circulating water to enable their distribution within the available habitat of connected vesicles. In view of the likely sporadic interconnectedness of the deep habitats in crustal rocks, a considerable diversity of biotopes is to be expected.

A decade ago Baross et al. (2004) pointed out that even after 40 years of scientifically coordinated drilling in the oceanic basement ‘…so far, we have not been able to visualize the microbial communities in their specific biotopes within the crust …’. Today, by detailed studies of the fossil record and with the aim of powerful 3D techniques, we are able to visualize this hidden biosphere. Fossil data provide an essential tool to understand the ecology and diversity of subseafloor biotopes that we cannot yet observe *in vivo*, as well as a window onto the ancient history of the deep biosphere.

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**AUTHOR CONTRIBUTIONS**

M.I. and S.B. conducted the design and analyses and wrote the paper. M.I. prepared the samples and acquired ESEM/EDS data. S.B., A.A., V.B., F.M., and M.S. performed SRXTM measurements. C.B. acquired Raman spectroscopic data. S.B. prepared light micrographs and tomography renderings.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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