

Comparison of Microbial Communities Inhabiting Ultramafic- and Basalt-Hosted Hydrothermal Systems:

Genetic Characterization of 16S rRNA Genes, Hydrogen Oxidation and CO₂ Fixation Pathways

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For Margot

GLOSSARY

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ABSTRACT

The host rock of a hydrothermal system defines that system's specific fluid chemical composition. Therefore the type of host rock (ultramafic rocks versus basalts) plays a major role in determining the most abundant energy sources which are available for microorganisms. This study was conducted to investigate the influence the host rock and resulting fluid chemistry have on the distribution of the indigenous microorganisms. For this purpose hydrothermal fluids were collected from the ultramafic-hosted Logatchev hydrothermal field (LHF) and the basalt-hosted Lilliput hydrothermal field. The microbial community analysis was based on 16S rRNA genes and fluorescence *in situ* hybridization. The functionality of chemoautotrophic communities was assessed in terms of putative CO₂ fixation pathways and energy sources used.

Ultramafic host rocks at the LHF result in fluids with extremely high hydrogen contents. Possibly as a consequence, phylogenetically diverse hydrogen-oxidizing chemolithotrophs were detected. High abundance in hydrogen concentrations seem to coincide with the diversification of putative hydrogen-oxidizing prokaryotes at the three sites investigated. By contrast, the fluids from the Lilliput field are very low in hydrogen but high in sulfide. Significantly less diverse hydrogen-oxidizing microorganisms were detected at the basalt-hosted Lilliput hydrothermal field. Nevertheless, diverse bacteria were related to organisms involved in sulfur cycling. This suggests that the host rock indirectly determines the presence of microorganisms with specific physiologies. However, in addition to the importance of the host rock it can be shown that mixing processes of hydrothermal fluids with ambient seawater have a significant impact on the distribution of microorganisms.

Contrary, for CO₂ fixation pathways the type of host rock is not relevant. Key enzymes of the two CO₂ fixation pathways investigated tolerate different levels of oxygen. The presence of multiple environmental niches with different oxygen contents is reflected in the phylogenetic diversity of Calvin Benson-Bassham and reverse tricarboxylic acid cycle genes detected at the LHF. At the Lilliput hydrothermal field the diversity of these different genes suggests that the chemoautotrophic community is better adapted to low oxygen contents. The generally high diversity of CO₂ fixation pathway genes demonstrates the ability of metabolically versatile prokaryotes to exhaust the potential of every available habitat.

ZUSAMMENFASSUNG

Die Gesteinszusammensetzung (Ultramafite versus Basalte) bestimmt die fluid-chemischen Parameter eines Hydrothermalsystems sowie die vorrangige Energiequelle, die für Mikroorganismen zur Verfügung steht. Die vorliegenden Untersuchungen wurden durchgeführt, um den Einfluss des Muttergesteins und dessen resultierende Fluidchemie auf die mikrobielle Lebensgemeinschaft an Hydrothermalsystemen zu untersuchen. Hierzu wurden hydrothermale Fluide von dem ultramafischen Logatchev Hydrothermalfeld (LHF) und dem basaltischen Lilliput Hydrothermalfeld entnommen. Die mikrobiellen Gemeinschaftsanalysen basieren auf 16S rRNA Genen und Fluoreszenz *in situ* Hybridisierung. Die Funktionalität der chemoautotrophen Gemeinschaften wurde an Hand von CO₂ Fixierungswegen und potentiell verwendeten Energiequellen untersucht.

Ultramafische Muttergesteine des LHF führen zu extrem hohen Wasserstoffkonzentrationen. Möglicherweise wurden deshalb phylogenetisch diverse wasserstoffoxidierende Chemolithotrophe entdeckt. An den drei untersuchten Standorten des LHF scheinen hohe Wasserstoffkonzentrationen mit einer Diversifizierung von potentiell wasserstoffoxidierenden Prokaryoten zu korrelieren. Im Gegensatz dazu wurde eine deutlich geringere Diversität von wasserstoffoxidierenden Mikroorganismen im Lilliput Hydrothermalfeld entdeckt. Stattdessen waren diverse Bakterien mit Organismen verwandt, die einen Anteil am Schwefelzyklus haben. Dies deutet darauf hin, dass das Muttergestein indirekt die Anwesenheit von Mikroorganismen mit spezifischen Physiologien vorbestimmt. Es wurde jedoch auch gezeigt, dass zusätzlich zu der Bedeutung des Muttergesteins Mischprozesse zwischen hydrothermalen Fluiden mit dem umgebenden Meerwasser einen signifikanten Einfluss auf die Verteilung der Mikroorganismen haben.

Im Gegensatz hierzu ist die Art des Muttergesteins für den verwendeten CO₂ Fixierungsweg nicht von Bedeutung. Schlüsselenzyme der zwei untersuchten CO₂ Fixierungswege tolerieren unterschiedliche Mengen an Sauerstoff. Die Diversität der Gene, die Enzyme des Calvin Benson-Bassham Zyklus und des reversen Citratzyklus kodieren zeigen, dass die LHF Fluide durch multiple Habitate mit verschiedenen Sauerstoffkonzentrationen hindurchfließen. Die chemoautotrophe Gemeinschaft im Lilliput Hydrothermalfeld hingegen scheint besser an geringe Sauerstoffkonzentrationen angepasst zu sein. Die generell hohe Gen-Diversität der verschiedenen CO₂ Fixierungswege verdeutlicht die Fähigkeit von metabolisch unterschiedlichen Prokaryoten jedes erdenklich potentielle Habitat zu nutzen.

1 INTRODUCTION

1.1 Deep-Sea Hydrothermal Vent Fields

Low-temperature venting was the first type of hydrothermal activity discovered along the global mid-oceanic ridge system (Edmond *et al.*, 1979). The associated environments were recognized as important habitats for microorganisms and macro-fauna (Corliss *et al.*, 1979, Karl *et al.*, 1980). The primary mechanisms responsible for the chemistry of diffuse discharge are mixing processes of hot, reduced hydrothermal fluids with seawater ingressing the crust (James & Elderfield, 1996). Additionally, conductive cooling (Cooper *et al.*, 2000), mineral precipitation/dissolution (James & Elderfield, 1996) and biological activity (Lilley *et al.*, 1983) strongly affect the chemical signature of the diffuse emissions.

Deep-sea hydrothermal vent environments comprise diverse biotopes which are characterized by steep physical and chemical gradients supporting the microbial communities (Kelley *et al.*, 2002). Physico-chemical conditions providing essentials for microbial life include temperature, pH, energy sources (reduced substrates e.g. hydrogen, sulfur compounds and methane) and oxygen (Kelley *et al.*, 2002). The most important physiological group are chemolithoautotrophic prokaryotes. They are responsible for the indigenous microbial primary production by using reduced compounds such as hydrogen or sulfur species to fix carbon dioxide (Shock & Holland, 2004). Their energy and carbon sources are supplied through either magma degassing and/or high temperature water-rock interactions (Shock & Holland, 2004).

1.2 Ultramafic- and Basalt-Hosted Hydrothermal Systems

Both ultramafic- and basalt-hosted hydrothermal systems are found in Mid-Ocean Ridge spreading areas. In hydrothermal fluids of magma driven hydrothermal systems (basalt-hosted) sulfide accounts for the most abundant reducing power (Kelley *et al.*, 2002). In contrast, fluids of hydrothermal systems hosted by ultramafic rocks reveal significantly higher hydrogen contents (Wetzel & Shock, 2000).

Ultramafic rocks are mainly composed of olivine and pyroxene (Mg and Fe²⁺ silicates). These minerals are thermodynamically stable at high pressures and temperatures present deep within the earth but react with water at moderate temperatures and pressures. The hydration of these rocks at near surface environments typically leads to the formation of serpentine minerals, brucite, and magnetite and is referred to as serpentinization. The oxidation of Fe²⁺ and the reduction of water through the net reaction $3\text{FeO} + \text{H}_2\text{O} \rightarrow \text{Fe}_3\text{O}_4 + \text{H}_2$ during serpentinization results in the release of molecular hydrogen. Basalts contain olivine, pyroxene and feldspar. Generally the sulfur concentrations in submarine mafic rocks are significantly higher than in ultramafic rocks (Alt & Shanks, 1998). This results in higher sulfide concentrations in basalt-hosted hydrothermal fluids (Alt & Shanks, 1998). It is thus clear that the type of host rock affects the chemical composition of the emanating fluids. In turn the hydrothermal fluids supply indigenous prokaryotes with energy and carbon sources, which are necessary to fuel primary production.

1.2.1 Ultramafic-Hosted: Logatchev Hydrothermal Field (LHF)

Hydrothermal habitats hosted in ultramafic rocks possibly represent our closest analogue to early earth environments (Holm & Charlou, 2001). They could have played a pivotal role in the origin and evolution of life (Shock & Schulte, 1998). To date only a few active ultramafic-hosted hydrothermal vents are known, e.g. the Lost City, Rainbow and Logatchev hydrothermal fields which are located along the northern Mid-Atlantic Ridge (Bogdanov *et al.*, 1997, Kelley *et al.*, 2001, Charlou *et al.*, 2002, Douville *et al.*, 2002, Kuhn *et al.*, 2004). From all active vent sites, the Rainbow vent field (36°N), with its geological setting and fluid physico-chemistry, is the one most closely resembling the Logatchev hydrothermal field (LHF). Both vent systems host black smoker chimneys with measured temperatures of the emanated fluids of up to 360°C (Donval *et al.*, 1997, Lackschewitz *et al.*, 2005). Serpentinization processes are evident by high concentrations of dissolved hydrogen and methane compared to fluids emanating from basalt-hosted systems (Kelley *et al.*, 2001). However, additional reaction of heated seawater with gabbroic rocks is indicated. The hydrothermal fluids are acidic (and characterized by moderate silica enrichment, high transition metal concentrations and significantly lower sulfide concentrations compared to basaltic systems) (Douville *et al.*, 2002, K. Schmidt, A. Koschinsky, D. Garbe-Schönberg, L. M. de Carvalho, and R. Seifert, submitted for publication 2006). Focused hot hydrothermal

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fluids emit from chimney structures (Irina II and Barad Dur) and areas of smoking craters (e.g. Irina I and Site B) (Fig. 1A, B, C). In addition, low temperature diffuse outflow exists.

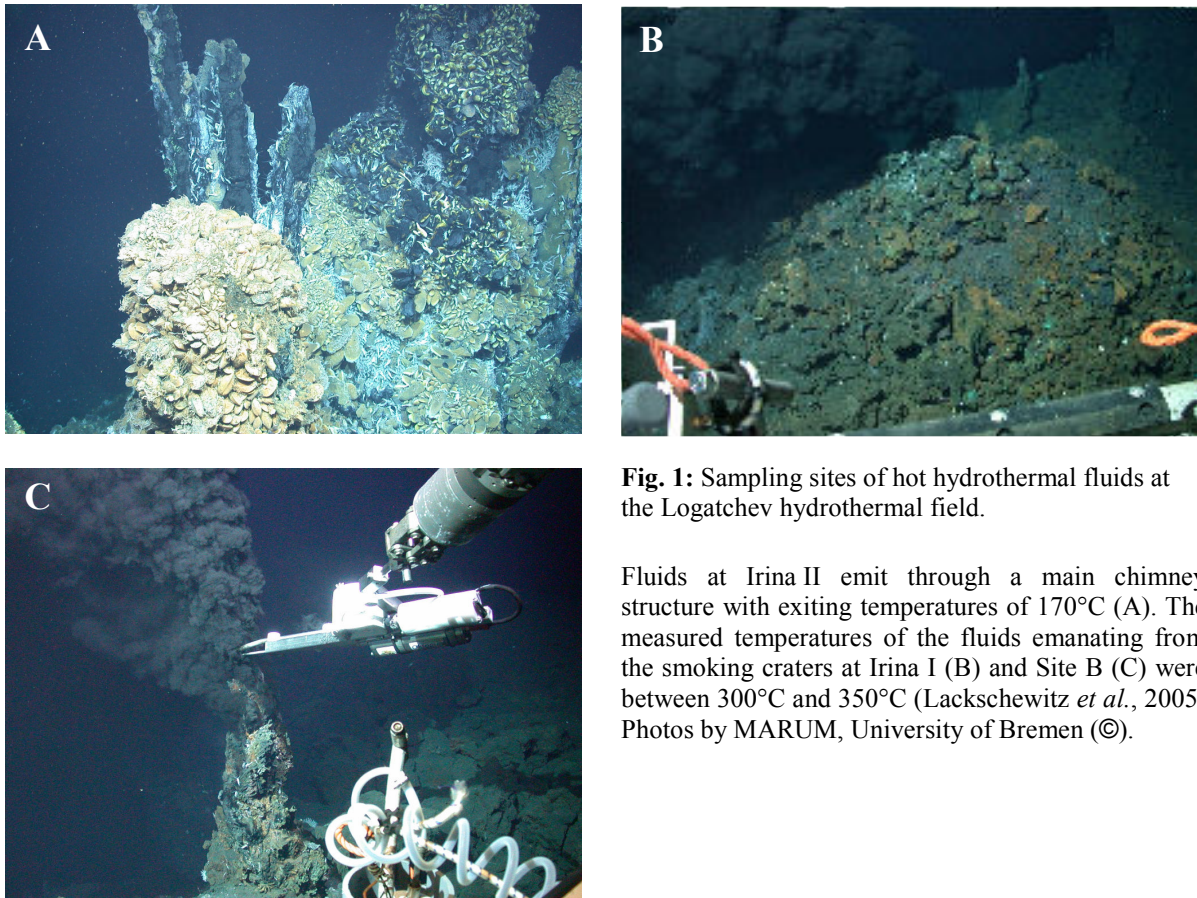


Fig. 1: Sampling sites of hot hydrothermal fluids at the Logatchev hydrothermal field.

Fluids at Irina II emit through a main chimney structure with exiting temperatures of 170°C (A). The measured temperatures of the fluids emanating from the smoking craters at Irina I (B) and Site B (C) were between 300°C and 350°C (Lackschewitz *et al.*, 2005). Photos by MARUM, University of Bremen (©).

1.2.2 Basalt-Hosted: Lilliput Hydrothermal Field

The Lilliput hydrothermal field was discovered in 2005 as the most southern known hydrothermal field on the Mid-Atlantic Ridge (9°33'S) (Haase *et al.*, 2005). It is located on a ridge segment with considerably thickened oceanic crust (11 km) (Bruguier *et al.*, 2003). It has been suggested that diffuse low-temperature venting may be typical for ridge segments with thickened crust because high-temperature vents appear to be very rare on such segments. For example, only one hydrothermal system (the Steinhóll field) was found in a detailed study covering some 600 km of the Reykjanes Ridge, which has a thickened crust comparable to that of the Mid-Atlantic Ridge at 9°33'S (German *et al.*, 1994). Possible explanations for the apparent lack of high-temperature venting on ridge segments with thickened crust are that melt lenses (representing the deep-seated heat source and reaction zone) may lie too deep to be reached by seawater circulation. Alternatively, deep-water circulation does not occur at

such sites due to the absence of deep faults in the thick, hot crust (German *et al.*, 1994, Chen, 2003).

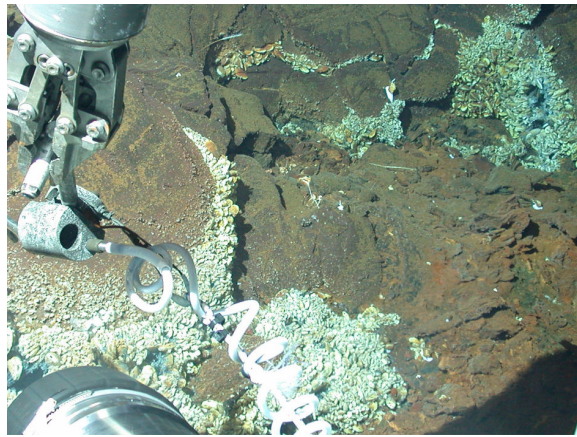


Fig. 2: Sampling site of low-temperature fluids at the Lilliput hydrothermal field. Outflow of fluids revealed temperatures of 5.5°C. Photos by MARUM, University of Bremen (©).

At the Lilliput hydrothermal field low-temperature diffuse fluids (<16.7°C) emit through cracks of basalt pillows overgrown with patchy assemblages of *Bathymodiolus* mussels (Haase *et al.*, 2005) (Fig. 2). The accumulation of low-temperature Fe-oxides/hydroxides has been observed (Fig. 2). So far, no evidence indicative of high-temperature processes (e.g. chimneys or sulfides) has been detected suggesting that temperatures at Lilliput do not exceed a few tens of degrees (Haase *et al.*, 2005). Additionally, the unsuccessful search of high-temperature emissions using the autonomous underwater vehicle “ABE” in 2006 supports the idea of low-temperature based hydrothermalism at Lilliput. Judging from the abundance of juvenile mussels (O. Giere, personal communication) it has been concluded that the Lilliput hydrothermal field might have recently been reactivated and subsequently populated by mussels.

1.3 Microorganisms Inhabiting Hydrothermal Environments

Several studies have been concerned with the diversity of microorganisms inhabiting geographically distinct basalt-hosted systems, while only three (including the present work) ultramafic-hosted hydrothermal vent environments (Lost City, Rainbow and Logatchev hydrothermal fields) have been investigated for their indigenous microbial community (López-García *et al.*, 2003, Brazelton *et al.*, 2006). The chemolithotrophic microbial communities of ultramafic- and basalt-hosted deep-sea hydrothermal environments show a

clear dominance of only a few phylogenetic lineages. From the domain archaea members of the order *Methanococcales* are apparently well adapted to these special habitats. Bacteria of the order *Aquificales* and the *Epsilonproteobacteria* represent equal matches within the domain bacteria. Chemolithoautotrophic organisms, like representatives of the *Methanococcales*, *Epsilonproteobacteria* or *Aquificales* have been repeatedly detected in various deep-sea hydrothermal vent environments (e.g., Huber *et al.*, 2002, Huber *et al.*, 2003, Takai *et al.*, 2003, Takai *et al.*, 2004a, Nakagawa *et al.*, 2005b).

Members of the order *Methanococcales* are strictly anaerobic and able to use hydrogen and carbon dioxide as a substrate for methanogenesis (Whitman *et al.*, 1992). Previous studies have implied that they inhabit hydrothermal subsurface environments (Huber *et al.*, 2002, Nakagawa *et al.*, 2005b). Hydrogenotrophic methanogenesis is independent of seawater derived oxidants (i.e. O₂ or NO₃) but controlled by gaseous substances (hydrogen and carbon dioxide) originating from hydrothermal fluids (Takai *et al.*, 2004a). Therefore, methanogens are probably the most important primary producers of subvent biospheres (Huber *et al.*, 2002).

The *Epsilonproteobacteria* have been shown to constitute up to 90% of microbial vent communities (Nakagawa *et al.*, 2006). They are the primary colonizers of newly founded vent emissions (Alain *et al.*, 2004, Higashi *et al.*, 2004, Nakagawa *et al.*, 2005c) and have been detected in diverse habitats of hydrothermal vent environments (Teske *et al.*, 2002, Hoek *et al.*, 2003, Huber *et al.*, 2003, Page *et al.*, 2004, Nakagawa *et al.*, 2005c). Nevertheless, they are the least well characterized class among the *Proteobacteria* (Campbell *et al.*, 2006). According to Corre and colleagues (Corre *et al.*, 2001) *Epsilonproteobacteria* have been classified into at least 6 subgroups: A, B, C, D, F and G. However, it was recently discovered that the group C sequences had been misclassified (Campbell *et al.*, 2006). As a majority of *Epsilonproteobacteria* is able to fix carbon dioxide (e.g., Inagaki *et al.*, 2004, Miroshnichenko *et al.*, 2004, Takai *et al.*, 2004c, Nakagawa *et al.*, 2005a) they are possibly responsible for major parts of primary production in vent environments (Campbell *et al.*, 2006). Several studies have revealed the metabolic diversity of *Epsilonproteobacteria* (for review, see Campbell *et al.*, 2006). Their ability to use a wide range of electron donors and electron acceptors (e.g. hydrogen, sulfur compounds, nitrate and oxygen) suggests their importance in hydrogen, sulfur and nitrogen cycling at hydrothermal vent environments (Takai *et al.*, 2003, Nakagawa *et al.*, 2005d, Campbell *et al.*, 2006). Other chemolithotrophic species involved in hydrogen oxidation include thermophilic organisms of deeply-rooted lineages e.g.

Persephonella spp. (Götz *et al.*, 2002) or *Desulfurobacterium* spp. (L'Haridon *et al.*, 1998). Sulfur oxidizing mesophiles like *Thiomicrospira* spp. have also been identified in hydrothermal vent emissions (Jannasch *et al.*, 1985, Takai *et al.*, 2004b).

Due to the focus on high-temperature black smokers in the past, comparatively few studies are available engaging in the microbiology and fluid chemistry of diffuse, low-temperature emissions and ambient habitats. Microbiology studies of low-temperature hydrothermal environments include diffuse discharge sites from the Juan de Fuca Ridge (Holden *et al.*, 1998, Huber *et al.*, 2002, Huber *et al.*, 2003), organic rich sediments from the Guaymas Basin (Teske *et al.*, 2002) and the Rainbow hydrothermal field (López-García *et al.*, 2003) as well as metazoan-symbiont associations and adjacent substrate (Alain *et al.*, 2002, Alain *et al.*, 2004, Goffredi *et al.*, 2004, Page *et al.*, 2004). In all cases a rich microbial fauna was observed. It included members of *Gamma*-, *Epsilon*- and *Deltaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Aquificales*, various candidate divisions as well as hyperthermophilic archaea (Alain *et al.*, 2002, Huber *et al.*, 2002, Teske *et al.*, 2002, Huber *et al.*, 2003, López-García *et al.*, 2003, Goffredi *et al.*, 2004, Page *et al.*, 2004). The frequent encounter of thermophilic and hyperthermophilic prokaryotes in low-temperature emissions has suggested the presence of a hot subsurface environment (Holden *et al.*, 1998, Huber *et al.*, 2002, Huber *et al.*, 2003).

1.3.1 Energy Sources

Dependent on the source rock hosting the hydrothermal systems different energy sources are available for fueling autotrophic growth. As opposed to ultramafic-hosted hydrothermal systems (e.g. LHF) where hydrogen and methane are the dominant inorganic energy sources supplied by the hydrothermal fluids (R. Seifert, personal communication) at basalt-hosted systems e.g. Lilliput hydrothermal field sulfide is the most abundant energy source (Haase *et al.*, 2005). In accordance with the petrological characteristics of the host rocks different inorganic energy sources are expected to be predominantly available and thus preferentially used as an electron donor by the indigenous chemolithoautotrophs to gain energy for carbon fixation.

Epsilonproteobacteria have frequently been encountered at hydrothermal vent environments (Campbell *et al.*, 2006). Even though their metabolism has been described as versatile (Campbell *et al.*, 2006), hydrogen oxidation seems to be widespread among this group (Nakagawa *et al.*, 2005c). The detection of soluble, hydrogenase activity and the successful amplification of NiFe-Uptake Hydrogenase (catalyzes the oxidation of molecular hydrogen) encoded by the gene *hynSL* has enabled genetic characterization of hydrogen-oxidizing chemolithotrophic *Epsilonproteobacteria* on a functional level (Takai *et al.*, 2005). This is especially important for resolving the impact petrological characteristics of the rocks (ultramafic- as opposed to basalt-hosted systems) have on the microbial community.

1.3.2 CO₂ Fixation Pathways

Autotrophic microorganisms at hydrothermal vent environments are responsible for the production of biomass. They are therefore fundamental for all other organisms inhabiting these ecosystems. To date four pathways are known which can be used for CO₂ fixation. This includes the Calvin Benson-Bassham (CBB) cycle, the reverse tricarboxylic acid (rTCA) cycle, the reductive acetyl coenzyme A pathway and the 3-Hydroxypropionate cycle (Madigan *et al.*, 2003).

The CBB cycle is widely distributed (Elsaied & Naganuma, 2001, Shively *et al.*, 2001). Functional genes encoding the key enzymes of the CBB cycle include the ribulose-1,5-bisphosphatecarboxylase/oxygenase (RubisCO) (Chase, 1993). Two distinct types of RubisCO (form I and form II) exist, which differ structurally from each other (Tabita, 1995). The large subunits of form I and form II RubisCO are encoded by genes designated *cbbl* and *cbbm* respectively (Kusian & Bowien, 1997). Form II RubisCO has a lower specificity for CO₂ versus O₂ than form I and is thus more effective in CO₂ rich environments (Tabita, 1995). Hence, it is believed that form II resembles the ancient form of RubisCO while form I is postulated to have evolved due to the decline of CO₂ and the emergence of oxygen in the atmosphere (McFadden & Tabita, 1974, McFadden *et al.*, 1986, Shively *et al.*, 1986). The CBB cycle is predominantly found in organisms with a high energy yield and a phototrophic or aerobic chemoautotrophic lifestyle (Madigan *et al.*, 2003).

1 INTRODUCTION

In contrast, prokaryotes inhabiting environments with more extreme parameters (temperature, pH, oxygen conditions) generally seem to use alternate pathways for CO₂ assimilation. Such an alternate CO₂ fixation pathway is the reverse tricarboxylic acid (rTCA) cycle, with key enzymes including the ATP citrate lyase (encoded by the *aclBA* gene). However, some affiliates of sulfate reducing *Deltaproteobacteria* growing on acetate also use ATP citrate lyase in the TCA cycle (Möller *et al.*, 1987). The rTCA cycle has been demonstrated to operate in several prokaryotes. They are characterized by microaerophilic or anaerobic growth i.e. *Aquificales*, anoxic phototrophic bacteria (*Chlorobium limicola*), *Epsilonproteobacteria*, sulfate reducing bacteria (*Desulfobacter* spp.), and sulfur-reducing *Crenarchaeota* (*Thermoproteus* spp.) (Evans *et al.*, 1966, Shiba *et al.*, 1985, Schauder *et al.*, 1987, Beh *et al.*, 1993, Hügler *et al.*, 2005, Takai *et al.*, 2005).

Epsilonproteobacteria are among the most abundant organisms at hydrothermal environments, are metabolically and thermally versatile (Campbell *et al.*, 2006) and autotrophic representatives have been shown to use the rTCA cycle (Hügler *et al.*, 2005, Takai *et al.*, 2005). Indeed, the rTCA cycle seems to be the predominant mechanism for autotrophic CO₂ fixation at hydrothermal vent environments (Campbell & Cary, 2004, Takai *et al.*, 2005, Campbell *et al.*, 2006). Advantages of using the rTCA cycle as opposed to the CBB cycle are reflected in the energy consumption. The operation of the CBB cycle is energetically more demanding than the rTCA cycle (Madigan *et al.*, 2003). Other differences between the two CO₂ fixation pathways include oxygen sensitivity/tolerance of their key enzymes (Tabita, 1995, Takai *et al.*, 2005).

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2.1 Sampling Sites and Fluid Characteristics

Hydrothermal fluid samples were retrieved during dives with the remotely operated vehicle (ROV) QUEST (MARUM, University of Bremen) during the HYDROMAR I (M60/3, 2004) and HYDROMAR II (M64/2, 2005) cruises to the Logatchev hydrothermal field (LHF) and during the MAR-SÜD II (M64/1, 2005) cruise to the Lilliput hydrothermal field (Fig. 3).

2.1.1 Logatchev Hydrothermal Field (LHF)

The LHF is located at $14^{\circ}45'N$ and $44^{\circ}58'W$ on the northern Mid-Atlantic Ridge (MAR) in water depths between 2960 m and 3060 m (Fig. 3, 4) (Bogdanov *et al.*, 1997, Kuhn *et al.*, 2004). It is an ultramafic-hosted hydrothermal system with distinct differences in rock

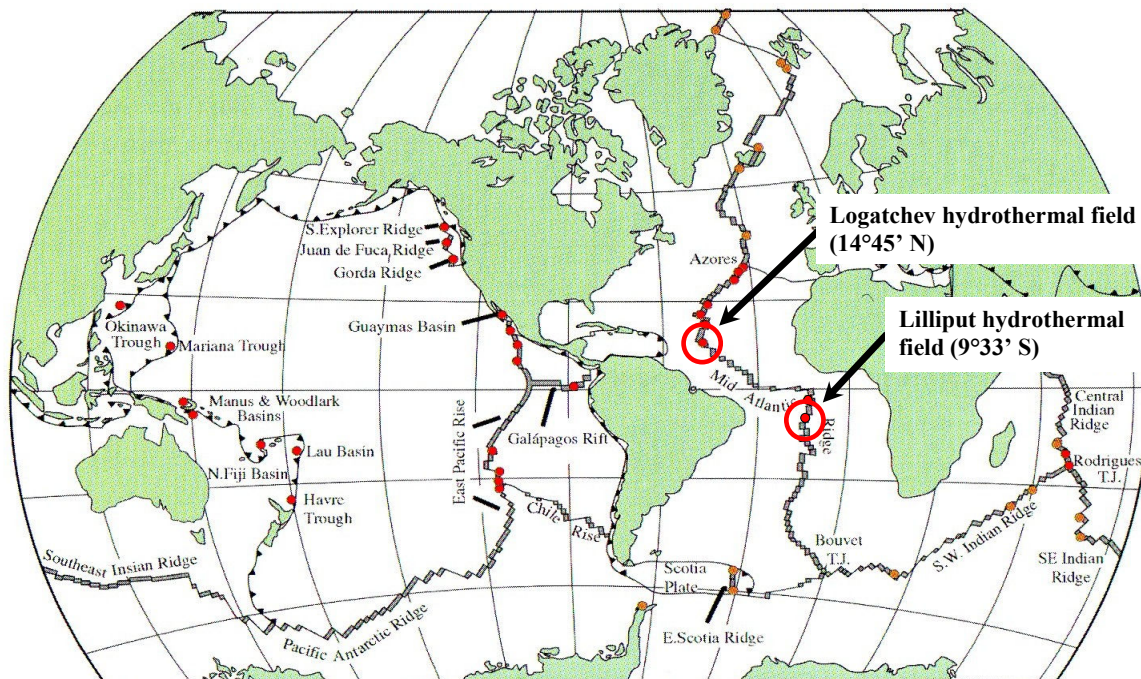


Fig. 3: Locations of known hydrothermal activity along the global mid-ocean ridge system. The Logatchev and Lilliput hydrothermal fields are highlighted by red circles. Red dots indicate active sites; orange dots denote midwater chemical anomalies. Modified after Baker & German (2004).

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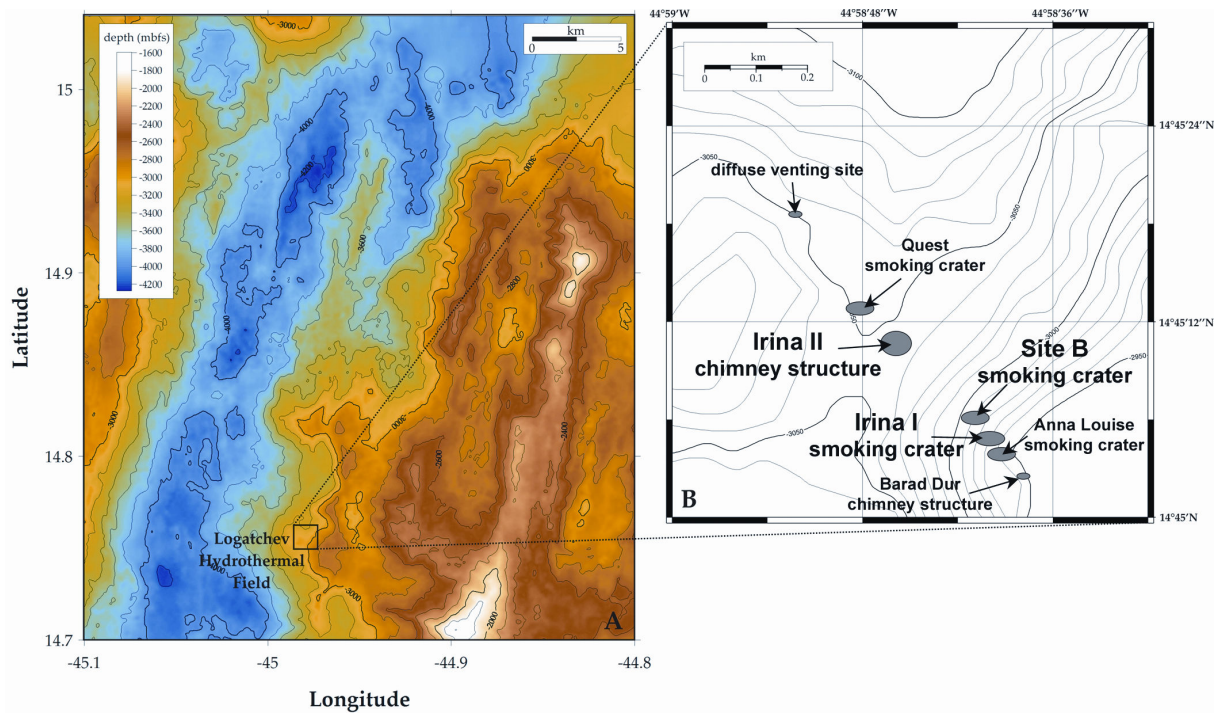


Fig. 4: Location of the Logatchev hydrothermal field (LHF) on the northern Mid-Atlantic Ridge. It is located at water depths between 2960 and 3060 m (A). The three sites where high-temperature fluids were sampled (Irina I, Irina II and Site B) at the LHF are highlighted (B). Modified after Kuhn *et al.* (2004).

mineralogy of mafic and ultramafic rocks (Kuhn *et al.*, 2004). Serpentinization processes at the LHF are responsible for extremely high hydrogen (≤ 19 mM) and methane concentrations (≤ 3.5 mM) in hydrothermal fluids with sulfide concentrations not exceeding 3.5 mM (all end-member corrected) (K. Schmidt, A. Koschinsky, D. Garbe-Schönberg, L. M. de Carvalho, and R. Seifert, submitted for publication). Sulfide concentrations are lower compared to basaltic systems, typically ranging between 4 mM and 6 mM (Douville *et al.*, 2002).

High temperature fluid emissions were sampled in an area composed of two smoking craters (Irina I and Site B) and at the chimney structure of Irina II (Fig. 1A-C, 4B). While fluid emissions at the smoking crater sites measured exiting temperatures of 300-350°C, temperatures recorded for fluid outflow at the main complex Irina II were lower (170°C) (Lackschewitz *et al.*, 2005). All fluids show a slight depletion in chlorinity, which is still within the range of chloride variability and might be due to water-rock interactions (K. Schmidt, A. Koschinsky, D. Garbe-Schönberg, L. M. de Carvalho, and R. Seifert, submitted for publication). In general, hot, highly reducing and acidic fluids emanate from black smoker chimneys as well as smoking craters (K. Schmidt, A. Koschinsky, D. Garbe-Schönberg, L. M. de Carvalho, and R. Seifert, submitted for publication).

2.1.2 Lilliput Hydrothermal Field

The Lilliput diffuse outflow area is located at 9°32.85'S and 13°13.54'W on the southern Mid-Atlantic Ridge (MAR) (Fig. 3). It is located in a water depth of 1500 m and is hosted by basalts. The low-temperature emissions at the Lilliput hydrothermal field were only slightly elevated in hydrogen (16 nM) and in methane (2.6 µM) when compared to ambient seawater (R. Seifert, personal communication). Sulfide in contrast was the most abundant energy source (0.5 mM) available (H. Strauss & M. Peters, personal communication).

2.2 Sample Collection

For sampling of hydrothermal fluids from high (Logatchev - Irina I, Irina II and Site B) and low temperature (Lilliput) vents, a pumped flow-through system (Kiel Pumping System KIPS) was specially designed for the ROV QUEST (Garbe-Schönberg *et al.*, 2006). The pumping system is composed of a titanium nozzle connected with 5-15 parallel PFA sampling flasks (675 ml volume/flask) via PFA tubings. The nozzle was placed into the hot vent orifice or diffuse fluids prior to collection of hydrothermal samples. To ensure a complete exchange of fluids actual pumping time of fluid sampling accounted for approximately one hour. Fluid chemical analysis and microbial diversity studies were conducted using hydrothermal liquids from aliquots of the same sample. For microbiology studies, the liquids were concentrated *in vitro* on 0.2 µm pore size polycarbonate filters (Sartorius).

2.3 Fluorescence *in Situ* Hybridization (FISH)

To examine the relative abundances of different archaeal and bacterial groups in the diffuse outflow at the Lilliput hydrothermal field fluorescence *in situ* hybridization was performed. The oligonucleotide probes used are summarized in Table 1. Filters were fixed on board with formaldehyde (4% v/v) for 4 hours, washed twice with 1xPBS (phosphate-buffered saline), fixed in 1 ml ethanol: 1xPBS (1:1) and stored at 4°C until further treatment. FISH analysis with CY3-labeled oligonucleotide probes (final concentration, 50 ng/µl) and DAPI coloring were conducted according to Glöckner and colleagues (1999). Filter sections were inspected

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using an Axiophot epifluorescence microscope (Zeiss, Jena, Germany). All counts were corrected by subtracting the number of signals obtained with the negative control (NON338).

Table 1: Oligonucleotide probes used in this study.

Probe	Specificity	Position in <i>E. Coli</i>	Probe sequence (5'→3')	Fa ^a (%)	Reference
EUB338	Most Bacteria	338-355	GCT GCC TCC CGT AGG AGT	35	(Amann <i>et al.</i> , 1990)
EUB338II	<i>Planctomycetales</i>	338-355	GCA GCC ACC CGT AGG TGT	35	(Daims <i>et al.</i> , 1999)
EUB338III	<i>Verrucomicrobiales</i>	338-355	GCT GCC ACC CGT AGG TGT	35	(Daims <i>et al.</i> , 1999)
NON338	Negative control	338-355	ACT CCT ACG GGA GGC AGC	0	(Wallner <i>et al.</i> , 1993)
ALF968	<i>Alphaproteobacteria</i>	968-985	GGT AAG GTT CTG CGC GT	20	(Neef, 1997)
Beta42a	<i>Betaproteobacteria</i>	1027-1043	GCC TTC CCA CTT CGT TT	35	(Manz <i>et al.</i> , 1992)
cBeta42a	Competitor	1027-1043	GCC TTC CCA CAT CGT TT	35	(Manz <i>et al.</i> , 1992)
GAM42a	<i>Gammaproteobacteria</i>	1027-1043 [§]	GCC TTC CCA CAT CGT TT	35	(Manz <i>et al.</i> , 1992)
cGam42a	Competitor	1027-1043 [§]	GCC TTC CCA CTT CGT TT	35	(Manz <i>et al.</i> , 1992)
EP404	<i>Epsilonproteobacteria</i>	404-420	AAA KGY GTC ATC CTC CA	30	(Macalady <i>et al.</i> , 2006)
DELTA495a	Most <i>Deltaproteobacteria</i> , some <i>Gemmimonas</i> group	495-512	AGT TAG CCG GTG CTT CCT	45 ^b	(Loy <i>et al.</i> , 2002)
cDELTA495a	Competitor	495-512	AGT TAG CCG GTG CTT CTT	45	(Macalady <i>et al.</i> , 2006)
CF319a	<i>Cytophaga-Flavobacterium</i> cluster	319-336	TGG TCC GTG TCT CAG TAC	35	(Manz, 1996)
ARCH915	Archaea	915-934	GTG CTC CCC CGC CAA TTC CT	20	(Stahl & Amann, 1991)
Cren512	Most Crenarchaeota	512-527	CGG CGG CTG ACA CCA	0	(Jürgens <i>et al.</i> , 2000)
Eury498	Most Euryarchaeota	498-511	CTT GCC CRG CCC TT	0	(Burggraf <i>et al.</i> , 1994)

All positions are located on the 16S except “§” which indicates 23S. ^a Values represent percent formamide in the hybridization buffer; ^b formamide concentrations adapted for FISH (Macalady *et al.*, 2006).

2.4 DNA Extraction and PCR Amplification

Environmental DNA was extracted from filters (stored at -20°C) and from an isolate, *Thiomicrospira* sp. strain Lilliput-1, (courtesy of J. Kuever) using the Ultra Clean Soil DNA Isolation Kit (MoBio) according to the manufacturer’s instructions.

2.4.1 Archaeal and Bacterial 16S rRNA Genes

Archaeal and bacterial 16S rRNA genes were PCR amplified for all sampled locations by using the oligonucleotide primer sets consisting of 21F and 958R (DeLong, 1992) and 27F and 1492R (Lane, 1991) respectively. Primers (50 pmol/μl), 1 μl (bacteria) and 1.5 μl (archaea) of DNA template, and sterile water were added to PuReTaq Ready-To-Go-PCR

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Beads (Amersham Biosciences) to a total volume of 25 μ l. An initial denaturation step (92°C for 2 min) was followed by 20 cycles of 92°C for 40 s, 50°C for 40 s, 72°C for 1 min for amplification of bacterial DNA and 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for amplification of archaeal DNA. Final extension was 5 min at 72°C. To minimize PCR bias 20 cycles were conducted (Qiu *et al.*, 2001). The amplified product was purified by the Roche PCR purification kit according to manufacturer's instructions and reamplified as described above using 1 μ l of the purified extracts.

2.4.2 Genes Encoding Key Enzymes of the CBB and rTCA cycles

Next to the *cbbL* gene of the *Thiomicrospira* sp. strain Lilliput-1, the following CO₂ fixation genes were amplified for fluid samples originating from two LHF sites (Irina II and Site B) and one location at the Lilliput hydrothermal field. The form I RubisCO (*cbbL*), the form II RubisCO (*cbbM*) (key enzymes of the Calvin Benson-Bassham cycle) and the beta subunit of the ATP Citrate lyase (*aclb*) (one of the key enzymes of the reverse tricarboxylic acid cycle) were PCR amplified by using the primers *cbbL* F and *cbbL* R, *cbbM* F and *cbbM* R (Campbell & Cary, 2004) and 892F and 1204R (*aclb*) (Campbell *et al.*, 2003), with conditions described previously (Campbell *et al.*, 2003, Campbell & Cary, 2004).

2.4.3 Genes Encoding NiFe-Uptake Hydrogenase

Studies on the gene *hynL* were conducted on fluid samples from the LHF (Irina II and Site B) and Lilliput emissions as well as on the *Thiomicrospira* sp. strain Lilliput-1. For amplification of NiFe-Uptake hydrogenase the primers *hynL*110F and *hynL*410R and conditions according to Takai and colleagues (2005) were used.

2.5 Cloning and Sequencing

All PCR products were repurified as described above and cloned with a TOPO-TA cloning kit (Invitrogen, Carlsbad, Calif.). For screening of 16S rRNA genes, 100 clones were randomly

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picked for Irina I, Irina II and Site B (LHF) and one location at the Lilliput hydrothermal field and were resuspended in 25 µl of sterile water. For screening of the functional genes 25 clones were picked for *cbbL* and *hynL* genes of the *Thiomicrospira* sp. strain Lilliput-1. For two sites at the LHF (Irina II and Site B) and one location at the Lilliput hydrothermal field as well as each functional gene (*cbbM*, *cbbL*, *aclb* and *hynL*) 40 clones were picked. The clones were checked for correct insert size by PCR with the vector primers M13F and M13R. PCR products of the correct size were screened and partially sequenced by using the primers 27F or 21F for bacteria or archaea, respectively, or the forward primers according to the functional gene studied (*cbbM*, *cbbL*, *aclb* or *hynL*). Sequencing was performed using the ABI PRISM® BigDye™ Terminator Ready Reaction Kit (Applied Biosystems) and an ABI PRISM® 310 Genetic Analyzer (Perkin Elmer Applied Biosystems) or a 3730xl DNA Analyzer (Applied Biosystems). To clarify phylogenetic affiliation of specific bacterial sequences full sequences were obtained by additionally using the primers 342F and 1492R (Lane, 1991), 534R (Muyzer *et al.*, 1993) 1094R (Munson *et al.*, 1991).

2.6 Diversity Estimation of Clone Libraries

The proportion of prokaryotic and functional genetic diversity represented by the clone libraries was estimated by rarefaction analysis combined with non-linear regression. Rarefaction analysis calculations were performed with the program aRarefactWin (<http://www.uga.edu/strata/software/Software.html>), applying the algorithm as previously described (Hurlbert, 1971). Rarefaction curves were plotted and regressions performed using the following equation: $y = a(1 - e^{-bx})$, with sample size x , observed number of phylotypes y and a displaying estimated total diversity. SigmaPlot v6.0 (SPSS) was used for plotting and regression analysis. Coverage of clone libraries was calculated by division of the number of phylotypes with the estimated diversity.

2.7 Phylogenetic Analysis

Sequences were edited and assembled with Lasergene Software SeqMan (DNASTar Inc.). Chimeric sequences were identified by using the Chimera-Check software available from

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Ribosomal Database Project (Cole *et al.*, 2003) and eliminated. Sequences were compared with DNA and/or amino acid sequences in the public domain through BLAST searches (Altschul *et al.*, 1997). Sequence data were compiled by using ARB software (www.arb-home.de) and aligned with sequences obtained from the GenBank database by using the ARB FastAligner utility (Ludwig *et al.*, 2004). Resulting alignments were manually checked and for 16S rRNA genes verified against known secondary structure regions. Maximum-Likelihood based trees were constructed with 16S rRNA gene sequences and amino acid sequences using PhyML (Guindon & Gascuel, 2003). The Phylip version 3.65 package (J. Felsenstein, University of Washington, Seattle) was used additionally to construct a Maximum-Parsimony tree (DNAPARS) with 16S rRNA gene sequences of deeply-rooted lineages from LHF sequences. Bootstrap analysis (SEQBOOT) was used to provide confidence estimates for Maximum-Parsimony tree topologies. All trees were constructed using 100 bootstrap replicates and full length sequences (for 16S rRNA genes). Phylogenies for *cbbL*, *cbbM*, *aclb* and *hynL* genes were calculated from partial (140, 91, 299 and 241 amino acids respectively) sequences. The trees were imported into ARB and shorter sequences added subsequently to trees using the Parsimony Quick and Add option.

2.8 Nucleotide Sequence Accession Numbers

All sequences obtained in this study were submitted to DDBJ/EMBL/GenBank database. The 16S rRNA gene sequences from the LHF and the Lilliput hydrothermal field were assigned the accession numbers AM268531-AM268882, AM279649, AM279650 and AM295190-AM295249 respectively. Sequences encoding the *cbbL* and *cbbM* genes of the form I and form II RubisCO of the CBB cycle were assigned the accessions numbers AM403032-AM403045 (*cbbL*) and AM403008-AM403020 and AM403059-AM403062 (*cbbM*). Sequences encoding the beta subunit of the ATP citrate lyase (*aclb* gene) of the rTCA cycle were given the numbers AM403102-AM403120 and sequences encoding the large subunit of the NiFe-Uptake Hydrogenase (*hynL*) were designated AM403021-AM403031 and AM403046-AM403058. The *cbbL* genes *cbbL*-1 and *cbbL*-2 of *Thiomicrospira* sp. strain Lilliput-1 were designated AM404076 and AM404077.

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3.1 Logatchev Hydrothermal Field (LHF)

3.1.1 Diversity Estimation of Clone Libraries

Three archaeal and three bacterial 16S rRNA gene clone libraries were constructed from hot hydrothermal fluids at three sites of the ultramafic-hosted Logatchev hydrothermal field (LHF) (Table 2). The clones constituted for 65-93 bacterial and 34-44 archaeal clone sequences (Table 2). Sequences with similarities of $\geq 97\%$ were defined as a phylotype. Between 24 and 32 bacterial and 7 to 14 archaeal phylotypes were retrieved (Table 2). The estimated coverage of the clone libraries ranged between 60% and 100% of the total diversity (Table 2). This coverage is also demonstrated by rarefaction curves (Fig. 5A, B).

Table 2: Diversity estimation of 16S rRNA gene clone libraries from hot fluids at the Logatchev.

Hydrothermal field	sites	16S rRNA gene	
		bacteria	archaea
Logatchev (northern MAR)	Irina I	24 (80) [94%]	7 (35) [60%]
	Irina II	32 (65) [74%]	6 (44) [100%]
	Site B	30 (93) [86%]	14 (34) [100%]

Numbers of phylotypes precede number of total clone sequences, indicated in parenthesis. Estimated coverage values are shown in square brackets. Phylotypes are defined as $\geq 97\%$ sequence similarity.

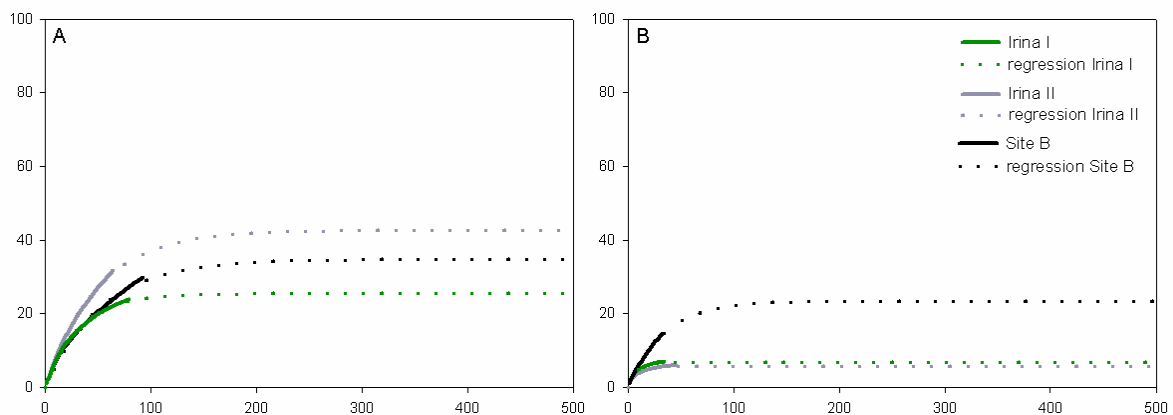


Fig. 5: Rarefaction curves for bacteria (A) and archaea (B) from the LHF locations Irina I, Irina II and Site B. The x axis indicates the number of clones investigated and the y axis denotes the expected number of phylotypes.

3.1.2 Phylogenetic Analysis

Sequences retrieved from fluid emissions at the LHF, but also found in the open water column (e.g., Acinas *et al.*, 1999, Long & Azam, 2001, Bano & Hollibaugh, 2002, A. Gärtner, unpublished data), were not taken into consideration. Their presence was assumed to be caused by mixing with ambient seawater during collection of fluid samples. Bacterial sequences not considered for this reason amount to 73%, 30% and 32% in fluid emissions at Irina I, Irina II and Site B respectively. These mainly include *Gammaproteobacteria* (e.g. *Vibrionales*, *Alteromonadales*, *Oceanospirillales*), *Alphaproteobacteria* (*Rhodobacterales*), and *Bacteroidetes* as well as very few *Betaproteobacteria* (*Burkholderiales*), *Planctomycetales*, *Clostridia* or *Actinobacteria*. Among the archaea 26%, 33% and 47% of sequences retrieved from fluids originating from Irina I, Irina II and Site B, respectively were excluded from analyses for the same reason as mentioned above. This includes a major faction affiliated to the Crenarchaeotic Marine Benthic Group I, which occurs at various locations. However, percentages of phylotypes were calculated from the total number of all bacterial or archaeal clone sequences obtained per vent site.

A large part of the LHF's indigenous microbial community (bacteria and archaea) was related to organisms known as autotrophic hydrogen-oxidizers. The limitations to inferring physiological properties from the analysis of 16S rRNA gene sequences have previously been demonstrated (e.g., Kashefi *et al.*, 2002). Nevertheless, in some instances, it may be possible to infer physiological traits of uncultured organisms from the physiology of very closely related microorganisms that are available in culture or from group-specific characteristics. For example, all cultured *Epsilonproteobacteria* affiliated to group D are capable of oxidizing hydrogen (for review, see Campbell *et al.*, 2006).

3.1.2.1 Bacteria

Epsilonproteobacteria. At Irina I, Irina II and Site B, affiliates of the *Epsilonproteobacteria* constituted 19%, 45% and 49%, respectively, of all retrieved bacterial clone sequences. Phylogenetically diverse representatives of *Epsilonproteobacteria* were detected in emissions from Irina I and Irina II (Fig. 6). This included members of groups B, D, F and

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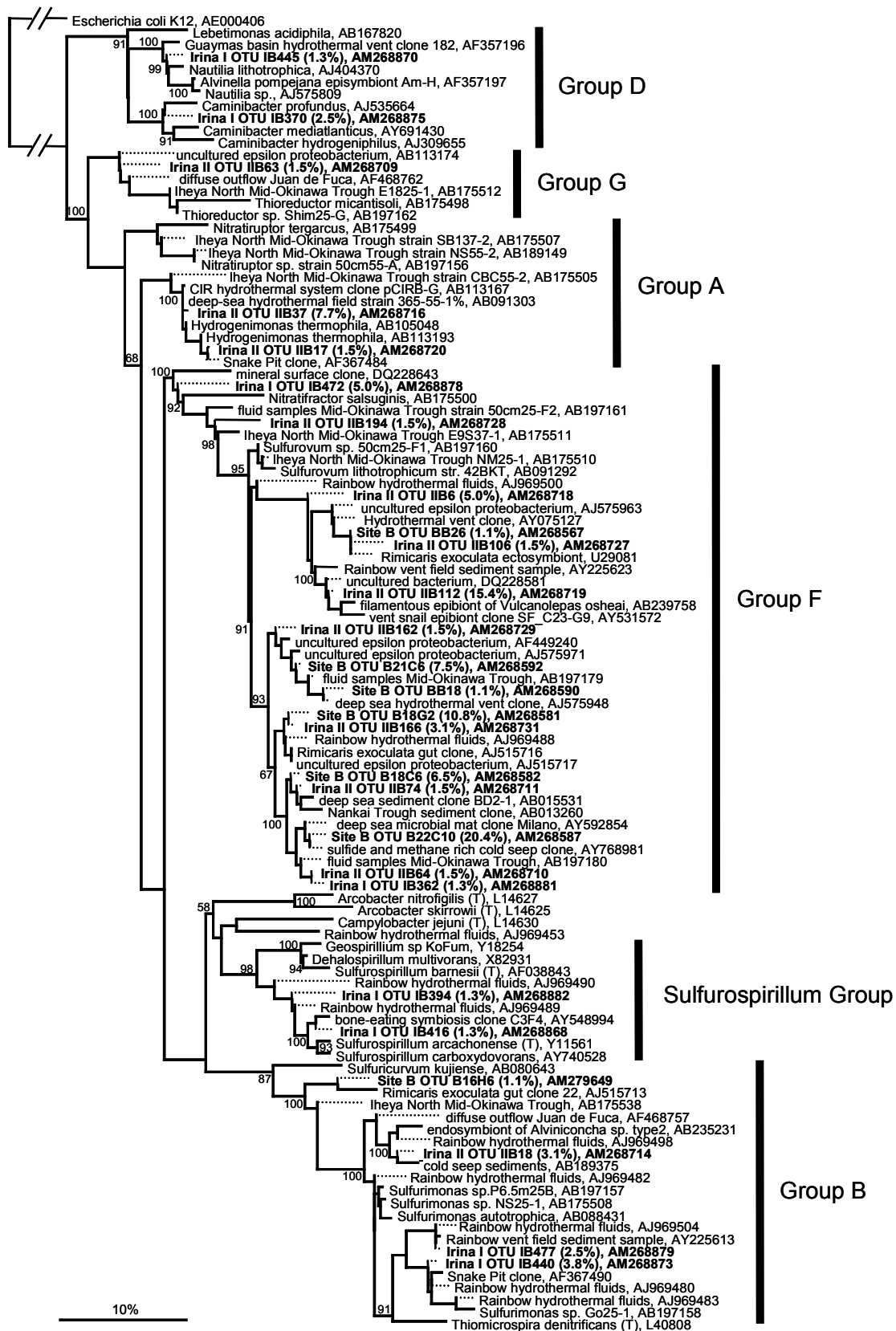


Fig. 6: Phylogeny of 16S rRNA gene sequences of *Epsilonproteobacteria* determined by Maximum-Likelihood analysis. Percentage of bootstrap resamplings above 50% is indicated. Dotted lines mark shorter sequences added subsequently to the tree. Sequences from Irina I, Irina II and Site B are printed in bold. Numbers in parenthesis following the phylotypes indicate percentage of sequences belonging to one phylotype. The scale bar represents the number of changes per nucleotide position.

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Sulfurospirillum spp. (Irina I) and affiliates of groups A, B, F and G (Irina II) (Fig. 6). By contrast, Site B epsilonproteobacterial sequences were restricted to groups B and F (Fig. 6).

At Irina I only 6% of bacterial sequences were related to members of group F. In contrast, they contributed significantly to the clone libraries recovered from fluids at Irina II and Site B (31% and 47% respectively). The majority of these sequences clustered into a deeply diverging group with no known cultured representatives (Fig. 6). They were related exclusively to sequences originating from vent environments. The closest described isolate of these LHF sequences was *Sulfurovum lithotropicum* (sequence similarities 86-98%). Only a minority of clone sequences at Irina I (6%), Irina II (3%) and Site B (1%) were associated with group B (Fig. 6). Sequences affiliated to *Sulfurospirillum* spp. were restricted to Irina I (Fig. 6). At Irina II, representatives of group A comprised 9% of the clone sequences and were closely related (99%) to *Hydrogenimonas thermophila* (Fig. 6).

Deeply-Rooted Lineages. Sequences of deeply-rooted lineages constituted a minor fraction of clones (Fig. 7). At Irina I 5% of the bacterial clone sequences were closely related to members of deeply-rooted lineages such as *Desulfurobacterium* sp. (99%), *Persephonella* sp. (98%) and *Oceanithermus profundus* (99%) (Fig. 7).

A single sequence originating from fluids emitted at Irina I and 9% of sequences at Irina II were affiliated with sequences of the group C *Epsilonproteobacteria*, recently recognized as having been misclassified (Campbell *et al.*, 2006). New tree calculations place them as a new group (RE1) in close proximity to the candidate division SR1 (Fig. 7). High bootstrap values support this position in Maximum-Likelihood and Maximum-Parsimony tree topologies (Fig. 7). The phylotypes were related to sequences from different hydrothermal environments such as the Guaymas Basin (Dhillon *et al.*, 2003), the East Pacific Rise (Alain *et al.*, 2004) or the Mid-Atlantic Ridge (Corre *et al.*, 2001) (Fig. 7). Two sequences from Site B were grouped in the uncultured candidate divisions SR1 and OD1, which were only distantly related (94%) to their closest relative (Fig. 7).

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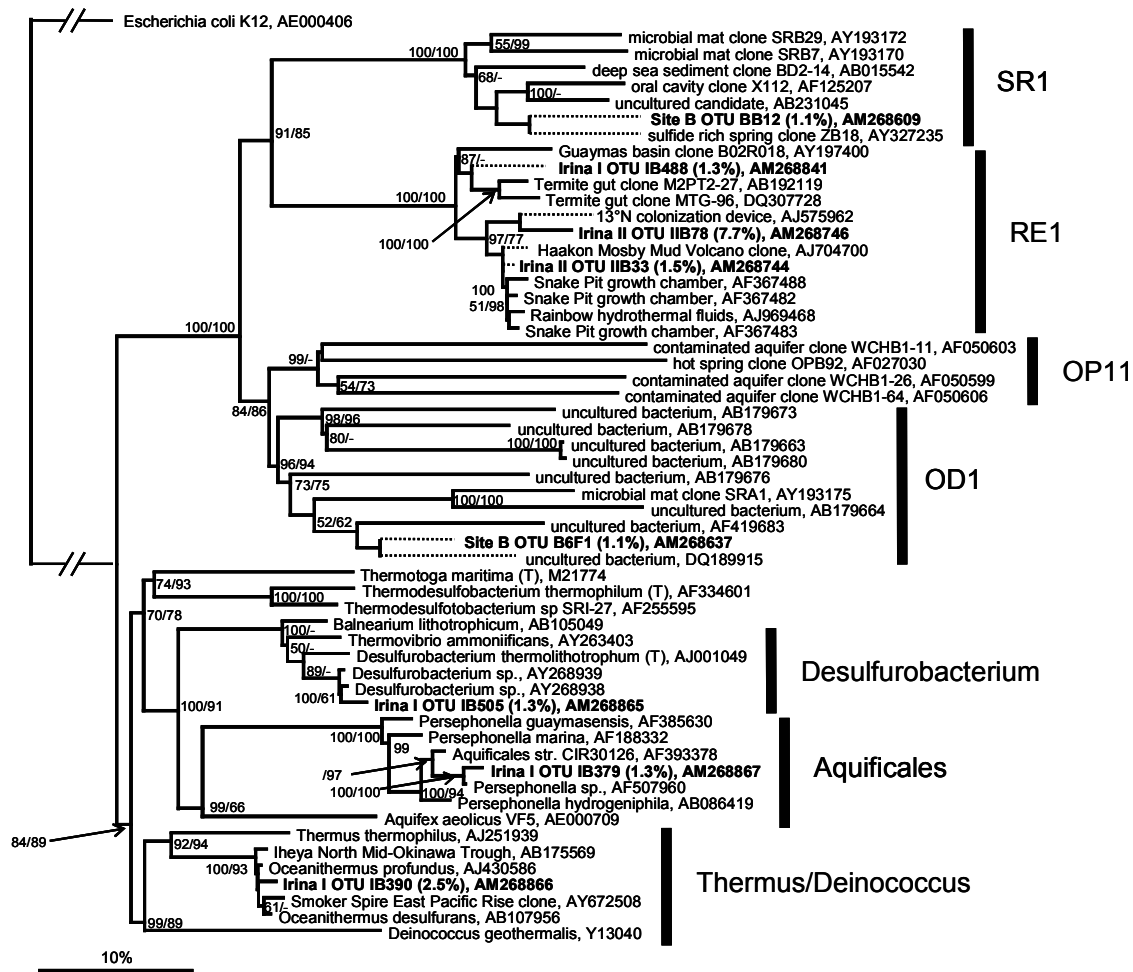


Fig. 7: Phylogeny of 16S rRNA gene sequences of deeply-rooted bacterial lineages as determined by Maximum-Likelihood analysis (ML) and Maximum-Parsimony analysis (MP). The percentage of bootstrap resamplings above 50% is indicated. Bootstrap probabilities estimated by ML and MP analyses are displayed as ML/MP. Tree topologies not supported by MP are indicated by “-“. Dotted lines mark shorter sequences added subsequently to tree. Sequences obtained from Irina I, Irina II and Site B are printed in bold. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. The scale bar represents the expected number of changes per nucleotide position.

Gammaproteobacteria. At Irina II 8% of the bacterial sequences were identical to thioautotrophic and methylotrophic symbionts of *Bathymodiolus* spp.. A single sequence at Irina I clustered with methylotrophic symbionts of these vent mussels. In contrast at Site B, no sequences were related to symbionts of *Bathymodiolus* spp.. However, at this site two gammaproteobacterial sequences were found which resembled symbiont sequences of *Escarpia spicata* (Di Meo *et al.*, 2000) and *Codakia orbicularis* (Gros *et al.*, 1996).

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Deltaproteobacteria. Fluids at Irina II and Site B included members of the *Deltaproteobacteria* (8% and 15% respectively). These were exclusively associated with the *Desulfobulbaceae* family (Fig. 8). The majority of fluid sequences at Irina II (5%) and at Site B (11%) were related to *Desulfocapsa sulfexigens* (Fig. 8).

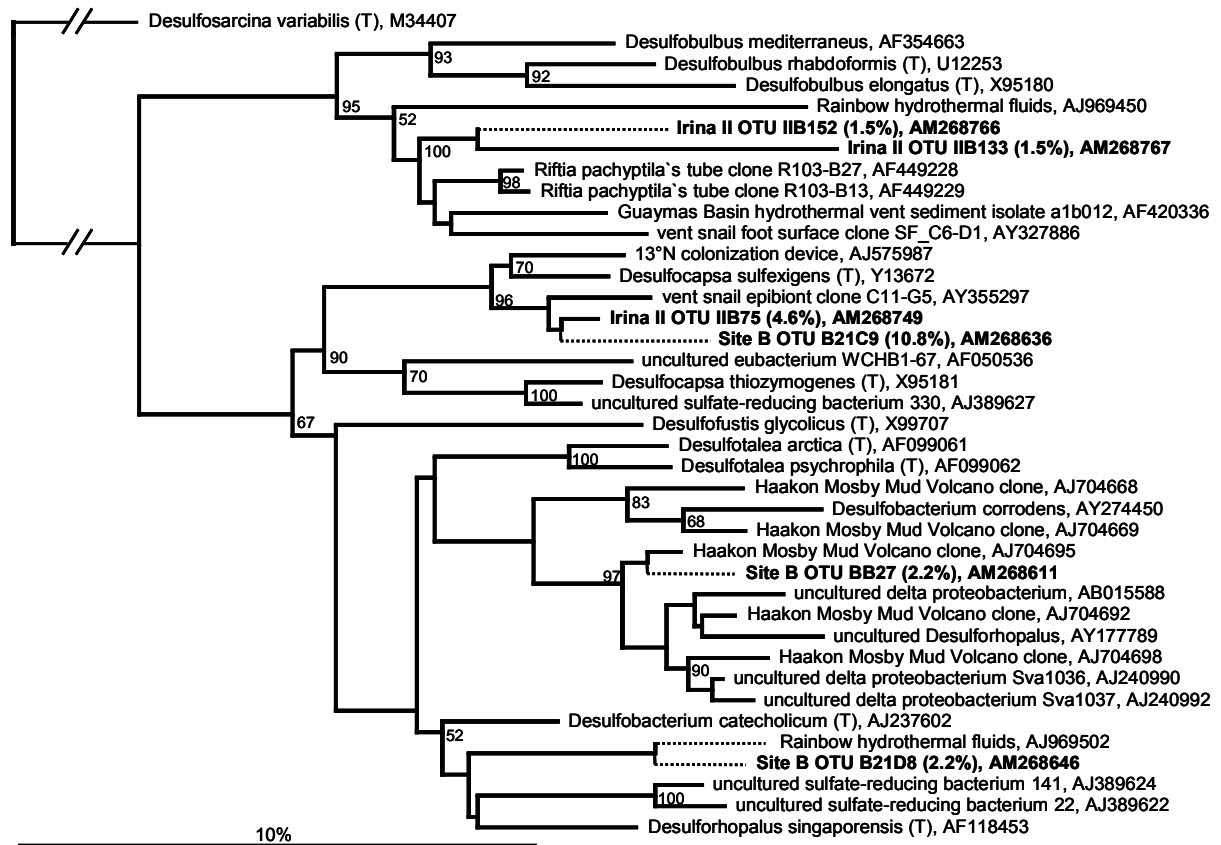


Fig. 8: Phylogeny of 16S rRNA gene sequences of *Deltaproteobacteria* determined by Maximum-Likelihood analysis. Percentage of bootstrap resamplings above 50% is indicated. Dotted lines mark shorter sequences added subsequently to the tree. Sequences from Irina I, Irina II and Site B are printed in bold. Numbers in parenthesis following the phylotypes indicate percentage of sequences belonging to one phylotype. The scale bar represents the number of changes per nucleotide position.

3.1.2.2 Archaea

At the Irina I, Irina II and Site B vent locations, members of the order *Methanococcales* accounted for 74%, 68% and 50% of archaeal clone sequences, respectively (Fig. 9). Only a few species of the Deep-Sea Hydrothermal Vent Euryarchotic Groups I and II, with no cultured representatives, were identified (data not shown).

Sequences related to “*Methanococcus aeolicus*” prevailed among the archaeal clone libraries at Irina I (43%), Irina II (54%) and Site B (47%) (Fig. 9). Representatives related to

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Methanocaldococcus infernus, were also present at Irina I (14%), at Irina II (4%) and at Site B (3%) (98-99% sequence similarities). Other methanogens found at Irina I (11%) include *Methanothermococcus thermolithotrophicus* (99% sequence similarity). At Irina II, 9% of the sequences were only distantly related (94%) to *Methanocaldococcus janaschii*. Additionally at Site B a single sequence was identified as a member of the ANME-2 lineage.

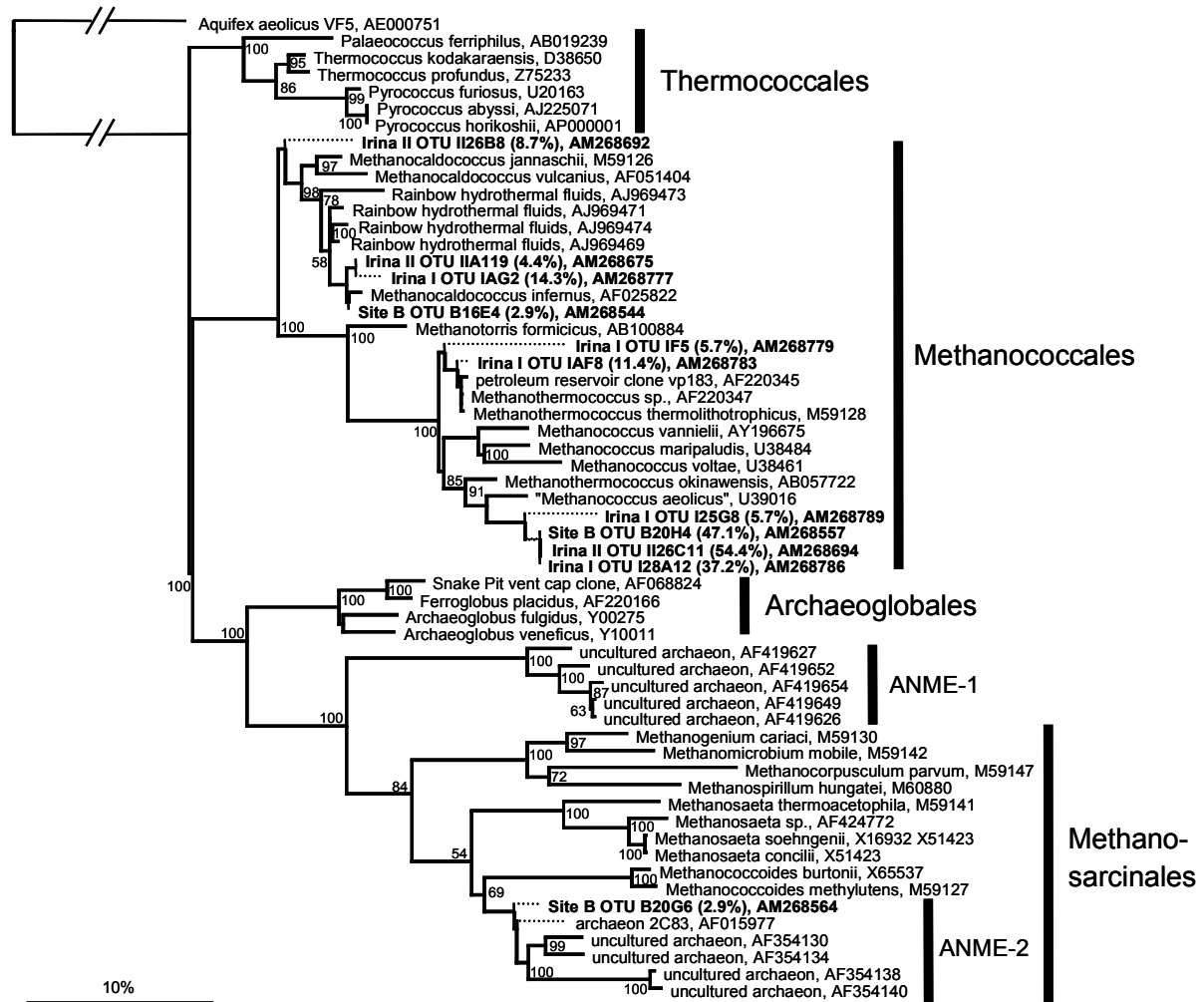


Fig. 9: Phylogeny of 16S rRNA gene sequences of Archaea determined by Maximum-Likelihood analysis. Percentage of bootstrap resamplings above 50% is indicated. Dotted lines mark shorter sequences added subsequently to the tree. Sequences from Irina I, Irina II and Site B are printed in bold. Numbers in parenthesis following the phylotypes indicate percentage of sequences belonging to one phylotype. The scale bar represents the number of changes per nucleotide position.

3.2 Lilliput Hydrothermal Field

3.2.1 Diversity Estimation of Clone Libraries

From the 100 bacterial and 100 archaeal clones investigated, 81 bacterial and 21 archaeal sequences were recovered (Table 3). Of the 81 bacterial sequences 51 phylotypes ($\geq 98\%$ sequence similarity was defined as a phylotype) were identified (Table 3). Rarefaction analyses demonstrated that the census is far from complete with an estimated coverage value of 54% for bacteria (Fig 10, Table 3). However, fluorescent *in situ* hybridization conducted with domain- and class-specific probes revealed a good coverage of the microbial community (Table 4). Rarefaction curves also indicated a high bacterial diversity due to the high number of non-repetitive phylotypes (75%) at this hydrothermal field (Fig 10, Table 3). Only 12 phylotypes were closely related to known sequences (similarity $\geq 97\%$). In part the remaining 39 phylotypes only distantly resembled known sequences (similarities 76.2-96.8%).

Table 3: Diversity estimation of 16S rRNA gene clone libraries from low-temperature fluids at Lilliput

Hydrothermal field	site	16S rRNA gene	
		bacteria	archaea
Lilliput (southern MAR)	Lilliput	51 (81) [54%]	10 (21) [73%]

Numbers of phylotypes precede number of total clone sequences, indicated in parenthesis. Estimated coverage values are shown in square brackets. Phylotype defined as $\geq 98\%$ sequence similarity.

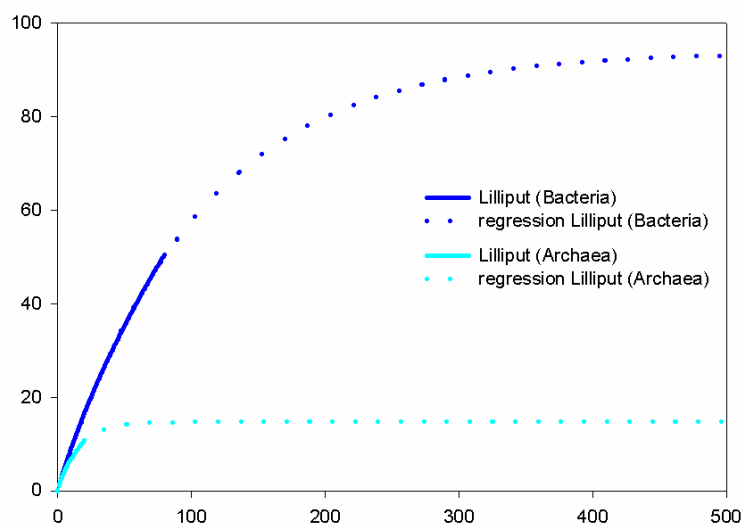


Fig. 10: Rarefaction curves for bacteria and archaea from the Lilliput hydrothermal fluids. The x axis indicates the number of clones investigated and the y axis denotes the expected number of phylotypes.

3.2.2 Fluorescence *in Situ* Hybridization (FISH)

The diffuse outflow at the Lilliput hydrothermal field was dominated by bacteria (89% of DAPI stained cells). Members of the *Cytophaga-Flavobacterium* cluster, *Epsilon*-, *Gamma*- and *Deltaproteobacteria* constituted for 13%, 28%, 22% and 12% of DAPI stained cells respectively (Table 4). Archaea (3 different probes used), *Alpha*- and *Betaproteobacteria*, were not detected by group-specific probes which are listed in Table 1.

Approximately 11% of DAPI stained cells neither hybridized with probes specific for the archaeal nor the bacterial domain (Table 4). These cells had a coccoidal shape and were smaller compared to all other cells. Moreover, 14% of DAPI stained bacterial cells hybridized with none of the class-specific probes used (see Table 1). Among cells hybridizing with the *Epsilonproteobacteria*-specific probe various morphologies were observed. This included

Table 4: Abundance of specific prokaryotes relative to DAPI signals

Probe	Specificity	Abundance ^a
EUB338I, II, III	most Bacteria	89
ARCH915	Archaea	<
Eury498	Euryarchaeota	<
Cren512	Crenarchaeota	<
ALF968	<i>Alphaproteobacteria</i>	<
Beta42a	<i>Betaproteobacteria</i>	<
GAM42a	<i>Gammaproteobacteria</i>	22
EP404	<i>Epsilonproteobacteria</i>	28
DELTA495a	most <i>Deltaproteobacteria</i> , some <i>Gemmimonas</i> group	12
CF319a	<i>Cytophaga-Flavobacterium</i> cluster	13
-----	not hybridized	11

< indicates number of signals below detection limit. ^a percentage of DAPI stained cells showing a signal with specific probe.

rods, coccoids, and filaments (Fig. 11). Chains consisting of coccoidal or filamentous cells (e.g. Fig. 11B and E) exhibited lengths of up to 132 μm and possibly longer. The width of

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these chains did not exceed 2 μm . Single filaments reached a width of 3 μm and a length of 20 μm (Fig. 11C and F).

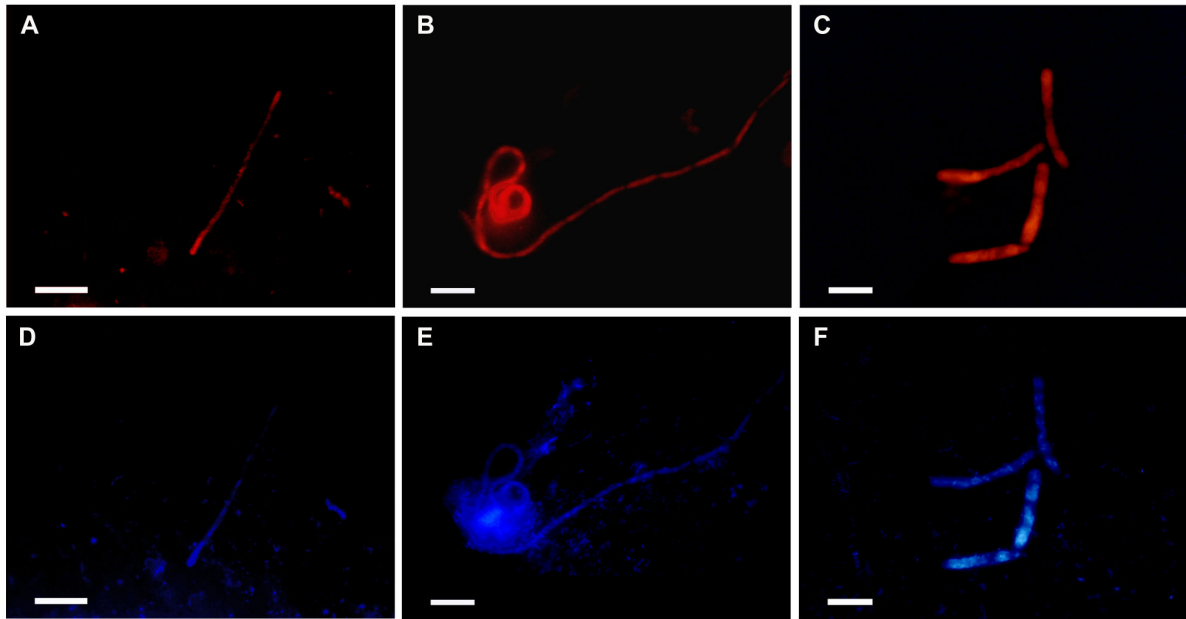


Fig. 11: Epifluorescence microscopy of whole cell FISH analysis and DAPI of Lilliput bacterial cells. For FISH Cy3-labelled oligonucleotide probes specific for *Epsilonproteobacteria* (A-C) were used and stained with DAPI (D-F). The scale bars represent 10 μm .

3.2.3 Phylogenetic Analysis

A bacterial and an archaeal clone library were constructed from low-temperature diffuse emissions at the Lilliput hydrothermal field. Bacterial representatives included members of the *Beta*-, *Gamma*-, *Epsilon*- and *Deltaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria* and candidate divisions TM6, OP8, SR1, WS5, WS6, OP11 and OD1 (Fig. 12). The few archaeal sequences were associated with the Deep-Sea Hydrothermal Vent Euryarchaeota Group (DHVE) and the Marine Group I (MG I) of the *Crenarchaeota* (Fig. 13).

3.2.3.1 Bacteria

Sequences of the clone library affiliated with the *Epsilonproteobacteria* accounted for 24% of bacterial clone sequences and were mainly assigned to group F (8%) and the *Arcobacter* group (13%) (Fig. 12). *Deltaproteobacteria* constituted for 17% of all bacterial sequences and were affiliated with *Desulfobacterales* and *Desulfovibrionales* (Fig. 12). High similarities of

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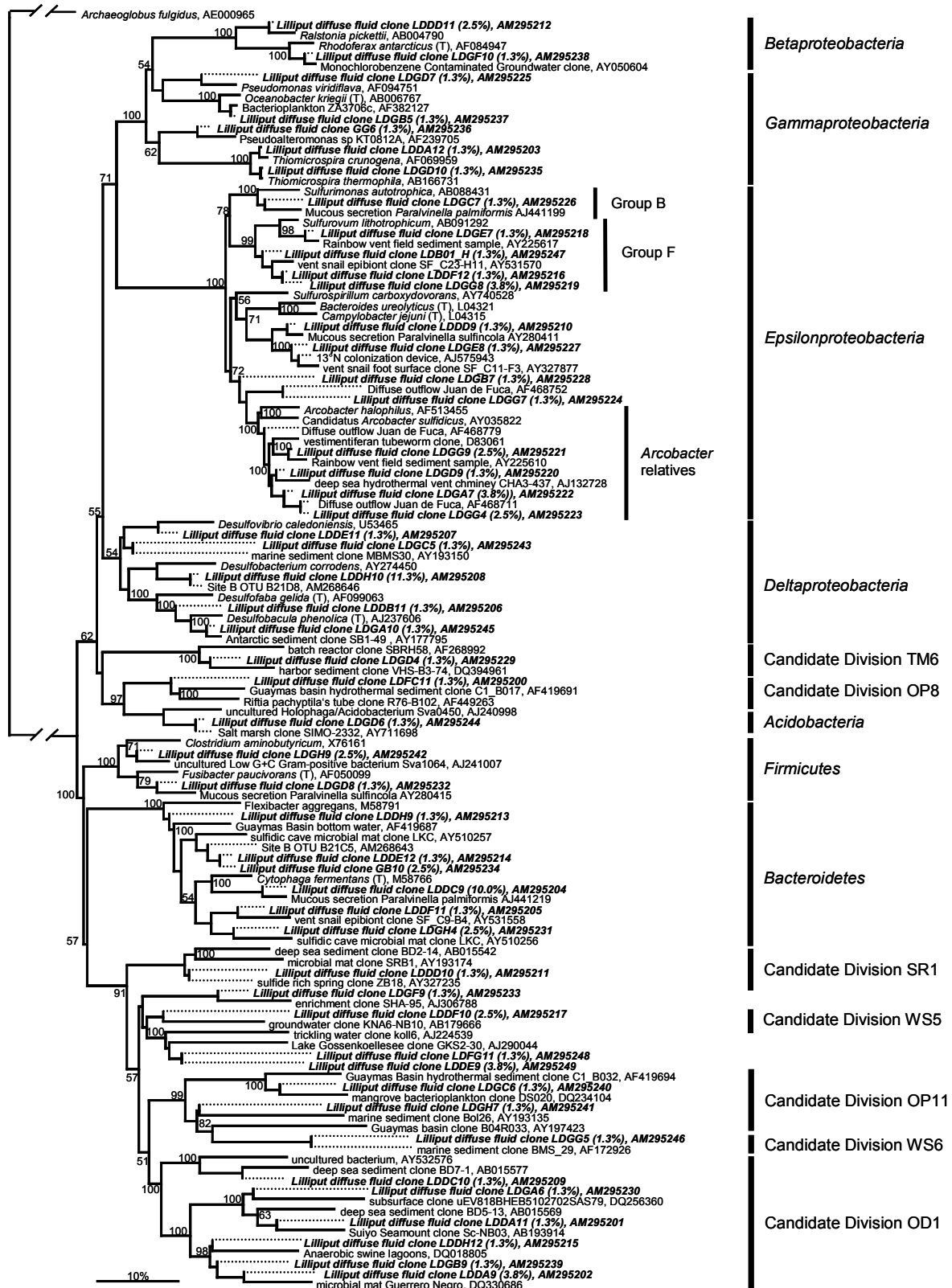


Fig. 12: Phylogeny of 16S rRNA Lilliput bacterial sequences determined by Maximum-Likelihood analysis. The percentage of bootstrap resamplings above 50% is indicated. Dotted lines mark shorter sequences added subsequently to the tree. Sequences from the Lilliput hydrothermal field are printed in bold/italic. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. Scale bar represents the number of changes per nucleotide position.

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gammaproteobacterial clone sequences with *Thiomicrospira thermophila* (99.8%) and *Thiomicrospira crunogena* (99.1%) were observed. Single sequences were associated with the candidate divisions TM6 and OP8 with the closest relatives being a harbor sediment clone (DQ394961) and a clone retrieved from *Riftia pachyptila*'s tube (López-García *et al.*, 2002). These clone sequences revealed similarities of 94.0% and 87.0% respectively. The clone sequence LDGD6 was identified as an *Acidobacterium*, related to a salt marsh clone (96.0%) (AY711698).

Bacteroidetes accounted for 19% of clone sequences and were affiliated with sequences originating from hot fluid emissions of the LHF, from mucous secretions of *Paralvinella palmiformis* (Alain *et al.*, 2002), from vent snail epibionts (Goffredi *et al.*, 2004), from Guaymas Basin sediments (Teske *et al.*, 2002) and from a sulfidic cave microbial mat (Engel *et al.*, 2004). Sequence similarities ranged between 87.5 and 95.6%. Bacteria of the phylum *Firmicutes* represented 4% of the clone sequences. Their next relatives originated from Svalbard sediments (Ravenschlag *et al.*, 1999) and mucous secretions of *Paralvinella sulfincola* (Page *et al.*, 2004) respectively.

A major fraction of Lilliput clone sequences (21%) was associated with representatives of candidate divisions of deeply-rooted lineages, i.e. candidate divisions OD1, OP11, SR1, WS5 and WS6 (Fig. 12). This is the first report on a member of the candidate division WS5 in a hydrothermally influenced setting. The closest relatives of different clones included sequences retrieved from a sulfide rich spring (Elshahed *et al.*, 2003), an enrichment culture (AJ306788), deep groundwater (Miyoshi *et al.*, 2005), mangrove bacterioplankton (DQ234104), marine sediment (Harris *et al.*, 2004), deep-sea sediment (Li *et al.*, 1999), subsurface water (DQ256360), a microbial mat (Ley *et al.*, 2006) and an anaerobic swine lagoon (DQ018805) with similarities between 76.2 and 93.8%.

3.2.3.2 Archaea

The majority of the archaeal sequences of the clone library was affiliated with the Marine Group I (MG I) (86%) (Fig. 13), of which 62% were closely related to different sequences obtained from deep-sea hydrothermal vent environments (Takai & Horikoshi, 1999). Some members of the MG I (19%) were closely related (98.5% similarity) to the autotrophic

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ammonia oxidizer *Nitrosopumilus maritimus* (Könneke *et al.*, 2005). Others (5%) were associated with sequences obtained from a carbonate chimney at Lost City vent field (Schrenk *et al.*, 2004). Within the Deep-Sea Hydrothermal Vent Euryarchaeota Group (DHVE) the Lilliput clones were distributed among DHVE 3 and DHVE 1 (Fig. 13). The only cultivated representative of the DHVE, the thermoacidophilic *Aciduliprofundum boonei* (DHVE 2) (Reysenbach *et al.*, 2006) was distantly related to the Lilliput clones LDG12C (77%) and LDG12E (73%). Their closest relatives were sequences found in tidal flat sediments (Kim *et al.*, 2005) and a diffuse outflow along the Juan de Fuca Ridge (Huber *et al.*, 2002), respectively (Fig. 13).

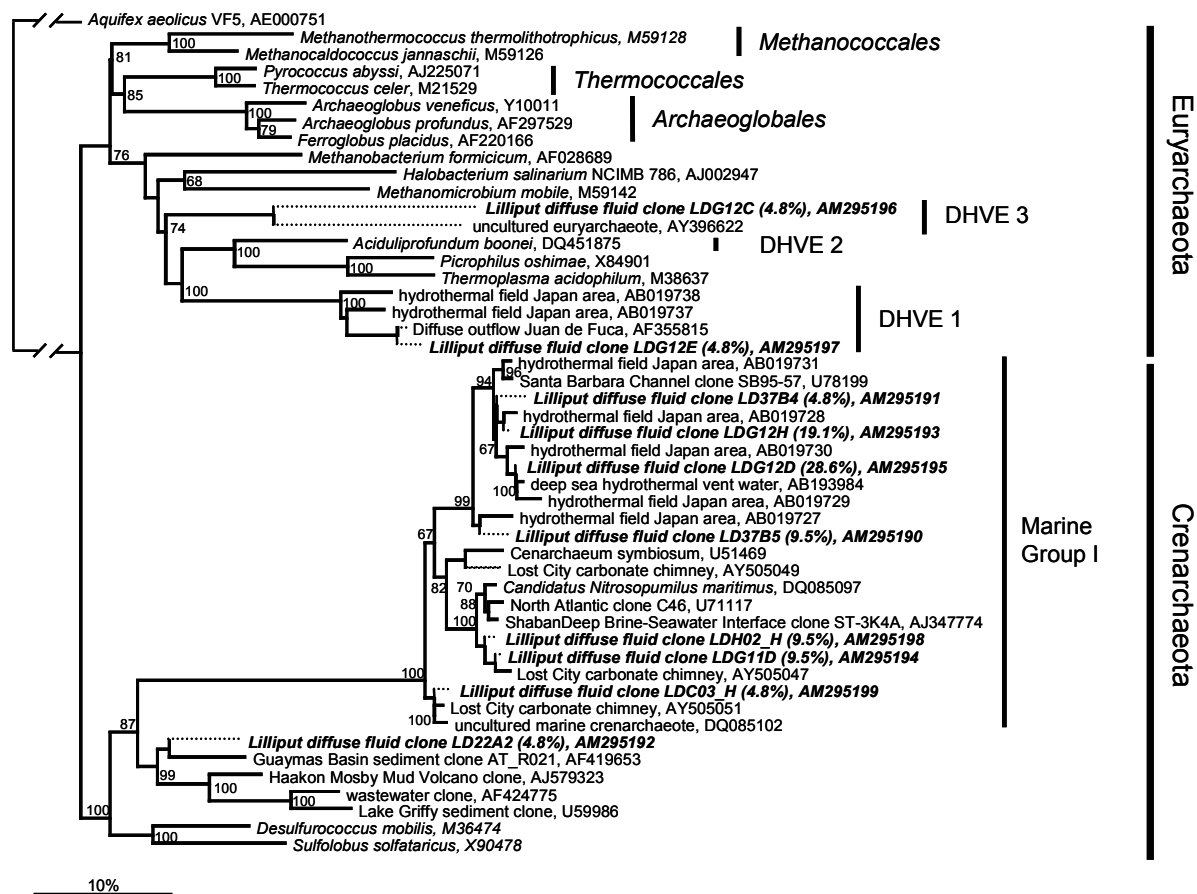


Fig. 13: Phylogeny of 16S rRNA Lilliput archaeal sequences determined by Maximum-Likelihood analysis. The percentage of bootstrap resamplings above 50% is indicated. Dotted lines mark shorter sequences added subsequently to the tree. Sequences from the Lilliput hydrothermal field printed in bold/italic. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. Scale bar represents the number of changes per nucleotide position.

3.3 Functional Genes at the Logatchev and Lilliput hydrothermal fields

3.3.1 Diversity Estimation of the Clone Libraries

Clone libraries of functional genes (*cbbL*, *cbbM*, *acIb* and *hynL*) were constructed from samples of the hot fluid emissions at Irina II and Site B of the Logatchev hydrothermal field (LHF) and of the low-temperature outflow at the Lilliput hydrothermal field. The genes *cbbL* and *cbbM* encode the key enzymes RubisCO form I and II of the Calvin Benson-Bassham (CBB) cycle. The *acIb* gene encodes the beta subunit of the ATP citrate lyase which is a key enzyme of the reverse tricarboxylic acid cycle (rTCA). The *hynL* gene encodes the large subunit of the NiFe-Uptake Hydrogenase which catalyzes the oxidation of molecular hydrogen.

Table 5: Diversity estimation of the *cbbL*, *cbbM*, *acIb* and *hynL* gene clone libraries from Logatchev and Lilliput

Hydrothermal field	sites	PCR detection of functional genes			
		CBB cycle	rTCA cycle	H ₂ oxidation	
		<i>cbbL</i>	<i>cbbM</i>	<i>acIb</i>	<i>hynL</i>
Logatchev (northern MAR)	Irina II	6 (28) [100%]	6 (27) [76%]	10 (39) [95%]	9 (30) [83%]
	Site B	5 (38) [100%]	5 (36) [100%]	2 (29) [100%]	11 (38) [100%]
Lilliput (southern MAR)	Lilliput	3 (30) [100%]	6 (34) [98%]	7 (33) [100%]	4 (38) [100%]

Libraries were constructed from fluids originating from Irina II and Site B of the LHF and from fluids of the Lilliput hydrothermal field. Numbers of phylotypes precede number of total clone sequences, indicated in parenthesis. Estimated coverage values are shown in square brackets. Identical amino acid sequences are defined as a phylotype. *CbbL* and *cbbM* denotes genes encoding the key enzymes Rubisco form I and II, respectively, of the Calvin Benson-Bassham cycle (CBB); The *acIb* gene encodes one of the key enzymes of the reverse tricarboxylic acid cycle (rTCA) (beta subunit of the ATP citrate lyase) and the *hynL* gene encodes the large subunit of the NiFe-Uptake Hydrogenase, which catalyzes the oxidation of molecular hydrogen.

All the functional gene sequences referred to denote amino acid sequences. Identical amino acid sequences were defined as a phylotype, disregarding variations in the DNA sequence. The coverage of the clone libraries was good (Fig. 14A-D, Table 5). With the exception of *cbbM* and *hynL* genes recovered from fluids at Irina II (coverage of 76% and

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83% respectively) the clone coverage was estimated between 95% and 100% for the different clone libraries (Table 5).

At Irina II and Site B the RubisCO form I and II sequences were diversely distributed. A total of 28 and 38 (*cbbL*), 27 and 36 (*cbbM*) sequences were recovered from these locations respectively. They constituted for 5 and 6 different phylotypes (Table 5). Only three phylotypes of *cbbL* genes and 6 of *cbbM* genes were retrieved from Lilliput fluids (Table 5). The number of genes encoding the beta subunit of the ATP citrate lyase (*acIb* gene) differed significantly between Irina II and Site B revealing 10 and 2 phylotypes of 39 and 29 sequences respectively (Fig. 16, Table 5). At Lilliput 7 phylotypes of 33 recovered sequences were identified (Table 5). Major differences in the diversity of the *hynL* genes were also observed between the LHF and the Lilliput field. While at the LHF between 30 and 38 sequences with 9-11 phylotypes were recovered, fluids sampled at the Lilliput hydrothermal field yielded merely 4 phylotypes (38 sequences) (Fig. 17, Table 5).

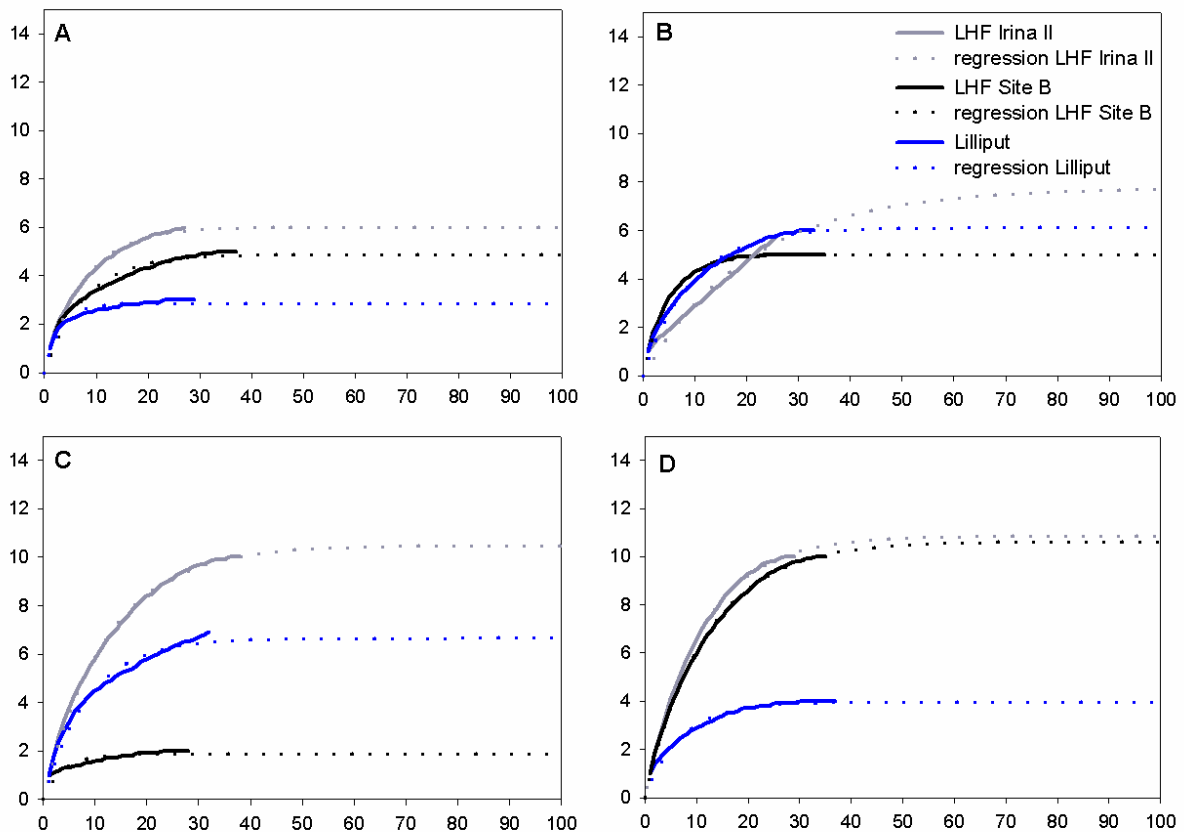


Fig. 14: Rarefaction curves for *cbbL* (A) and *cbbM* (B), *acIb* (C) and *hynL* (D) genes from the LHF locations Irina II and Site B, and of Lilliput hydrothermal emissions. The x axis indicates the number of clones investigated and the y axis denotes the expected number of phylotypes.

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The sequences affiliated with the CBB and rTCA cycles and NiFe-Uptake Hydrogenases extend the existing database. As already highlighted by Hügler and colleagues (2005) the tree topology data of the *acIb* gene is in good agreement with the 16S rRNA gene phylogeny (Fig. 6, 12, 16) implying that the gene might not only be useful as a functional marker but may also represent a phylogenetic marker.

3.3.2 The CBB (*cbbL* and *cbbM* Genes) and the rTCA (*acIb* Gene) Cycles

From hydrothermal fluids collected at Lilliput, *Thiomicrospira* sp. strain Lilliput-1 was isolated (J. Kuever, unpublished data). Based on 16S rRNA gene data it resembles *Thiomicrospira thermophila* by 100%. The *Thiomicrospira* sp. strain Lilliput-1 revealed two types of *cbbL* genes (*cbbL*-1 and *cbbL*-2) which were affiliated with other *Thiomicrospira* spp. (92.6-98.6% amino acid similarity) (Fig. 15, Table 6).

The majority of Rubisco form I (*cbbL*) amino acid sequences was associated with endosymbiont sequences of the *Solemya* clam (AY531637) or the *Bathymodiolus* mussel (AY945760) (Fig. 15) irrespective whether originating from the LHF or the Lilliput hydrothermal field. At Site B, LHF, solely amino acid sequences affiliated with endosymbionts were detected. In addition, the form I RubisCO of *Thiomicrospira thermophila* was observed at Irina II of the LHF and the Lilliput hydrothermal field (Fig. 15, Table 6). At Irina II the number of *Thiomicrospira cbbL*-1 or *cbbL*-2 genes constituted 61% of all form I amino acid sequences compared to a mere 40% at the Lilliput hydrothermal field.

A major part of Rubisco form II amino acid sequences at the Lilliput hydrothermal field were affiliated with either *Thiomicrospira thermophila* or *Thiomicrospira crunogena* (Fig. 15, Table 6). The remaining sequences from the LHF and the Lilliput hydrothermal field were related to sequences originating from hydrothermal plumes (Elsaied & Naganuma, 2001), groundwater (AY099396) and aquifer as well as a hypersaline anoxic basin (van der Wielen, 2006) and were not directly affiliated with cultured representatives (Fig. 15, Table 6).

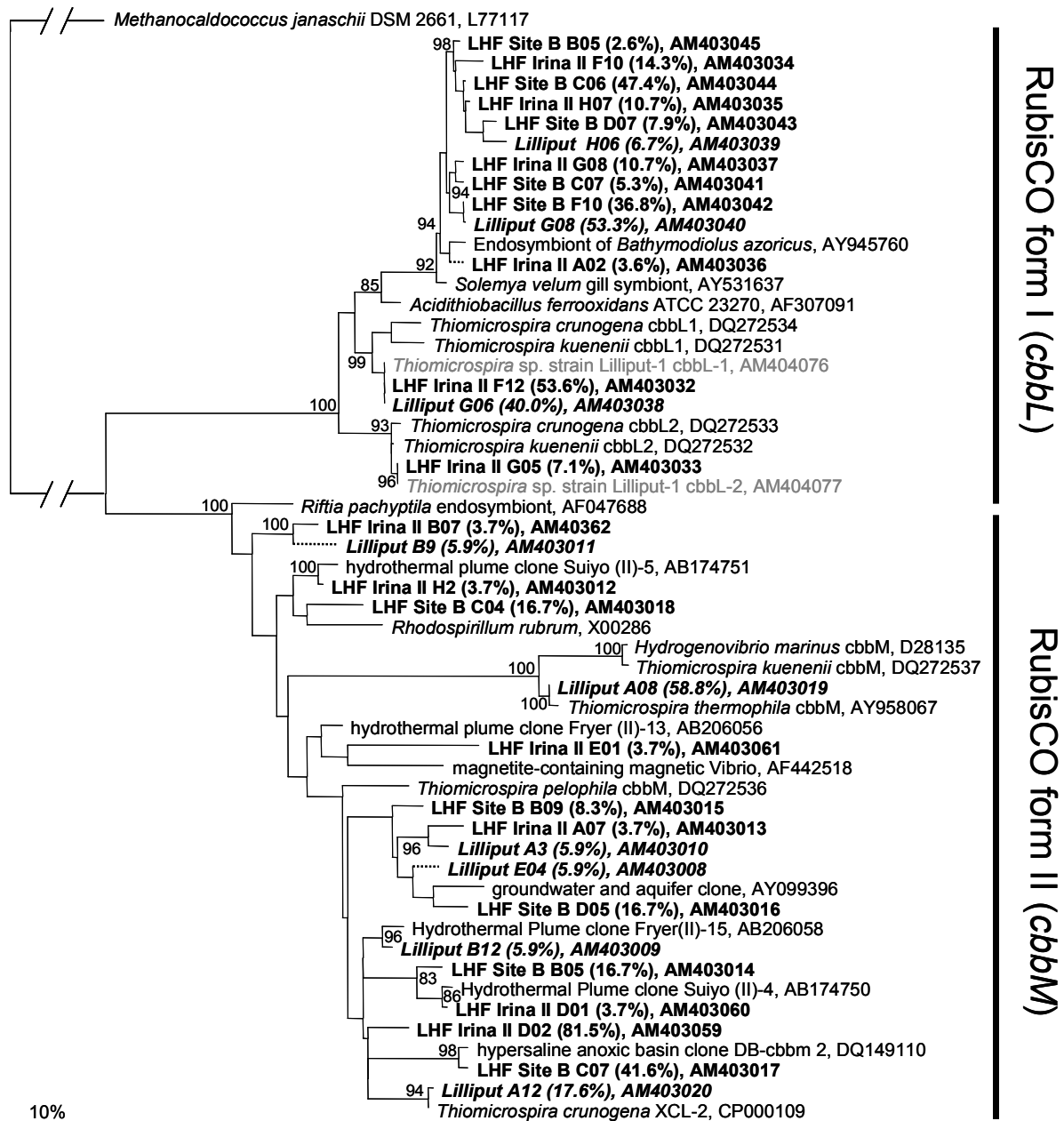


Fig. 15: Phylogeny of genes encoding the key enzymes RubisCO form I (*cbbL*) and form II (*cbbM*) of the Calvin Benson-Bassham cycle as determined by Maximum-Likelihood analysis. The percentage of bootstrap resamplings above 75% is indicated. Dotted lines mark shorter sequences added subsequently to tree. Sequences obtained from the LHF sites (Irina II and Site B) and the Lilliput hydrothermal field are printed in bold and in italic respectively. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. The scale bar represents the expected number of changes per nucleotide position.

ATP citrate lyase beta subunit (*aclb* gene) encodes one of the key enzymes of the reverse tricarboxylic acid (rTCA) cycle. Major differences in the distribution of the *aclb* gene sequences exist between the two sites within the LHF as well as compared to the Lilliput field (Fig. 16). The largest diversity of *aclb* sequences was observed at Irina II, LHF, including potential affiliates of groups A, B, D, F, G and the *Arcobacter* group of the

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Epsilonproteobacteria. In contrast, fluids from Site B (LHF) displayed solely two phylotypes (Table 6) possibly associated with groups B and F (Fig. 16). In Lilliput fluids 7 phylotypes were observed (Table 5) and were associated with affiliates of probably the *Arcobacter* group and group B of the *Epsilonproteobacteria* (Fig. 16, Table 6).

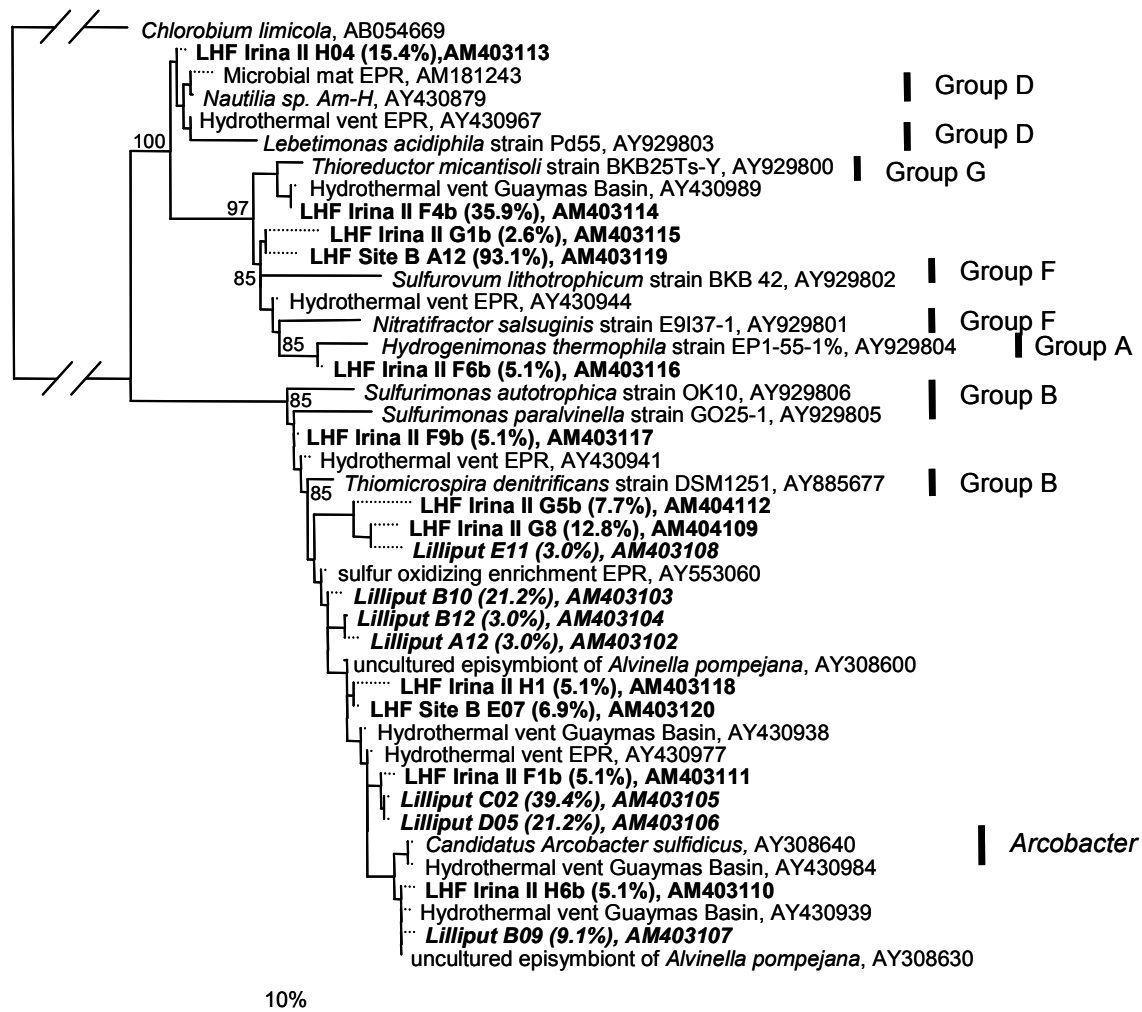


Fig. 16: Phylogeny of genes encoding the key enzyme the beta subunit of the ATP Citrate Lyase (*aclb*) of the rTCA cycle, as determined by Maximum-Likelihood. The percentage of bootstrap resamplings above 75% is indicated. Dotted lines mark shorter sequences added subsequently to tree. Sequences obtained from the LHF sites (Irina II and Site B) and the Lilliput hydrothermal field are printed in bold and in italic respectively. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. The scale bar represents the expected number of changes per nucleotide position.

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Table 6: List of phylogenetic affiliations of functional gene sequences from the LHF and Lilliput field

Clone Name	Number of representatives in fluid (percentage)		Closest relative (accession number)	Identity amino acid sequence (%)	
	LHF				
	Site B	Irina II			
<i>cbbl</i>					
Site B B05	1 (2.6)		Endosymbiont of <i>Solemya velum</i> (AY531637)	96.3	
Site B C06	18 (47.4)		Endosymbiont of <i>Solemya velum</i> (AY531637)	94.1	
Site B D07	3 (7.9)		Endosymbiont of <i>Solemya velum</i> (AY531637)	92.0	
Site B C07	2 (5.3)		Endosymbiont of <i>Solemya velum</i> (AY531637)	94.9	
Site B F10	14 (36.8)		Endosymbiont of <i>Bathymodiolus azoricus</i> (AY945760)	96.3	
Irina II F10		4 (14.3)	Endosymbiont of <i>Solemya velum</i> (AY531637)	92.0	
Irina II H07		3 (10.7)	Endosymbiont of <i>Solemya velum</i> (AY531637)	96.6	
Irina II G08		3 (10.7)	Endosymbiont of <i>Bathymodiolus azoricus</i> (AY945760)	95.6	
Irina II A02		1 (3.6)	Endosymbiont of <i>Bathymodiolus azoricus</i> (AY945760)	96.6	
Irina II F12		15 (53.6)	<i>Thiomicrospira</i> sp. strain Lilliput-1 <i>cbbl</i> -1 (AM404076)	100.0	
Irina II G05		2 (7.1)	<i>Thiomicrospira</i> sp. strain Lilliput-1 <i>cbbl</i> -2 (AM404077)	100.0	
Lilliput H06			2 (6.7)	Endosymbiont of <i>Solemya velum</i> (AY531637)	92.8
Lilliput G08			16 (53.3)	Endosymbiont of <i>Bathymodiolus azoricus</i> (AY945760)	95.6
Lilliput G06			12 (40.0)	<i>Thiomicrospira</i> sp. strain Lilliput-1 <i>cbbl</i> -1 (AM404076)	100.0
<i>cbbM</i>					
Site B C04	6 (16.7)		<i>Rhodospirillum rubrum</i> (X00286)	81.6	
Site B B09	3 (8.3)		uncultured bacterium clone RA13c10II (AY099396)	84.7	
Site B D05	6 (16.7)		uncultured bacterium clone RA13c10II (AY099396)	88.2	
Site B B05	6 (16.7)		uncultured prokaryote clone Suiyo (II)-4 (AB174750)	95.3	
Site B C07	15 (41.6)		uncultured prokaryote clone DB-cbbm 2 (DQ149110)	95.3	
Irina II B07		1 (3.7)	<i>Riftia pachyptila</i> endosymbiont (AF047688)	78.4	
Irina II H02		1 (3.7)	uncultured prokaryote clone Suiyo (II)-5 (AB174751)	95.4	
Irina II E01		1 (3.7)	magnetite-containing <i>Vibrio</i> (AF442518)	69.4	
Irina II A07		1 (3.7)	uncultured bacterium clone RA13c10II (AY099396)	83.5	
Irina II D01		1 (3.7)	uncultured prokaryote clone Suiyo (II)-4 (AB174750)	98.9	
Irina II D02		22 (81.5)	<i>Thiomicrospira crunogena</i> (CP000109)	87.1	
Lilliput B09			2 (5.9)	<i>Riftia pachyptila</i> endosymbiont (AF047688)	75.7
Lilliput A08			20 (58.8)	<i>Thiomicrospira thermophila</i> cbbM (AY958067)	98.9
Lilliput A03			2 (5.9)	uncultured bacterium clone RA13c10II (AY099396)	87.1
Lilliput E04			2 (5.9)	uncultured bacterium clone RA13c10II (AY099396)	84.2
Lilliput B12			2 (5.9)	uncultured prokaryote clone Fryer(II)-15 (AB206058)	94.4
Lilliput A12			6 (17.6)	<i>Thiomicrospira crunogena</i> (CP000109)	98.8
<i>acfb</i>					
Site B A12	27 (93.1)		<i>Sulfurovum lithotrophicum</i> (AY929802)	93.0	
Site B E07	2 (6.9)		episymbiont <i>Alvinella pompejana</i> clone 3713R-5 (AY308600)	96.5	
Irina II H04		6 (15.4)	<i>Nautilia</i> sp. (AY430879)	99.0	
Irina II F4b		14 (35.9)	uncultured prokaryote clone 2E10 (AY430989)	98.8	
Irina II G1b		1 (2.6)	<i>Sulfurovum lithotrophicum</i> (AY929802)	84.7	
Irina II F6b		2 (5.1)	<i>Hydrogenimonas thermophila</i> (AY929804)	96.5	
Irina II F9b		2 (5.1)	uncultured prokaryote clone 729-4_G4 (AY430941)	96.6	
Irina II G5b		3 (7.7)	<i>Thiomicrospira denitrificans</i> (AY885677)	78.7	
Irina II G08		5 (12.8)	uncultured epsilonproteobacterial clone 899-3 (AY553060)	89.5	
Irina II H01		2 (5.1)	episymbiont <i>Alvinella pompejana</i> clone 3713R-5 (AY308600)	92.0	
Irina II F1b		2 (5.1)	uncultured prokaryote clone 694-3 (AY430977)	95.5	
Irina II H6b		2 (5.1)	uncultured prokaryote clone 2a09 (AY430939)	96.6	
Lilliput E11			1 (3.0)	uncultured epsilonproteobacterial clone 899-3 (AY553060)	87.7
Lilliput B10			7 (21.2)	uncultured epsilonproteobacterial clone 899-3 (AY553060)	94.2
Lilliput B12			1 (3.0)	episymbiont <i>Alvinella pompejana</i> clone 3713R-5 (AY308600)	94.3
Lilliput A12			1 (3.0)	episymbiont <i>Alvinella pompejana</i> clone 3713R-5 (AY308600)	93.2
Lilliput C02			13 (39.4)	uncultured prokaryote clone 694-3 (AY430977)	96.6
Lilliput D05			7 (21.2)	uncultured prokaryote clone 694-3 (AY430977)	96.6
Lilliput B09			3 (9.1)	uncultured prokaryote clone 2a09 (AY430939)	95.5
<i>hynL</i>					
Site B 33F01	1 (2.6)		<i>Flavobacterium johnsoniae</i> (NZ_AAPM01000004)	67.3	
Site B 33E01	7 (18.4)		<i>Flavobacterium johnsoniae</i> (NZ_AAPM01000004)	71.3	
Site B 33H03	2 (5.3)		<i>Rhodobacter sphaeroides</i> (NZ_AAME01000007)	73.8	
Site B 33D01	2 (5.3)		<i>Hydrogenimonas thermophila</i> (AB206664)	60.6	
Site B 33F03	4 (10.5)		"Sulfurimonas paralvinella" (AB206665)	71.0	
Site B 33E02	2 (5.3)		"Sulfurimonas paralvinella" (AB206665)	70.1	
Site B 33D02	12 (31.6)		"Sulfurimonas paralvinella" (AB206665)	70.1	
Site B 33C01	2 (5.3)		<i>Thiomicrospira crunogena</i> (CP000109)	51.6	
Site B 33G03	2 (5.3)		<i>Thiomicrospira crunogena</i> (CP000109)	96.9	
Site B 33D03	3 (7.9)		<i>Thiomicrospira denitrificans</i> (NC_007575)	70.9	
Site B 33H01	1 (2.6)		<i>Thiomicrospira denitrificans</i> (NC_007575)	67.8	
Irina II 33F07		2 (6.7)	<i>Flavobacterium johnsoniae</i> (NZ_AAPM01000004)	70.1	
Irina II 33A08		1 (3.0)	<i>Oligotropha carboxydoverans</i> (X82447)	76.3	
Irina II 33E08		4 (13.3)	<i>Hydrogenimonas thermophila</i> (AB206664)	60.0	
Irina II 33C09		2 (6.7)	<i>Hydrogenimonas thermophila</i> (AB206664)	52.0	
Irina II 33F09		9 (30.0)	"Sulfurimonas paralvinella" (AB206665)	71.9	
Irina II 33A09		2 (6.7)	"Sulfurimonas paralvinella" (AB206665)	70.1	
Irina II 33C08		1 (3.3)	"Sulfurimonas paralvinella" (AB206665)	71.9	
Irina II 33F08		4 (13.3)	<i>Thiomicrospira denitrificans</i> (NC_007575)	70.4	
Irina II 33H10		4 (13.3)	<i>Thiomicrospira denitrificans</i> (NC_007575)	74.0	
Lilliput 33B05			2 (10.5)	<i>Azotobacter vinelandii</i> (L23970)	79.5
Lilliput 33D05			2 (10.5)	<i>Oligotropha carboxydoverans</i> (X82447)	76.9
Lilliput 33A06			14 (73.7)	<i>Thiomicrospira crunogena</i> (CP000109)	96.9
Lilliput 33B04			1 (5.3)	<i>Lebetimonas acidiphila</i> (AB206667)	74.0

3.3.3 Hydrogen Oxidation (NiFe-Uptake Hydrogenase gene: *hynL*)

The significantly lower diversity of NiFe-Uptake Hydrogenase phylotypes in the basalt-hosted Lilliput fluids compared to fluids from the ultramafic-hosted Logatchev field is evident (Fig. 17, Table 6). In Lilliput fluids, merely four *hynL* gene phylotypes with solely one phylotype associated with *Epsilonproteobacteria* were detected (Fig. 17). This sequence was distantly related to *Campylobacter jejuni*, *Thioreductor micantsoli* and *Lebetimonas acidiphila* (60.0-73.9%). In contrast, LHF fluids from Irina II and Site B yielded 9-11 phylotypes of which seven and six phylotypes, respectively, were affiliated with diverse hydrogen-oxidizing *Epsilonproteobacteria* (Fig. 17, Table 6). With respect to the distribution

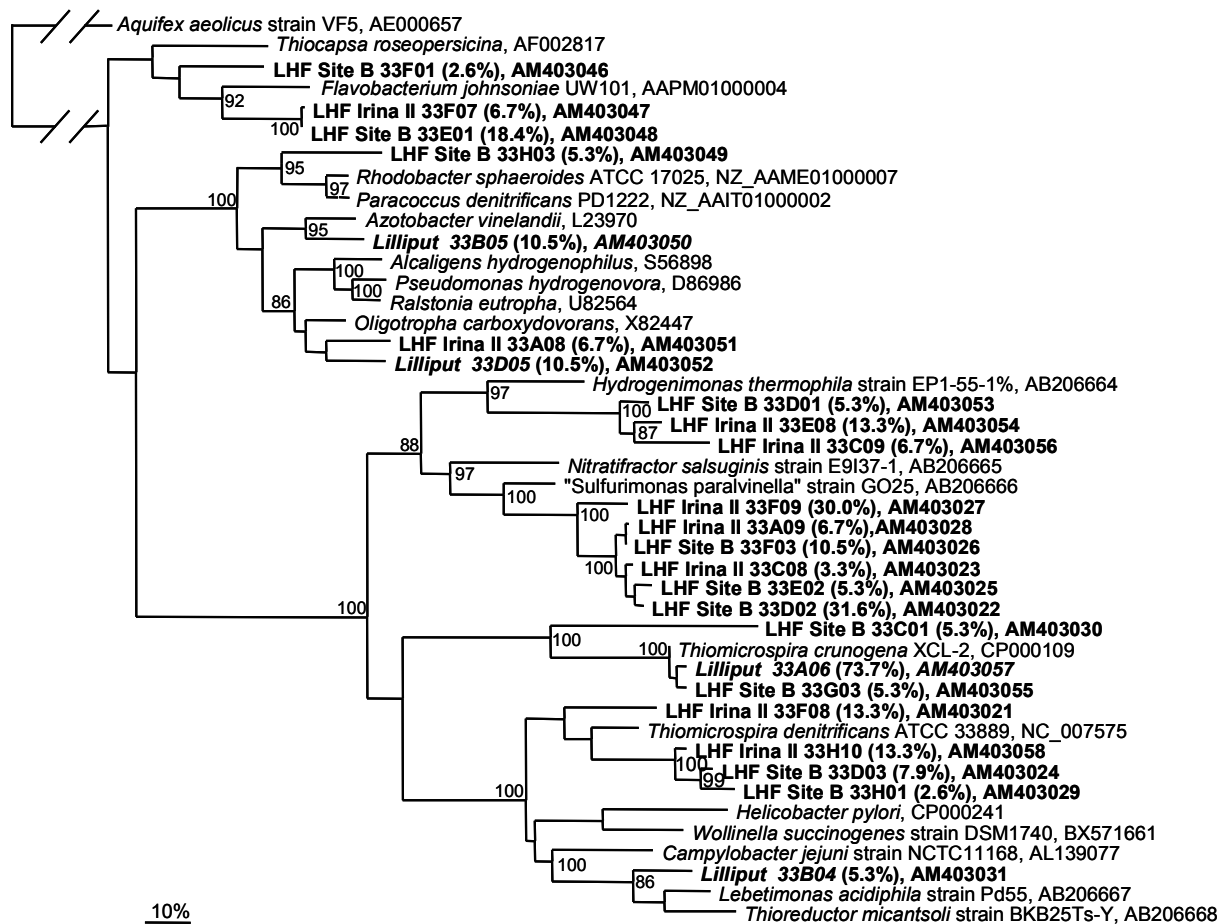


Fig. 17: Phylogeny of *hynL* genes encoding the large subunit of the NiFe-Uptake Hydrogenase determined by Maximum-Likelihood analysis. Bootstrap resamplings above 75% are indicated. Sequences obtained from the LHF sites (Irina II and Site B) and the Lilliput hydrothermal field are printed in bold and in italic respectively. Numbers in parenthesis indicate percentage of sequences belonging to one phylotype. The scale bar represents expected number of changes per nucleotide positions.

of sequences detected at the LHF, site-specific differences were clearly discernible. Yet, sequences were generally related to the same members of *Epsilonproteobacteria*, including

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representatives of groups A, B and F. However, sequence data on the gene encoding NiFe-Uptake Hydrogenase, especially concerning the *Epsilonproteobacteria*, is very limited. In addition to the epsilonproteobacterial sequences, Site B contained a sequence which was associated with *Thiomicrospira crunogena* (Fig. 17, Table 6). Sequences related to *Thiomicrospira crunogena* were also detected at the Lilliput hydrothermal field (Fig. 17). They constituted for 74% of the Lilliput *hynL* genes. Other *hynL* sequences found in fluids from both hydrothermal fields include affiliates of *Alpha-* and *Gammaproteobacteria* and *Bacteroidetes* (Fig. 17, Table 6). However, these are not necessarily associated with energy generation through hydrogen oxidation for autotrophic growth (Madigan *et al.*, 2003).

4 DISCUSSION

When identifying factors responsible for the presence or absence of microorganisms numerous parameters apart from host-rock lithology and mixing processes of hydrothermal fluids with ambient seawater need to be taken into consideration. At present, little data is available on microbial communities of ultramafic-hosted systems influenced by serpentinization processes (Schrenk *et al.*, 2004, Nercessian *et al.*, 2005, Brazelton *et al.*, 2006). In addition, only few microbiology studies have incorporated fluid chemical data of energy sources other than sulfide in their studies (e.g., Takai *et al.*, 2004a). However, these fluid chemical data as well as in situ measurements are essential for understanding the impact of specific environmental parameters on the occurrence of microorganisms.

Misinterpretation of microbial data could be caused by various sampling techniques applied in different studies. Furthermore, the microbial intra-field variability observed at distinct hydrothermal vent environments might reflect small spatial differences apparent in a single vent location. Nakagawa and colleagues (2005c) documented substantial compositional variations of microorganisms found in the fluids as well as in close and distant proximity of the emission site.

Apparent spatial variability in microbial diversity at different vent locations could also reflect successional stages of the vents. However, time series studies are scarce (e.g., Huber *et al.*, 2002, Huber *et al.*, 2003). The importance of temporal variations of fluid chemical composition and associated shifts in the microbial assemblage, especially concerning the *Epsilonproteobacteria*, have been shown to coincide with the progressing of the vent maturity following a volcanic eruption (Huber *et al.*, 2003).

4.1 Logatchev Hydrothermal Field (LHF)

This study is the first assessment of the microbial community inhabiting the Logatchev hydrothermal field (LHF). The geological setting, notably an ultramafic-hosted hydrothermal field, results in high hydrogen concentrations within the emanating fluids (Table 7). Possibly as a consequence, a large fraction of the microorganisms inhabiting the LHF is associated with hydrogen-oxidizers (Fig. 6, 7, 9, 17).

The archaea recovered at all vent locations were mainly associated with *Methanococcales* (Fig 9). In contrast, the bacterial communities varied at the three studied sites. It seemed that an increase in hydrogen results in a diversification of bacteria putatively capable of oxidizing hydrogen. Nonetheless, the microorganisms detected were associated with cultured prokaryotes requiring different growth temperatures and tolerances towards oxygen. This underlines the importance of mixing processes next to the abundance of available energy sources for the inhabitation of the hydrothermal biotopes.

4.1.1 The Role of Host Rocks

Methanococcales use hydrogen and CO₂ as a substrate for hydrogenotrophic methanogenesis (Whitman *et al.*, 1992). The prevalence of archaeal sequences associated with the *Methanococcales* at all investigated LHF sites (Fig. 9) might indicate a causal relationship between the presence of methanogenic archaea and the setting of an ultramafic-hosted system. Stable carbon isotope signatures determined for methane do not indicate a significant proportion of biogenic methane (R. Seifert, personal communication). This argues against a metabolic significance of *Methanococcales* in the LHF. Nevertheless, a biogenic methane signature might be masked due to a high background level of nonbiogenic methane.

Representatives of the *Epsilonproteobacteria* and of deeply-rooted lineages have been associated with hydrogen oxidation (e.g., L'Haridon *et al.*, 1998, Götz *et al.*, 2002, Campbell *et al.*, 2006). In contrast to the archaeal community, the fluids at each of the three vent emission sites support different bacterial communities (Fig. 6, 7, 9). It seemed that an increase in hydrogen concentration led to a more diverse community of putative hydrogen-oxidizing bacteria. For example, at Irina I, where the highest hydrogen concentrations were found in this study (5.9 mM) (R. Seifert, personal communication) (Table 7), potential hydrogen-oxidizing affiliates were distributed throughout the entire bacterial domain. These included *Aquificales* and *Epsilonproteobacteria* (group D) (Fig. 6, 7). At Irina II, where significantly lower hydrogen concentrations were found (2.2 mM) (R. Seifert, personal communication), less diverse potential hydrogen-oxidizing bacteria were present (Fig. 6, Table 7). They were limited to the epsilon subdivision of the *Proteobacteria* (groups A and G). In contrast, at Site B, which had only slightly lower hydrogen contents than Irina II, no typical hydrogen-oxidizing bacteria were observed. The organisms detected here were affiliated with groups B

and F of the *Epsilonproteobacteria* (Fig. 6). Only some associates of these groups have been shown to be capable of hydrogen oxidation, while others are able to utilize reduced sulfur compounds (Campbell *et al.*, 2006). However, studies conducted on the NiFe-Uptake Hydrogenase (catalyzes the oxidation of molecular hydrogen), in fluids originating from Irina II and Site B, demonstrated the potential of diverse *Epsilonproteobacteria* to be involved in hydrogen oxidation (Fig. 17). The presence of putative hydrogen-oxidizers at all LHF locations might indicate that the ultramafic rock hosting the hydrothermal system selects for organisms with specific physiologies (hydrogen oxidation). If the nature of the host rock is the only factor influencing the distribution of microorganisms, one would expect this to be reflected in microbial diversity analyses obtained from other ultramafic-hosted systems. However, this is not the case (Table 7).

Out of all active venting sites, the Rainbow vent field is the one with its geological setting and fluid-physico-chemistry most similar to the LHF (Donval *et al.*, 1997, Lackschewitz *et al.*, 2005). Nonetheless, typical hydrogen-oxidizers such as *Aquificales* or members associated with e.g. groups A or D (*Epsilonproteobacteria*) were not detected at the Rainbow hydrothermal field (López-García *et al.*, 2003) (Table 7). However, the three LHF locations have shown that bacterial diversity can differ significantly even though general chemical parameters seem to be similar (Table 7). In contrast, *Methanococcales* were found at the Rainbow field using functional gene analysis (Nercessian *et al.*, 2005). This is in agreement with archaeal communities observed in the LHF fluids (Fig. 9).

Although the Lost City vent field is ultramafic-hosted and fluids have high hydrogen concentrations, the physico-chemical features of the emitting fluids are very different to those found in the Logatchev and Rainbow fields (for references see Table 7). The role of methane as a microbial energy source seems to be pronounced in the Lost City environment (Brazelton *et al.*, 2006). But despite high hydrogen contents, no typical hydrogen-oxidizing organisms were apparent (Brazelton *et al.*, 2006). However, hydrogen oxidation has been suggested for some prokaryotes (Brazelton *et al.*, 2006).

In contrast to the ultramafic-hosted systems described above, basalt-hosted hydrothermal systems typically reveal lower hydrogen contents (e.g., van Dover *et al.*, 2001, von Damm & Lilley, 2004). Despite the low hydrogen concentrations in basalt-hosted environments, microbial communities consist in part of organisms related to phylogenetically diverse hydrogen-oxidizing microorganisms (e.g., Hoek *et al.*, 2003, Kormas *et al.*, 2006) (Table 7).

As shown for Site B, even relatively high concentrations of hydrogen (1.8 mM) do not necessarily result in phylogenetically diverse hydrogen-oxidizing communities (Table 7). This set of findings indicates that other parameters in addition to host-rock composition are essential in controlling the structure of the microbial population. The large number of phylogenetically diverse species affiliated with organisms characterized by various temperature and oxygen requirements suggests that mixing processes (hydrothermal fluids and oxygenated seawater) play an important role for the microbial community.

4.1.2 The Importance of Mixing Processes

The frequent encounters of prokaryotes related to cultured representatives with different temperature and oxygen requirements suggest that mixing processes might be important at the three vent locations studied at the LHF. As hydrothermal fluids rise to the surface they mix with ambient seawater, resulting in physico-chemical gradients. The physico-chemical framework is reflected in the physiologies of organisms adapted to these environments. Therefore, the presence of specific groups of organisms can provide an indication of the intensity of these mixing processes.

The highest phylogenetic diversity throughout the bacterial domain was observed at Irina I. It included members of *Epsilonproteobacteria* and deeply-rooted lineages (Fig. 6, 7). A thermophilic lifestyle is a group-specific characteristic of some of these representatives (L'Haridon *et al.*, 1998, Götz *et al.*, 2002, Campbell *et al.*, 2006). In fluids from Irina II, merely organisms were detected which were linked to thermophilic *Epsilonproteobacteria*. However, they are characterized by slightly lower growth temperatures compared to members of the deeply-rooted lineages. This implies that the habitable environments at Irina I allow less intense mixing processes than the biotopes at Irina II do. At Site B, no prokaryotes were observed related to thermophilic bacteria. The only microorganisms typically known for a thermophilic lifestyle included archaea of the order *Methanococcales* (Fig. 9). *Epsilonproteobacteria* inhabiting Site B biotopes were exclusively affiliated to members of groups B and F. Their cultured representatives have been described as mesophiles (for review, see Campbell *et al.*, 2006). This could indicate that bacterial life at this location is only possible where dilution of the hydrothermal fluids is ensured.

The presence of chemical gradients is also reflected in different levels of oxygen requirements characteristic for certain cultivated groups. As shown, several organisms at Irina I were related to bacteria with a thermophilic lifestyle. This suggests less intense mixing with oxygenated water. However, in addition to sequences related to strictly anaerobic organisms some were also associated with aerobic and microaerophilic bacteria e.g. members of the epsilonproteobacterial group F (Fig. 6). Therefore, oxygen must be available in some areas. The absence of *Deltaproteobacteria* at Irina I might indicate a limitation of sulfate or other oxidized sulfur species, or the influence of oxygen on the bacterial community.

Group-specific characteristics of group F *Epsilonproteobacteria*, include not only lower growth temperatures (30-37°C) but also tolerance towards oxygen (microaerobic) (Campbell *et al.*, 2006). In contrast to Irina I, members of this group were detected in great diversity at Irina II and Site B. Their occurrence in the fluids next to anaerobic *Desulfobulbaceae* implicates advanced mixing processes which have caused multiple biotopes to arise along the fluid pathways.

The differences of oxygen levels in the various biotopes are also reflected in the genes detected encoding key enzymes of the two investigated CO₂ fixation pathways, Calvin-Benson-Bassham (CBB) and reverse tricarboxylic acid (rTCA) cycles. Compared to RubisCO form II (CBB) and ATP citrate lyase (rTCA), the RubisCO form I enzyme is best adapted to high oxygen concentrations (Tabita, 1995, Takai *et al.*, 2005). A high diversity of RubisCO form I genes was demonstrated for both fluid samples of Irina II and Site B at the LHF (Fig. 15). *Bathymodiolus* and *Solemya* symbionts and *Thiomicrospira* spp. were the closest relatives of the identified microorganisms (Fig. 15, Table 6). This indicates that symbionts or symbiont related organisms together with *Thiomicrospira* spp. are well adapted to an efficient carbon fixation in oxic habitats. The presence of *Bathymodiolus* spp. symbionts was also verified based on 16S rRNA genes. Their encounter in outflows at Irina II is not surprising, as *Bathymodiolus* assemblages colonize the entire surroundings (Kuhn *et al.*, 2004, Lackschewitz *et al.*, 2005). Predation or natural death of *Bathymodiolus* spp. could explain the occurrence of symbionts among free-living prokaryotes of the area. In contrast to Irina II, hydrothermal fluids from Site B displayed a higher genetic diversity among the CBB genes than *acIb* genes of the rTCA cycle (Fig. 15, 16). Nevertheless, this does not implicate the irrelevance of the rTCA cycle at Site B as the organisms represented by the two *acIb* gene phylotypes could quantitatively be dominant and significantly contribute to CO₂ fixation. The

multiple *cbmM* sequences present in Site B fluid emissions, argue against a high oxygen content as being responsible for the low diversity of autotrophic *Epsilonproteobacteria* (Fig. 15). Hence, it is presumed that other parameters (e.g. temperature) account for the composition of the chemoautotrophic community at Site B, favoring diverse bacteria operating the CBB cycle (Fig. 15)

Because thermophilic *Methanococcales* have been detected in low-temperature fluids, it has been argued that they might originate from subsurface environments (Huber *et al.*, 2002, Takai *et al.*, 2004a, Nakagawa *et al.*, 2005b). They are independent of seawater-derived oxidants. The uniformity of the archaeal community at the LHF could suggest that parts of the *Methanococcales* originate from the subsurface.

4.1.3 Other Environmental Parameters

A single sequence at Irina I and 10% of bacterial sequences from Irina II fluids were affiliated with the new group RE1 (Fig. 7), of which no cultured representatives exist. As all sequences of this group are derived from reducing environments, the name of RE1 for Reduced Environment is proposed. It is conspicuous that, with the exception of sequences retrieved from termites (Hongoh *et al.*, 2005), all others originate from hydrothermal vent environments (Fig. 7). The chemical conditions i.e. low sulfide concentrations and high hydrogen concentrations (Table 7) could be favorable for the occurrence of RE1 members. However, several sequences of this group are derived from Snake Pit (Corre *et al.*, 2001) where significantly higher sulfide concentrations (6 mM) have been determined (Douville *et al.*, 2002) than at the LHF (Table 7).

For the first time, a sequence affiliated with the ANME-2 lineage of the *Methanosarcinales* is reported from hot hydrothermal vents (Fig. 9). Interestingly, it originates from Site B, where the lowest methane concentrations were measured (0.6 mM) (Table 7). Affiliates of ANME-2 mediate the anaerobic oxidation of methane and have been found in anoxic sediments, seep environments and also hydrothermally active sediment (e.g., Boetius *et al.*, 2000, Orphan *et al.*, 2001). Whether the sequence affiliated with the ANME-2 group is associated with methane oxidation remains open, as no significant methane oxidation rates were measured in fluids at the LHF (J. Felden, personal communication).

Table 7: Fluid physico-chemical parameters and selected microorganisms at different hydrothermal vents

Geological setting		Basaltic		Ultramafic				
		EPR	CIR	MAR				
Mid-Ocean Ridge		NT^a	Edmond^b	Rainbow^c	Logatchev^d	Lost City^e		
Hydrothermal vent field		Near Bio 9	Fuzzy toothpick	Irina I	Site B	Atlantis massif		
Vent site of microbiological sample								
Physico-chemical parameters	T (°C)	350	70	360*	300-350	40-91		
	pH (25°C)	ND	ND	2.9-3.1*	6.2	3.8		
	H₂S (μmol kg⁻¹)	≤ 3.300*	ND	≤ 2.500	277	1,241		
	H₂ (mmol kg⁻¹)	≤ 0.3*	0.2*	13.0*	2.2	1.8		
	CH₄ (mmol kg⁻¹)	≤ 0.015*	0.4*	2.2*	0.7	0.6		
BACTERIA	γ	<i>Thiomicrospira</i>	-	-	-	+		
		<i>Methylococcales</i>	-	-	-	-	+	
	δ	<i>Proteobacteria</i>	Group A	-	-	+	-	
			Group B	+	+	+	+	
			Group D	-	+	-	-	-
			Group F	+	+	+	+	+
			Group G	-	-	+	-	-
		<i>Sulfurospirillum</i>	+	+	-	+	+	
	ε	<i>Aquificales</i>		+	+	+	-	
			<i>Desulfurobacterium</i>	-	-	-	-	-
ARCHAEA	<i>Methanococcales</i>		+	+	+	-		
		<i>Methanosarcinales</i>	-	-	ND	-	+	
	ANME Group		-	-	ND	-	+	
			-	-	-	-	+	

Fluid data not obtained from identical microbiological sampling sites are marked by “*”. All chemical compositions of vent fluids are end-member concentrations. EPR, CIR and MAR denote East Pacific Rise, Central Indian Ridge and Mid-Atlantic Ridge respectively; NT denotes Northern Transect; ND corresponds to not determined; Microorganisms detected/not detected are indicated by a “+/-”. ^a Von Damm & Lilley, 2004; Kormas *et al.*, 2006; ^b Van Dover *et al.*, 2001; Hoek *et al.*, 2003; ^c Donval *et al.*, 1997; López-García *et al.*, 2003; Nercessian *et al.*, 2005; ^d Lackschewitz *et al.*, 2005; fluid chemical data A. Koschinsky, R. Seifert & H. Strauss, personal communication; H₂S, H₂ and CH₄ concentrations are minimum values; ^e Kelley *et al.*, 2001, Schrenk *et al.*, 2004, Brazelton *et al.*, 2006.

4.2 Lilliput Hydrothermal Field

Low temperatures of discharging fluids ($\leq 16.7^{\circ}\text{C}$) (Haase *et al.*, 2005) and the absence of hyperthermophilic and thermophilic prokaryotes (Table 8) implicate hydrothermal fluids of low to moderate temperatures in the subsurface at the Lilliput hydrothermal field. Judging from a higher Fe (II) fraction and additionally relative high oxygen contents an oxic/anoxic zonation throughout the mussel assemblage is assumed. This is reflected in prokaryotes related to cultured bacteria with different oxygen requirements. Hence a scheme which places the detected organisms is suggested, displaying their potential processes and interactions in a context at this site (Fig. 18). Contrary to the LHF, the main energy sources for chemolithotrophic bacteria at Lilliput are probably reduced sulfur compounds. Group-specific characteristics of bacteria using this metabolism include *Thiomicrospira* spp. and members of the *Epsilonproteobacteria* (Fig. 12, Table 8).

Table 8: Detection of specific microorganisms in the diffuse fluids at the Lilliput hydrothermal field

		Microorganisms	Lilliput hydrothermal field
BACTERIA	<i>Proteobacteria</i>	γ <i>Thiomicrospira</i>	+
		<i>Methylococcales</i>	-
		δ	+
		Group A	-
		Group B	+
		Group D	-
		ϵ Group F	+
		Group G	-
		<i>Arcobacter</i>	+
		<i>Aquificales</i>	-
<i>Desulfurobacterium</i>	-		
ARCHAEA	<i>Methanococcales</i>	-	
	<i>Methanosarcinales</i>	-	

4.2.1 Novelty of the Ecosystem

The high number of non-repetitive bacterial sequences indicates a high phylogenetic diversity at the Lilliput hydrothermal field (Fig. 12). This is consistent with reports from diffuse emissions at the East Pacific Rise (e.g., Huber *et al.*, 2002, Huber *et al.*, 2003). This diverse

bacterial community is accompanied by a variety of metabolic features (deduced from group-specific characteristics) and reflects the multiple niches along the fluid pathway (Fig. 18). Of the 51 phylotypes 76% showed little similarity to other known sequences (76.2-96.8%). This suggests that comparable habitats have never been investigated before. The novelty of the prokaryotic assemblage detected at the Lilliput hydrothermal field could be due either to the geographically distinct location or to the low-temperature influence of the hydrothermal field hosted by an exceptionally thick oceanic crust. However, sequence novelty could also reflect the multiple habitats associated with the *Bathymodiolus* assemblages. To date *Bathymodiolus* assemblages have not been studied with respect to the influence they have on the free-living microbial community. This is surprising considering the functionality of the mussels (i) in providing secondary substrate and (ii) as their accumulation hinders fast resuspension of sedimented organic matter. Although no studies have specifically been conducted on excretions from *Bathymodiolus* mussels, analogues like the *Mytilus* mussels (without symbionts but closely related to *Bathymodiolus* spp.), have been shown to release dissolved organic carbon, ammonia, and organic dissolved nitrogen which in turn, are utilized by the microbial population (Tupas & Koike, 1990). If this were the case also for *Bathymodiolus* mussels they could be responsible for a major part of the nutrient supply to the endemic heterotrophic community. Therefore they could be assigned a new role in terms of determining the structure of the ecosystem.

4.2.2 Habitats Characterized by Microorganisms

Lilliput fluids comprise various organisms associated with aerobic, microaerophilic and anaerobic bacteria (Fig. 12), which are unlikely to occur in immediate proximity. This suggests different oxic/anoxic environments (Fig. 18). The oxygen variability of various cultured representatives related to Lilliput bacteria is also reflected in the oxygen sensitivities of key enzymes typical for the different CO₂ fixation pathways that were detected by the genetic approach (Fig. 15, 16). However, genes encoding the *cbbL* gene (form I RubisCO) revealed less diversity compared to the *cbbM* (form II RubisCO) and *aclb* (rTCA cycle) genes (Fig. 15, 16) which indicate that fewer organisms in the emissions are adapted to potentially fixing CO₂ in the presence of oxygen. This is in contrast to previous reports, claiming that form I RubisCO is more diverse compared to the form II RubisCO in hydrothermal samples (Elsaied & Naganuma, 2001, Campbell & Cary, 2004). The rTCA cycle seems to be the

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predominant mechanism for autotrophic CO₂ fixation at hydrothermal vent environments (Campbell & Cary, 2004, Takai *et al.*, 2005, Campbell *et al.*, 2006). This is corroborated by findings of diverse *aclb* genes in the diffuse emissions at Lilliput (Fig. 16).

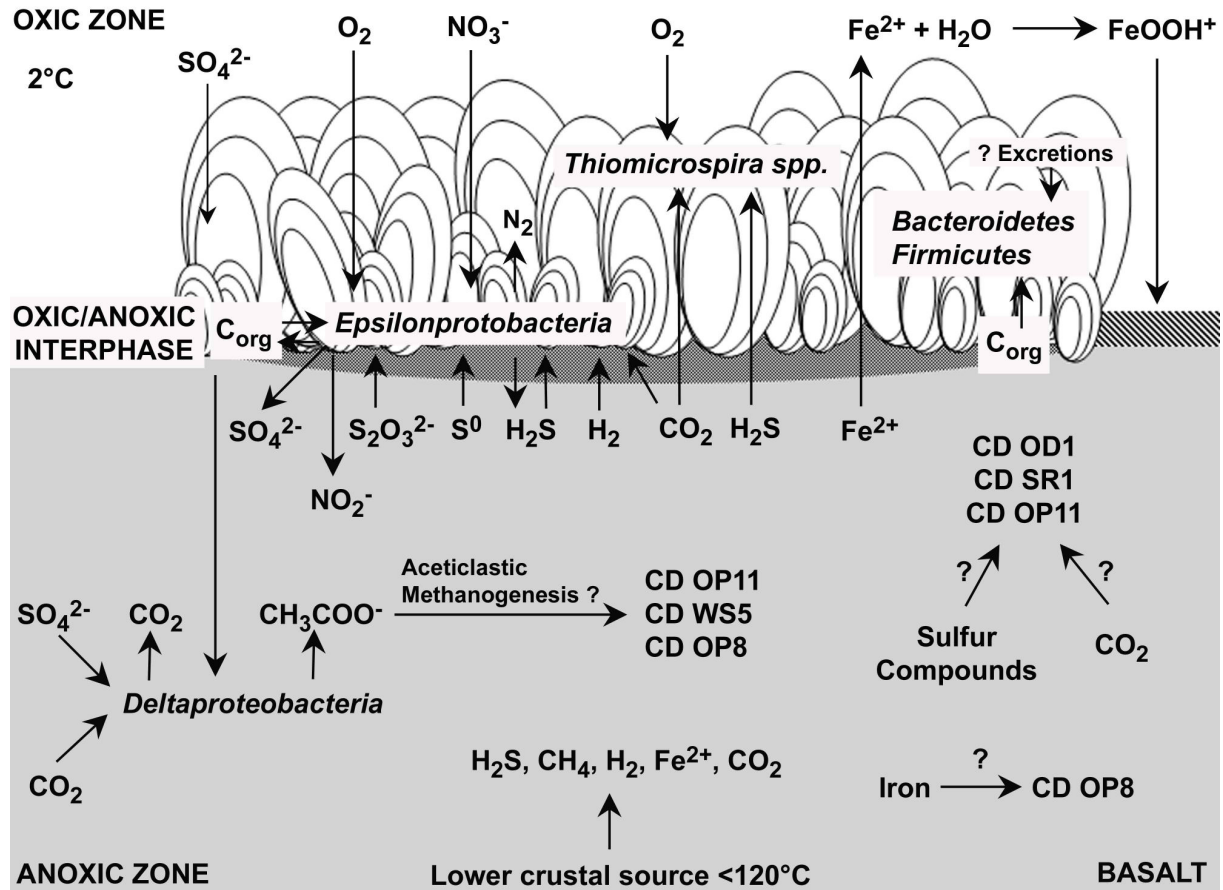


Fig. 18: Hypothetical sketch of the oxic/anoxic interface established by the mussel assemblage and the underlying anoxic zone (subsurface) at the Lilliput hydrothermal field. The flow of reduced compounds derived from hydrothermal fluids and oxidants from the ambient seawater is indicated. The location and potential metabolic mechanisms of bacteria are suggested. CD indicates candidate division.

The elevated oxygen concentrations (3.12 ml/L) (D. Garbe-Schönberg, personal communication) determined at the point of discharge are inconsistent with the reduced features (e.g. excess of Fe II compared to Fe III fraction) typically known for hydrothermal fluids. However, assuming mixing processes of the oxygenated ambient seawater and reduced hydrothermal fluids taking place in the oxic/anoxic interphase of the mussel assemblage, a clear physico-chemical zonation can be expected. It is in fact reflected in the prokaryotes inhabiting the distinct layers (Fig. 18). Based on the microbial community structure and the

chemical characteristics of the diffuse fluids three separated environmental zones are envisioned: the oxic zone, the oxic/anoxic interphase and the anoxic zone (Fig. 18). The oxic zone is characterized by 2°C ambient seawater with prokaryotes being unaffected by fluid discharge and is thus not further discussed. The area of the mussel patch comprises the oxic/anoxic interface (Fig. 18). This community is influenced by low-temperature fluid emissions supplying reduced inorganic compounds from below and oxidants i.e. O₂, SO₄²⁻ and NO₃⁻ derived from ambient seawater. Hence, a strong physico-chemical gradient can be expected across the mussel patch. The subsurface environment is considered to be the anoxic zone. It is characterized by reduced compounds from the diffuse fluids (Fig. 18).

Prokaryotes detected in hydrothermal emissions disclose physico-chemical parameters characteristic for habitats through which the hydrothermal fluid has passed on the way to the surface. For example, thermophilic and hyperthermophilic prokaryotes have been used as microbial indicators for the existence of a hot subsurface biotope and have been frequently observed in diffuse emissions (e.g., Holden *et al.*, 1998, Huber *et al.*, 2002, Huber *et al.*, 2003). However, these microbial tracers were absent in Lilliput emissions arguing against the presence of a hot fluid in the near subsurface. This finding is corroborated by abiotic low-temperature indicators, i.e. low-temperatures of the fluid emissions (<16.7°C), the absence of chimney structures and sulfide deposits, and the presence of low-temperature formations like iron oxide/hydroxides (Fig. 2). Further support for this conclusion stems from low concentrations of H₂ and CH₄ and the particularly low H₂/CH₄ ratios (Table 7). Especially the latter should be much higher assuming the emanating fluids to be derived from oxygenated, cold ambient seawater admixed in the subsurface with a small portion of hot hydrothermal fluid (R. Seifert, personal communication).

4.2.2.1 Archaea of an Oxic Environment

Repeated hybridizations conducted with fluorescently labeled probes specific for Archaea, *Crenarchaeota* and *Euryarchaeota* (Table 1) showed that the numbers of detected signals remained below the detection limit (<1% of total cells). This finding is consistent with the difficulties in amplification of archaeal DNA and the low number of retrieved archaeal sequences. Archaea of the Lilliput hydrothermal field were predominantly associated with the *Crenarchaeota* Marine Group I (MG I), which are distributed widespread in the water column

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of the world's oceans (e.g., DeLong, 1992). The presence of members of the *Crenarchaeota* MG I suggest an ambient seawater contribution to the fluids, which is supported by temperature measurements (Table 8) and oxygen concentrations (D. Garbe-Schönberg, personal communication) of the diffuse fluid emissions. As suggested this mixing process takes place at the oxic/anoxic interface. Part of the MG I sequences (19% of the archaeal clone library) was closely affiliated with the chemolithoautotrophic, ammonia-oxidizing *Nitrosopumilus maritimus* (Könneke *et al.*, 2005). Considering the option of mussels excreting bioavailable nitrogen this could indicate an involvement of the MG I *Nitrosopumilus*-like representatives in the local nitrogen and carbon cycle. The only cultured member of the Deep-Sea Hydrothermal Vent Euryarchaeota Group (DHVE) to date is the thermoacidophilic *Aciduliprofundum boonei* (Reysenbach *et al.*, 2006). However, clone sequences at Lilliput affiliated with the DHVE were too distantly related to this thermophilic isolate, prohibiting assumptions on the physiological features of these uncultured archaea.

Even though affiliates of the MG I might be involved in recycling mussel excretions and contribute to the local nitrogen and carbon cycling, archaea generally seem to play a minor role at Lilliput. In consistence with their extremely low abundance, the presence of archaea is caused most likely by the entrainment of ambient seawater. Together with the absence of hyperthermophilic methanogens and heterotrophs it is concluded that no indigenous archaeal community exists in the oxic/anoxic interface or immediate subsurface at Lilliput, which significantly influences the fluid chemistry or the bacterial community.

Table 9: Measured physical and chemical properties of the diffuse fluids at the Lilliput hydrothermal field

Physico-chemical parameters	ambient seawater	Lilliput hydrothermal field
End-member fluid portion (%)	0	4
T (°C)	2	5.5
pH	8.1	6.4
H ₂ (nM)	< 0.5	16
CH ₄ (nM)	< 1	2662
δ ¹³ C _{methane} (‰)	ND	-32.6
H ₂ S (μM)	< 1*	500 [§]
Fe ²⁺ (μM)	< 1.8*	22.2
Fe ³⁺ (μM)	ND	0.5

Data originate from Perner *et al.* (submitted for publication 2007b). The pH was measured at 25°C. Concentrations below the detection limit of the method used are indicated with “*”. “§” denotes that the sample was collected from the same field a year later. ND = not determined.

4.2.2.2 Bacteria of the Oxic/Anoxic Interface

Microbial life at the oxic/anoxic interface is fueled by reduced diffuse emissions supplying inorganic compounds (i.e. H_2S , H_2 , CH_4 and Fe^{2+}) and seawater-derived oxidants, i.e. O_2 , SO_4^{2-} and NO_3^- . While the low-temperature discharge supplies electron donors (energy sources) the ambient seawater delivers electron acceptors. *Gorgonaria* and other biological colonization (Fig. 2) indicate a strong source of suspended organic material in the Lilliput area (Haase *et al.*, 2005). Furthermore, increased accumulation of organic matter within the mussel patches can be assumed as well as possible organic excretions of the vent mussels contributing to the organic matter budget. This diverse and massive supply of inorganic and organic energy sources is reflected in the phylogenetically diverse bacterial community (Fig. 12).

Typical organisms of the oxic/anoxic interface are *Thiomicrospira* spp.. They have been isolated from different hydrothermal environments and require reduced sulfur compounds as well as oxygen (Jannasch *et al.*, 1985). In fluids emitting from the Lilliput hydrothermal field, *Thiomicrospira* spp. were detected on the basis of the 16S rRNA gene (Fig. 12, Table 8) and genes encoding form I and II RubisCO (Fig. 15). The presence of *Thiomicrospira* spp. was also asserted by culture dependent techniques (J. Kuever, personal communication). Among the genes encoding the two types of RubisCO, environmental sequences were predominantly affiliated with *Thiomicrospira* spp. detected in the diffuse fluids. This indicates that among the chemoautotrophic community putatively capable of operating the Calvin Benson-Bassham cycle for CO_2 fixation, their role is pronounced. Quantitative FISH analyses corroborate that *Gammaproteobacteria* are a substantial part of the community (Table 4).

Next to *Thiomicrospira* spp. other prokaryotes known to be widespread in hydrothermal environments and involved in the oxidative part of the sulfur cycle include *Epsilonproteobacteria* (Campbell *et al.*, 2006). However, unlike *Thiomicrospira* spp. their affiliates are microaerophilic or anaerobic (Campbell *et al.*, 2006). This would place them in an area of the oxic/anoxic zone orientated towards the subsurface (Fig. 18). Nevertheless, Huber and colleagues have shown that the diversity of *Epsilonproteobacteria* is favored by conditions of increased mixing of oxygenated seawater with reduced fluids (Huber *et al.*, 2003). *Epsilonproteobacteria* observed at Lilliput include affiliates of the *Arcobacter* lineage, groups B and F (Fig. 12, Table 8). While oxidation of reduced sulfur compounds has been demonstrated for some members of these groups other representatives of these groups

additionally or exclusively oxidize hydrogen (Campbell *et al.*, 2006). Because of the high abundance of sulfide measured in Lilliput fluids (Table 8) it is assumed to be the prominent energy source for chemolithoautotrophs. However, other chemical compounds such as hydrogen and methane can not be excluded to be of importance. While no typical hydrogen-oxidizers (e.g. *Aquificales* and representatives of groups A and D of the *Epsilonproteobacteria*) were detected (based on 16S rRNA genes) (Fig. 12, Table 8), potential hydrogen-oxidizing candidates remain within the *Epsilonproteobacteria*. To identify epsilonproteobacterial hydrogen-oxidizers directly, the gene encoding NiFe-Uptake Hydrogenase of *Epsilonproteobacteria* was studied (Fig. 17). For this purpose specifically designed primers were used (Takai *et al.*, 2005). Even though the NiFe-Uptake Hydrogenase is phylogenetically not very well conserved and primers usually cover only a limited phylogenetic range (e.g., Wawer *et al.*, 1997), the specific primers used have shown to successfully amplify genes throughout the group of hydrogen-oxidizing *Epsilonproteobacteria* (Takai *et al.*, 2005). While for Lilliput fluids a single phylotype of the epsilonproteobacterial lineage putatively involved in hydrogen oxidation was detected no other epsilonproteobacterial NiFe-Uptake Hydrogenase genes were identified (Fig. 17). Hence, in agreement with low hydrogen concentrations (Table 8) this points to the minor role hydrogen probably plays as an energy source at the Lilliput hydrothermal field. With methane contents being only slightly elevated compared to ambient seawater (Table 8) the absence of known methane oxidizers is not surprising. Hence, sulfide likely represents the most important energy source for chemolithoautotrophs at Lilliput.

Many *Epsilonproteobacteria* are able to fix carbon dioxide (e.g., Inagaki *et al.*, 2004, Miroshnichenko *et al.*, 2004, Takai *et al.*, 2004c, Nakagawa *et al.*, 2005a) and are possibly responsible for major parts of the primary production in vent environments (Campbell *et al.*, 2006). In contrast to *Gammaproteobacteria* like *Thiomicrospira* spp., *Epsilonproteobacteria* use the rTCA cycle for CO₂ fixation (e.g., Hügler *et al.*, 2005, Takai *et al.*, 2005). The *acIb* gene (encoding a key enzyme of the rTCA cycle) phylogeny (Fig. 16) is in agreement with the 16S rRNA data (Fig. 12). This shows that representatives of probably group B and *Arcobacter* relatives are putatively involved in CO₂ fixation by using the rTCA cycle. Additionally, this might also indicate heterotrophy for Lilliput sequences affiliated with members of group F and *Campylobacter* spp.. These organisms were detected based on the 16S rRNA gene (Fig. 12) but were absent from the *acIb* clone library (Fig. 16). As

calculations on the coverage of the *acfb* clone library showed (Table 5) it remains unlikely that other *acfb*-gene phylotypes were not detected (Table 5).

Epsilonproteobacteria comprised 28% of all DAPI stained cells. Compared to studies from other hydrothermal environments their abundance is relatively low (Nakagawa *et al.*, 2006). However, *Epsilonproteobacteria* were the predominant prokaryotes of the Lilliput microbial community (Table 4). As they exceeded other prokaryotes in size (Fig. 11), their impact on the ecosystem regarding uptake or release of substances will be higher compared to smaller cells of equal abundance. Therefore, their role might be more pronounced than expected by simple cell counts. Furthermore the ability of coccoidal or filamentous epsilonproteobacterial cells to form chains (e.g. Fig. 11B, E) is beneficial for their survival as chain formations increase attachment abilities and possibly shelters the cells from predation by metazoans.

Next to inorganic compounds fueling chemoautotrophic life, organic matter functions as energy and carbon source for heterotrophic organisms. Only recently elevated concentrations of dissolved organic carbon (DOC), compared to ambient seawater, have been measured in diffuse emissions and were correlated with high microbial cell counts (Lang *et al.*, 2006). Despite elevated DOC in diffuse emissions (Lang *et al.*, 2006) members of the *Cytophaga-Flavobacterium* cluster, which are specialized in polymer degradation (Kirchman, 2002), were as abundant at Lilliput as in the open ocean (Glöckner *et al.*, 1999). Representatives of the phylum *Firmicutes* are also involved in degradation of organic compounds (Madigan *et al.*, 2003). Although they were detected in the fluids at Lilliput by sequencing, their abundance remains uncertain.

4.2.2.3 Bacteria of the Anoxic Zone

Epsilonproteobacteria, proposed to inhabit the lower area of the Lilliput mussel bed, have previously been associated with the seafloor biotope (Huber *et al.*, 2003). With the majority of the species capable of using nitrate or sulfur compounds as electron acceptors (Campbell *et al.*, 2006) their dispersal into the anoxic zone remains likely. The importance of sulfur cycling at this hydrothermal environment has been highlighted above and is further corroborated by the presence of *Deltaproteobacteria* (Table 8). Their occurrence next to

Epsilonproteobacteria has also been reported from hydrothermal sediments (Teske *et al.*, 2002). The co-occurrence of both groups could result from their complementary roles in the sulfur cycle (sulfate reduction for *Deltaproteobacteria* and sulfur oxidation for *Epsilonproteobacteria*) (Campbell *et al.*, 2006). Sulfate reducing bacteria use a broad range of electron donors (e.g. primary alcohols, fatty acids, carboxylic acids, glucose) with organic compounds being oxidized completely to CO₂ (e.g. *Desulfobacula* spp.) or incompletely to acetate (e.g. *Desulfovibrio* spp.). Others are capable of chemolithoautotrophic growth with H₂ and CO₂ (e.g. *Desulfobacterium* spp.). Considering their role in carbon and sulfur cycling their abundance (12% of DAPI stained cells) might be fundamental to the indigenous subsurface inhabitants of the Lilliput field.

Members of the deeply-rooted candidate divisions OD1, OP11, SR1, or WS6 have been observed in hydrothermal environments (Reysenbach *et al.*, 2000, Teske *et al.*, 2002, Huber *et al.*, 2003, Page *et al.*, 2004, Nakagawa *et al.*, 2005b). Very little is known in respect to their function in these ecosystems. However, they have been exclusively recovered from anoxic environments (Dojka *et al.*, 1998, Hugenholtz *et al.*, 1998, Dojka *et al.*, 2000, Harris *et al.*, 2004). An involvement in sulfur cycling has been postulated for members of the candidate divisions OD1, OP11 and SR1 (Harris *et al.*, 2004). With regard to Lilliput, their presence is consistent with relative high sulfide contents (Table 8).

Determination of the stable carbon isotope signature of CH₄ gave a $\delta^{13}\text{C}$ of -32.6‰ (R. Seifert, personal communication) (Table 8). This signature is typically found for methane produced by thermal degradation of organic matter from phototrophic organisms. However, considerable amounts of sedimented organic matter within the basalt rocks hosting the Lilliput field are unlikely. Alternatively, the methane might be produced by abiotic, mineral-catalyzed CO₂ reduction in the presence of H₂. Laboratory experiments have recently shown the possibility to generate methane about 35‰ depleted in ¹³C relative to CO₂ by abiotic synthesis under hydrothermal conditions (McCollom & Seewald, 2006). However, the low concentrations of H₂ detected in the Lilliput fluid (Table 8) argue against abiotic synthesis as a principal source of methane (R. Seifert, personal communication). Assuming that the majority of the methane present stems from methanogens, methyl-type fermentation appears to be a more likely pathway than abiotic carbonate reduction. Methanogenesis from acetate involves a depletion in ¹³C on the order of 25‰ to 35‰ for $\delta^{13}\text{C}_{\text{methane}}$ relative to $\delta^{13}\text{C}_{\text{acetate}}$ while a significantly greater depletion in ¹³C (more than 55‰ for $\delta^{13}\text{C}_{\text{methane}}$ relative to

4 DISCUSSION

$\delta^{13}\text{C}_{\text{carbon dioxide}}$) is observed for methanogenesis by carbonate reduction (Whiticar, 1999). The $\delta^{13}\text{C}$ of both potential substrates, CO_2 and acetate, are not enriched in ^{13}C (i.e., $\delta^{13}\text{C} < 0\text{‰}$). Hence, a measured $\delta^{13}\text{C}_{\text{methane}}$ of -32.6‰ is consistent with $\delta^{13}\text{C}_{\text{methane}}$ of acetoclastic methanogenesis. In this context, the respective acetate should possess a rather heavy $\delta^{13}\text{C}$ signature close to that of sea water CO_2 suggesting its synthesis associated with small isotope fractionation as given by the rTCA or the 3-hydroxypropionate cycles but not the acetyl-CoA pathway (Hayes, 2001, House *et al.*, 2003).

Members of the candidate divisions OP11, WS5 and OP8, observed at the Lilliput hydrothermal field (Fig. 12), have previously been described for different redox zones of an aquifer, including a methanogenic zone where acetoclastic methanogenesis has been proposed as the dominant microbial mechanism (Dojka *et al.*, 1998). With acetate generally being available in the subsurface environment and a carbon isotope signature for methane suggesting acetoclastic methanogenesis as principal pathway for methane production, members of this group might be involved in the same type of metabolism at the Lilliput hydrothermal field (Fig. 18). Representatives of the candidate division OP8 have further been detected in the iron/sulfate reducing zone of that aquifer (Dojka *et al.*, 1998).

A fraction of all DAPI stained cells (11%) was not targeted by the domain specific probes used (Table 4). These cells exhibited a smaller size compared to the other prokaryotes. Only recently, Miyoshi and colleagues demonstrated that members of the candidate divisions OD1, OP11, WS5 and WS6 are smaller than $0,2 \mu\text{m}$ (Miyoshi *et al.*, 2005). Interestingly, the bacteria-specific probes used here discriminate against Lilliput sequences affiliated with the candidate divisions OD1, OP11 and WS6 (up to 2 mismatches). By contrast, the EUB probe set matched with Lilliput affiliates of the candidate division WS5. With members of the candidate divisions OD1, OP11 and WS6 contributing 13% to the Lilliput clone library, these observations could indicate that these cells are representatives of these groups. If this were the case, this would implicate that their abundance and hence ecological role in terms of substrate turnover in their immediate environment has been underestimated.

5 ULTRAMAFIC- AND BASALT-HOSTED HYDROTHERMAL SYSTEMS

– A COMPARISON -

5.1 Microorganisms and Energy Sources

The chemistry of hydrothermal fluids differs as a consequence of the rock-type hosting the hydrothermal system. High hydrogen and methane contents characterize ultramafic-hosted systems whereas high sulfide concentrations are typical for basalt-hosted environments (Kelley *et al.*, 2002). Therefore it can be assumed that the microbial community structure varies in accordance with the local type of host rock. In consistence with the ultramafic nature of the LHF, diverse potential candidates of hydrogen-oxidizing *Aquificales*, *Epsilonproteobacteria* and *Methanococcales* were found (Fig. 6, 7, 9, 17). By contrast, at the basaltic setting of the Lilliput hydrothermal field these typical hydrogen-oxidizing prokaryotes were absent (Fig. 12, 13, Table 8) and potential hydrogen-oxidizing affiliates identified on the basis of NiFe-Uptake Hydrogenase (catalyzes the oxidation of molecular hydrogen) were limited to a single epsilonproteobacterial phylotype (Fig. 17). This indicates the importance of the host-rock in terms of supplying specific energy sources and, hence, selecting for specific microbial physiologies. At the three LHF locations a diversification of the hydrogen-oxidizing community seemed to coincide with an increase in hydrogen concentration (energy source) in the fluids (Table 7). However, comparison of hydrogen-oxidizing communities in hydrothermal fluids hosted by different types of host rocks and, hence, diverse fluid compositions revealed no apparent correlations (Table 7) (e.g., López-García *et al.*, 2003, Nercessian *et al.*, 2005, Kormas *et al.*, 2006).

At present time, using available data, it appears that the occurrence of chemolithotrophic prokaryotes is based on minimum concentrations of specific energy sources supplied by the host hydrothermal systems. However, other parameters such as temperature and oxygen levels have a stronger impact on the composition of the indigenous hydrothermal vent microbial populations. Therefore, it is concluded that while the host rock affects to a certain degree the microbial diversity the presence of microorganisms in specific habitats is more strongly influenced by mixing processes of hydrothermal fluids and seawater, rendering respective physico-chemical gradients.

Unfortunately the data on functional genes are limited and restricted to single point examinations. To date this is the only study available engaging in the subject of correlating NiFe-Uptake Hydrogenase genes with the types of host rock. Thus, further comprehensive studies using corresponding samples (for fluid chemistry and microbiology studies) and applying identical analytical techniques are needed to clarify the individual factors influencing the distribution and composition of prokaryotes at hydrothermal vent environments with differing host rocks.

5.2 CO₂ Fixation Pathways

For an organism to operate the Calvin Benson-Bassham (CBB) cycle more energy is required than for prokaryotes using the reverse tricarboxylic acid (rTCA) cycle for CO₂ fixation (Madigan *et al.*, 2003). The oxidation of various inorganic energy sources yields different amounts of energy. For example, the oxidation of hydrogen releases more energy than the oxidation of sulfide does (Madigan *et al.*, 2003). Therefore, one might presume that in ultramafic-hosted systems, where more hydrogen is available (“high quality energy”) the detected microorganisms would be capable of fixing CO₂ by a more energy consuming metabolism such as the CBB. However, based on data from this study, the type of host rock seems irrelevant for favoring the operation of one of the two investigated CO₂ fixation pathways (Fig. 15, 16). Hydrothermal fluids of both, the ultramafic-hosted LHF and of the basalt-hosted Lilliput field, hydrothermal systems support bacteria using either the CBB or the rTCA cycle (Fig. 15, 16). As no significant differences in the diversity of genes were apparent in the one over the other investigated CO₂ fixation pathway putatively used at the two hydrothermal fields (Fig. 15, 16) it is suggested that energy is not a limiting factor for the operation of different CO₂ fixation pathways used by specific prokaryotes. Moreover, the broad phylogenetic diversity of the chemoautotrophic bacteria encountered, operating different CO₂ fixation pathways, reflects the multiple environments present. These habitats are characterized by various oxygen contents and hence different degrees of mixing. Therefore the diversity of different CO₂ fixing genes demonstrates the ability of metabolically versatile prokaryotes to exhaust every available habitat.

A high diversity of RubisCO form I genes was demonstrated for most fluid samples at all studied sites. This suggests that these organisms are well adapted to an efficient carbon

fixation in an oxic habitat. One might argue that members of form I and especially form II RubisCO with no close cultivated representatives might be contaminants from the ambient seawater. However, this is assumed not to be the case as highly similar affiliates would be expected, at least within the LHF. In contrast, phylogenetically diverse genes encoding the form II RubisCO were found within the LHF as well as compared to the Lilliput. The majority is associated with sequences typical for hydrothermal settings (e.g. *Thiomicrospira thermophila*, hydrothermal plume clones). Contrary to previous reports, which revealed form I RubisCO being more diverse than the form II RubisCO in hydrothermal samples (Elsaied & Naganuma, 2001, Campbell & Cary, 2004), the fluids from the LHF fields are fairly balanced in terms of diversity of the two types of RubisCO (Fig. 15). This again supports the multiple biotope-hypotheses of oxygen contents differing along the fluid pathway as an important parameter for biodiversity. The higher diversity of rTCA genes and of form II RubisCO of the CBB cycle compared to form I RubisCO genes suggests that the chemoautotrophic community at the Lilliput hydrothermal field is better adapted to low oxygen concentrations.

The multiple phylotypes of *acfb* genes (rTCA cycle) in fluids from Irina II (LHF) and Lilliput corroborate previous studies demonstrating the rTCA cycle to be the predominant mechanism for autotrophic CO₂ fixation at hydrothermal vent environments (Campbell & Cary, 2004, Takai *et al.*, 2005, Campbell *et al.*, 2006). Variations detected on the basis of the *acfb* genes (Fig. 16) reflect the 16S rDNA phylogeny (Fig. 6, 12). These variations are probably due to the extent of mixing processes causing temperature and oxygen gradients along the fluid pathway at the LHF and Lilliput hydrothermal field. The broad diversity of genes encoding different CO₂ fixation pathways has not been shown in the past for hydrothermal environments. The occurrence of a large variety of different RubisCO and *acfb* genes and the oxygen sensitivity or tolerance of specific key enzymes indicate that this diversification reflects the adaptation to specific habitats such as physico-chemical gradients along a hydrothermal vent system.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Die Promotion soll im Fach Mikrobiologie erfolgen. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

(Mirjam Perner)