

Cultivation of Sulfate-Reducing Bacteria from Deep Sediment Layers that are Influenced by Crustal Fluids (IODP Leg 301)

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Crustal fluids may fuel the deep biosphere

Microbiological studies on sediment cores collected during DSDP and ODP have consistently demonstrated the presence of a marine 'deep biosphere' (e.g. D'Hondt et al., 2004). Microbial communities were found to be present in sediments down to several hundreds of meters below the seafloor (Parkes et al., 2000). Furthermore, recent investigations indicated that the deep biosphere extends into the upper basaltic layers of the oceanic crust (Cowen et al., 2003; Huber et al., 2006; Nakagawa et al., 2006). These porous volcanic layers are characterized by the circulation of seawater, forming the largest aquifer on Earth. Due to their geochemical composition, the circulating fluids are supposed to fuel the deep biosphere by intrusion of oxidized compounds into overlying sediments (DeLong, 2004).

IODP Expedition 301 offered an excellent opportunity to test this hypothesis. Drilling was conducted at the Juan de Fuca Ridge, in the northeast Pacific Ocean. This location is one of the most intensively studied areas in terms of fluid flow hydrology and impact on sedimentological settings (Fisher et al. 2005). At IODP Site U1301 (water depth: 2650 m, sediment thickness: 265 m) sulfate diffuses into the sediment column from two sites, from bottom-seawater and the crustal aquifer, resulting in two sulfate-methane interfaces, and in an *upper* and a *lower* potential sulfate reduction zone (Fig.1a). For microbiological analyses high quality, non-contaminated sediment samples were obtained by advanced piston coring, as indicated by perfluorocarbon tracer (PFT) measurements (Lever et al., 2006).

Sulfate diffusion from below keeps microbes alive

Within the first phase of our investigations, we have quantified the abundance of microorganisms with various methods and determined microbial activities like sulfate reduction, anaerobic oxidation of methane, and exoenzyme activity at nearby *in situ* temperatures throughout the sediment column (Engelen & Ziegelmüller et al., 2008). In short, microbial cell densities decreased with sediment depth. Cell counts showed local peaks following geological settings and were enhanced in basement-near layers (data not shown). Potential metabolic rates (Fig. 1b) were elevated around the *lower* sulfate-methane transition zone (SMTZ). Using the semi-quantitative most probable number (MPN) technique, a significant fraction of the microbial community could be stimulated to grow *ex situ* from the lower sulfate-containing zone. Our findings clearly indicated that indigenous microbial populations are present, alive and metabolically active in deeply buried layers.

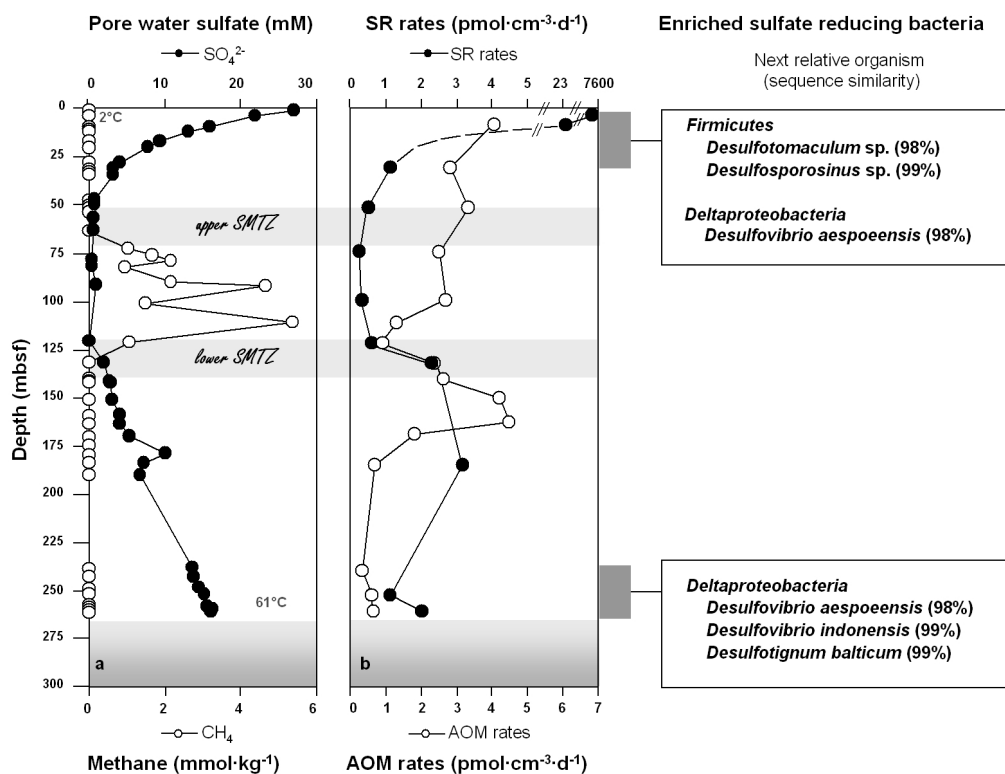


Fig.1 Depth profiles of (a) geochemical parameters and (b) metabolic activities. The phylogenetic affiliation of enriched sulfate-reducing bacteria from surface- and basement-near layers is indicated. SR, sulfate reduction, AOM, anaerobic oxidation of methane, SMTZ, sulfate-methane transition zone.

Molecular tools to monitor microbial enrichment cultures and subsequent isolations

Our investigations now focus on the analysis of cultivated members of the deep biosphere. Initial enrichments were performed in liquid dilution series and substrate gradient tubes. These cultures were started onboard the JOIDES Resolution immediately after core recovery. In order to stimulate growth of anaerobes, especially of sulfate-reducing bacteria, artificial seawater containing sulfate as terminal electron acceptor was amended with a defined substrate mixture in micromolar concentrations. Enrichment cultures were incubated at 20°C in the dark and at atmospheric pressure. Molecular screening was used to overview the diversity of cultivated microorganisms and to guide further isolation procedures via deep agar cultures and liquid dilution series.

For molecular screening, DNA was extracted from a) enrichment cultures that showed microbial growth determined by microscopic analysis or b) transferred colonies. 16S ribosomal RNA gene fragments were amplified by PCR and separated via denaturing gradient gel electrophoresis (DGGE). Distinct bands were excised, reamplified and sequenced. 16S rDNA sequences were phylogenetically identified using the BLASTn tool for the affiliation to their next relatives.

Due to low DNA-extraction yields, nested PCR was necessary. In spite of that a high diversity of growing bacteria was reflected in complex DGGE banding patterns. The screening revealed 22 operational taxonomic units (OTU) from seven eubacterial phyla, commonly found in natural environments: *Firmicutes*, *Actinobacteria*, *Beta*-, *Gamma*-, *Delta*- and *Epsilonbacteria*, *Cytophaga-Flavobacterium-Bacteroides*. Furthermore, DNA-signatures related to previously described sulfate-reducing bacteria (SRB) were detected in enrichments from 1.3, 31, 75 and even 260 mbsf.

Two different sulfate-reducing communities enriched from the sediment column

So far, the continuous use of microscopy, molecular screening of subcultures (Fig.2) and H₂S measurements led to a culture collection that is dominated by different sulfate reducers originating from top and bottom sediments (Fig. 1). *Desulfosporosinus*- and *Desulfotomaculum*-related *Firmicutes* were repeatedly enriched from the upper sulfate-reduction zone (1.3, 9 and 31 mbsf). These spore-forming SRB are widespread and previously isolated from both, oceanic and terrestrial habitats (eg. Moser et al., 2005, Detmers et al., 2004). From fluid-influenced sediments two different sulfate-reducing *Deltaproteobacteria* were isolated. The strains affiliated with *Desulfotignum balticum* (260 mbsf) and *Desulfovibrio indonensis* (239, 252 and 260 mbsf), respectively. While *Desulfovibrio* species are commonly found in the deep biosphere (e.g. Bale et al., 1997, Sass and Cypionka, 2004) the recently described genus *Desulfotignum* comprises four species, only. Two of them have been isolated from marine habitats (Kuever et al., 2001, Schink et al., 2002).

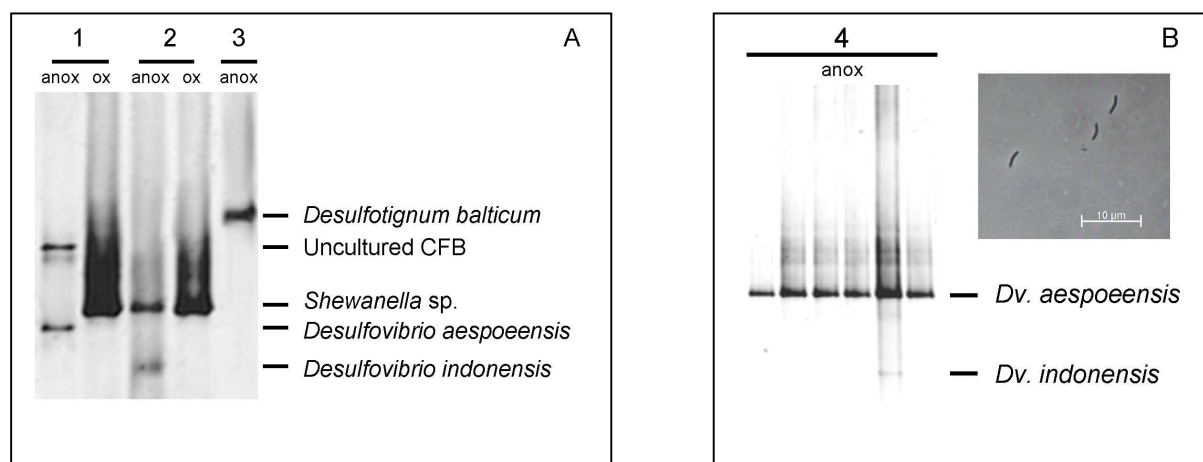


Fig.2 DGGE-fingerprints of different subcultures. DNA bands were assigned to the closest related bacteria. A) Anoxically incubated enrichment cultures and test for facultative growth under oxic conditions. A1+2) Anoxic subcultures, still containing different species. A3) Subculture of only one strict anaerobic strain. B) DGGE profiles of subcultures that exhibited vibrio-shaped cells.

More sulfate-reducing *Deltaproteobacteria* were enriched from both, seawater-influenced (1.3 mbsf) and crustal-fluids influenced sediment layers (239 and 260 mbsf). DGGE-band analysis resulted in an affiliation to *Desulfovibrio aespoeensis* (Fig.2B), a newly described sulfate reducer, supposed to be indicative for deep granitic rock aquifers (Motamedi and Pedersen, 1998). Currently we are working on the isolation of these strains into pure cultures to finally get access to their physiological properties.

Physiological experiments with sulfate-reducing isolates to unravel adaptations and lifestyles

In general, subsequent physiological characterization will elucidate the role of our isolates in biogeochemical cycles and their adaptations to this nutrient-limited habitat. We are especially interested in the substrate spectrum of the sulfate-reducing *Deltaproteobacteria* thriving in the deepest sediment layers. These strains are also tested for chemolithoautotrophy, i.e. growth on H₂/CO₂, since hydrogen may act as the key electron donor in basaltic environments (Stevens and McKinley,

1995). The use of alternative electron acceptors like manganese(IV), iron(III), nitrate or sulfur-compounds is examined as well. Furthermore, the maximum growth temperature of our *Desulfovibrio* strains was 45°C at ambient pressure. However, some of these isolates were obtained from basement-near layers with an *in situ* temperature of ~60°C (Fig.1). We suppose, that incubation experiments under *in situ* pressure (~300 bar) would lead to higher growth temperatures.

Conclusions

The enrichment of non-spore forming sulfate reducers from the crust-near layers indicates the presence of a viable and active deep biosphere and emphasizes the impact of crustal fluids on overlying sediments. Regarding the worldwide expansion of the crustal fluid aquifer, we assume that this impact is an important driving force for deep subsurface populations on a global scale.

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