Microbial diversity within the low-temperature influenced deep marine biosphere along the Mid-Atlantic-Ridge

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Versicherung

Hiermit versichere ich an Eides statt, dass die Dissertation mit dem Titel: „Microbial diversity within the low-temperature influenced deep marine biosphere along the Mid-Atlantic-Ridge“ selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, den Unterschrift:

„Wichtig ist, dass man nicht aufhört zu fragen.“

Albert Einstein
dt.- amerikan. Physiker

1879- 1955

Microbial diversity within the low-temperature influenced deep marine biosphere along the Mid-Atlantic-Ridge

by

Kristina Rathsack

Submitted in partial fulfillment of the requirements for the degree of

Doctor rerum naturalium

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ABSTRACT

The deep sea floor forms largest associated ecosystem on earth. With it´s numerous ecological niches such as mud-volcanoes, cold seeps and hot and low-temperature influenced vents it could provide a harbourage for a diverse microbial consortia. By several molecular and microbial approaches, basalt and sediment samples collected in the vicinity of low-temperature influenced diffuse vents along the Mid-Atlantic-Ridge, were investigated. Our study revealed that a diverse microbial population inhabiting the analysed rock samples, dominated by *Proteobacteria*. The isolated organisms are distinguishable from the overlaying deep sea water and by characterisation of selected isolates high tolerances against a broad range of temperatures, pH-values and salt concentrations were observed. Further chemotaxic analysis resulted in a reclassification within the *Aurantimonas* (*Alphaproteobacteria*). The examination of the polar lipid compositions of selected isolates revealed that the genus of *Aurantimonas* had to be divided and the descriptions of the genera *Aurantimonas* and *Fulvimarina* have to be emended.

TABLE OF CONTENTS

CHAPTER 1- INTRODUCTION

1.1 DEEP-SEA MICROBIAL COMMUNITIES

The deep-sea realm represents one of the most inhospitable habitats on Earth. Over half of the Earth's living space is assigned to the deep-sea environment with the major part of global biomass inhabiting this realm (Pfannkuche, 1992; Withman et al. 1998). With the discovery of the hot vent ecosystem near the Galapagos Island (Lonsdale, 1977) the research field of deep biosphere has been opened up. In the following decades, it was demonstrated that the deep biosphere is teeming with life (Joergensen & Boetius, 2007 and references therein). Besides higher life forms, microorganisms such as bacteria and archaea represent the main portion of the oceanic biomass (Oren, 2004). However, the distribution of microbes is not homogeneous. According to physicochemical conditions microbes distribute variably (Moeseneder et al. 2001). The top layer named epipelagic zone (0-200 mbsl, meter below sea level) is completely saturated by solar radiation. Based on photosynthesis as well as comfortable temperature and pressure conditions a diverse and abundant microbial fauna prosper within that thin oceanic surface layer. With increasing water depth, light incidence and temperature constantly decline while hydrostatic pressure is increasing. Solely the blue wavelength fraction penetrates the mesopelagic zone (200-1,000 mbsl) whereas the only light occurring in the bathypelagic zone (1,000-4,000 mbsl) derives from bioluminescence. As light intensity is too low to sustain photosynthesis in these regions, the inhabiting microbial consortia depend on organic matter produced in surface waters. Greater depths (abyssal zone; 4,000-6,000 mbsl) are characterised by water temperature close to the freezing point (-1°C-4°C) and the high hydrostatic pressure (1 bar per 10 meter water depth). At the deepest parts of the oceans (hadal zone; 6,000-11,000 mbsl), pressures of 110 MPa were observed (see review Lauro & Bartlett, 2008).

Barring of the upper photosynthetic layer of the oceans, the pelagic microbial cell number decreases rapidly with water depth (Lochte, 1992). In contrast, the biomass in the seabed and below seafloor, or at thermal discharge regions along the seafloor spreading centres outvalue the microbial population of the productive ocean surface water 10-10,000 fold (Parkes et al. 2000; Jörgensen & Boetius, 2007 and references therein). In higher depths of the sub-seafloor the porcaryotic cell number diminished again (Schippers et al. 2005).

Microbial dynamics along those extreme chemical and physical gradients are of significant interest for microbiologists.

1.2 GEOLOGICAL CONDITIONS AT THE OCEANIC RIDGES

The uppermost earth mantle and the overlaying oceanic crust constitute the oceanic lithosphere. The lower part of the lithosphere is called low-velocity zone, which separates the inelastic lithosphere from the more flexible asthenosphere. A different viscosity of these layers induces tectonic plate movement, whereas spreading zones occur at the border of two tectonic plates. Induced by the magmatic heat flux, molten mantel material of the asthenosphere forces it's way through the lithosphere. When the hot mantel material meets with the cold seawater, the thermal shock leads to the formation of basaltic rocks, which are generally covered by a glass layer (Fig. 1.1.1). Due to the high proceeding velocity, new oceanic floor basalt is continuously generated, such as 2 centimetres per year (cm/yr) (North Atlantic), up to 16 cm/yr (South Pacific). This process forms Mid-Oceanic-Ridges all along the tectonic plate boundaries, which are thus connected with each other and form the longest mountain range in the world, with a total length of about 70,000 km. The continuous formation of Mid-Ocean-Ridges leads to the generation of cracks and fissures within the lithosphere, where due to the high pressure cold sea water ingresses. At its way to the depth seawater is heated up by passing up-coming magma flows. During the flow-through metal

compounds from the surrounding mantle material are dissolved. The rising hydrothermal fluids, enriched with electron donors (e.g. methane, hydrogen, hydrogen sulphide, other sulphur compounds), meet the cold electron acceptor rich (oxygen, nitrogen compounds) ocean sea water (Jörgensen & Boetius, 2007) and thus providing the fundament for life relying on chemosynthesis (overview is depicted on Fig. 1.1).

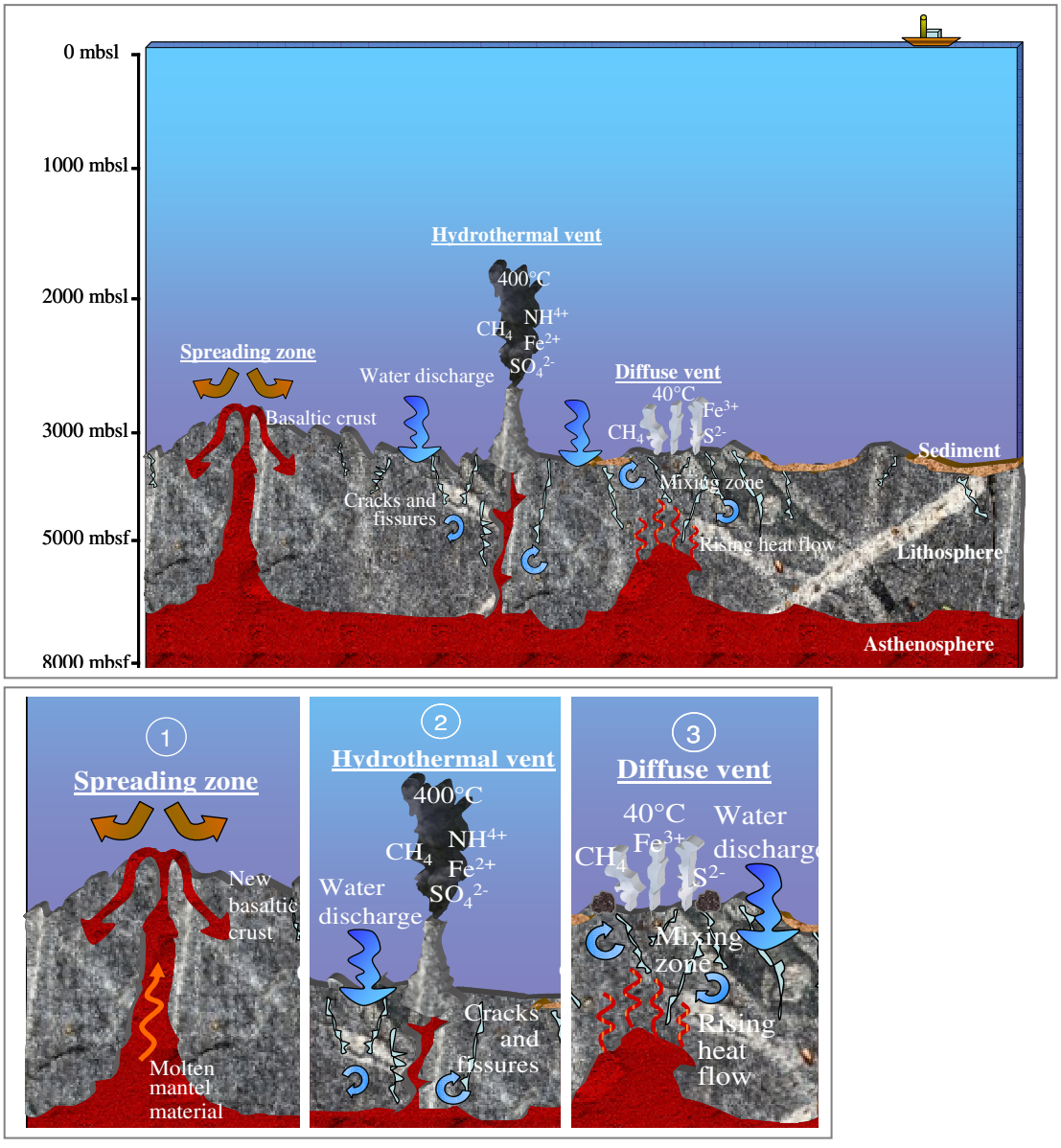

Figure 1.1

Schematic depiction of the geochemical conditions of different fluid discharge regions at the Mid-Ocean-Ridge. (1) Spreading zone, producing new ocean crust by rising molten mantel material. (2) Hot hydrothermal vent system with little interaction with penetrating sea water. (3) Diffuse discharge region based on sea water heated up by the contact with rising heat flows.

1.3 LOW-TEMPERATURE VENTS AND MICROBIAL POPULATION

When venting discharge occurs in a restricted area, the hot fluid is transported to the surface with no or little mixing with cold seawater (see Fig. 1.1). By precipitation of dissolved sulphides, impressing chimneys of the so-called “black and white smokers” are formed (Fig. 1.1.2). At hot vents or high-temperature vents these discharge fluids can reach temperatures more than 400 °C (Haase et al. 2007). In case of cracks and fissures in surrounding rocks, rising fluids may exit the seafloor over a widespread area. As continuously being cooled during the passage, their discharge temperatures only reach values of 8-40°C, forming the so-called low-temperature vents (Edmond & van Damm 1983; Jannasch 1994, see Fig. 1.1.3).

Hence, the key difference between hot vents and diffuse low-temperature venting is the plume temperature, therewith the amount of mixed seawater, and the extent of the pluming discharge region. In both systems the warm metal-rich water enables microorganisms to utilise this chemical energy, leading to highly diverse microbial consortia that colonise the center as well as the vicinity of the vent sites. Since longer time it has been suggested that the origin of life may have been influenced by such discharge areas (Russel et al. 1997; Nisbet & Sleep, 2001).

Low-temperature influenced venting systems were firstly discovered along Mid-Ocean-Ridges (Edmond et al. 1979), where the seafloor is not completely covered by sediment. These bare rocks are exposed to the ocean and could interact with seawater or could be influenced by thermal discharge processes. Approximately 1,000,000 km^2 of continuously formed basaltic seafloor (Edwards et al. 2005) provide an enormous potential for microbial colonisation. Thus, Mid-Ocean-Ridge systems are one of the most promising regions to investigate the diversity of microbial population within basaltic rocks. Just a few years ago at the occasion of a Meteor expedition (M64/1, 2005) new low-temperature influenced venting systems along the Acsension-Fracture-Zone (9°33'S- 4°48´S, Mid-Atlantic-Ridge) have been successfully discovered such as the Wideawake field, the Nibelungen field and the Lilliput

field. These three vent sites are characterised by spacious, diffuse discharge areas located in a water depth ranging from 3,000- 1,500 mbsl.

As the scientific community is most attracted by the investigation of the impressing high-temperature venting systems manifested in the "black and white smokers" pipes, only few studies deal with the microbial diversity of diffuse, low-temperature influenced venting sites. Actually, the knowledge about basalt-inhabiting microbes in relation to low-temperature influenced venting sites is very sparse.

Several studies addressed the diversity of microorganisms emanating from young, ridge flank ocean crust (Holden et al. 1998; Summit & Barros et al. 2001; Cowen et al. 2003; Huber et al. 2003; Huber et al. 2006). The authors applied cultivation dependent and molecular approaches as well as electron microscopy to reveal unique consortia of thermophiles among a mix of microorganisms generally found in sediments and seawater.

First hints for microbial colonisation of basaltic glass have been detected by scanning electron microscopy in the form of alteration traces along glass fractures (Giovannoni et al. 1996; Furnes et al. 1996; Fisk et al. 1998; Thorseth et al. 2001). Early suggestions for a microbial mediated dissolution of the glass creating this tunnel structures were given by Furnes & Staudigel (1999). According to their findings, 60- 85% of the glass alteration is of microbial origin (for review see: Staudigel et al. 2008).

Yet, very few studies have directly examined the correlation of basalt, basalt associated glass texture and weathered basaltic surface colonising microbial communities. Templeton and co-workers (2005) were the first capable to isolate Mn-oxidizing bacteria from weathered basalt of the Loihi Seamount. Recently Mason et al. (2007) and Santelli et al. (2008) demonstrated that basaltic rocks harbour a variety of distinguishable microbial phyla, displaying a higher diversity then the overlying deep sea water column.

But in fact, detailed information about phylogenetic, physiologic, activity and abundance of microorganisms existing on surfaces and within fractures and pore spaces of the basalt and associated textures is still sparse. Challenging questions remain, such as differences in abundance/ colonisation between basaltic rocks, sediment and oceanic seawater and what effect the microbial activity has on the deep sea rock texture and the adjacent sediment.

1.4 OUTLINE OF THIS STUDY

The exploration of low-temperature vents has garnered significant interest in the past years. In 2004 the DFG Priority Programme „From Mantel to Ocean: Energy, material and life cycles on spreading axes“ (SPP 1144) started for investigating venting sites and the related geochemical and biological processes. As a part of this programme basaltic rocks, glass, weathered basaltic surface, sediment and deep ambient seawater were collected in the vicinity of three diffuse venting areas along the Acsension-Fracture-Zone (Mid-Atlantic-Ridge; M64/1, 2005) in a depth ranging from 3,000 to 1,500 mbsl. The diffuse-flow of the mussel field Wideawake (around 3,000 mbsl) as well as the Lilliput field (around 1,500 mbsl) are characterised by an average temperature of 20°C. The Nibelungen field, located at a precipitous slope in a depth of 2,200 mbsl, is characterised by one hot hydrothermal plume in the south and numerous inactive vents nearby. By TV grab or using the remotely operating vehicle QUEST (MARUM, Bremen) samples of basaltic rocks and sediment were collected (see Fig. 1.2). The glassy, basaltic lava flows are enriched with reduced metal compounds (e.g. Fe, Mn, S). This energy source together with the cavernous structure of basaltic rocks and fractures within the glassy margins provide ideal conditions for a vast, endolithic microbial ecosystem.

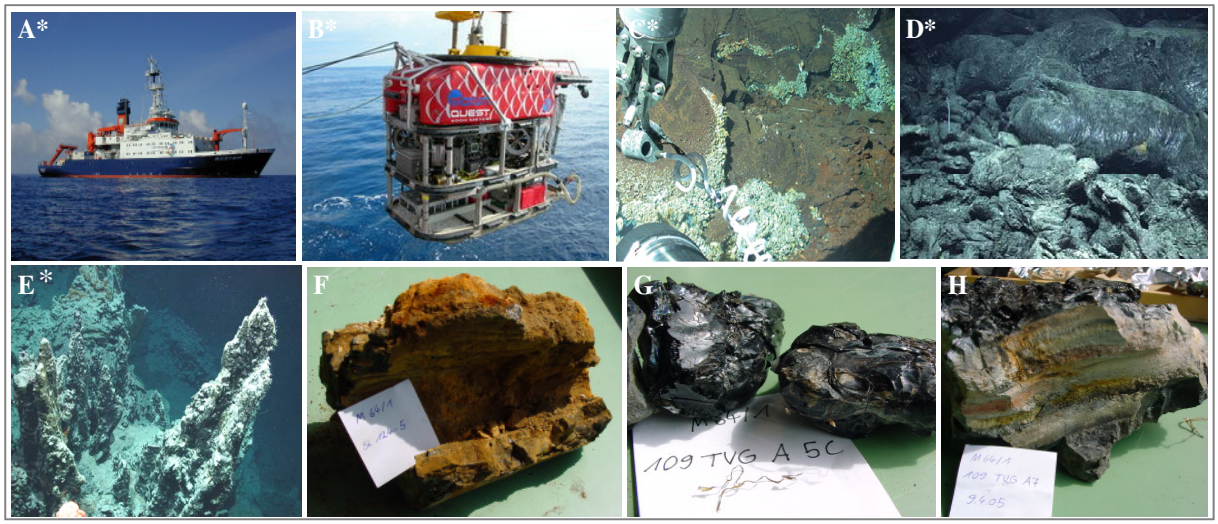

Figure 1.2

Photos illustrating the sample obtaining systems, the local conditions at the sampling sites along the Acsension-Fracture-Zone (Mid-Atlantic-Ridge) and examples of collected and determined rock textures. (A) the research vessel FS Meteor, (B) the remotely operated vehicle (ROV) QUEST (C) Lilliput diffuse venting field (9°33′S): low-temperature discharge emit through cracks of pillow lava, (D) Wideawake diffuse venting field (4°48´S): glassy lava tongues covering older sheet flows, (E) Nibelungen venting field (8°45`S): inactive chimneys, (F) investigated sediment sample, (G) investigated glassy sample (H) investigated basaltic rock with a glassy layer

*photo of MARUM, University of Bremen

The aim of this thesis was to determine the endolithic microbial diversity and abundance of microbial communities inhabiting the vesicles, fractures and veins of these sampled basaltic rocks and sediment samples. A combination of various molecular and microbiological methods was applied to achieve these objectives (Fig. 1.3).

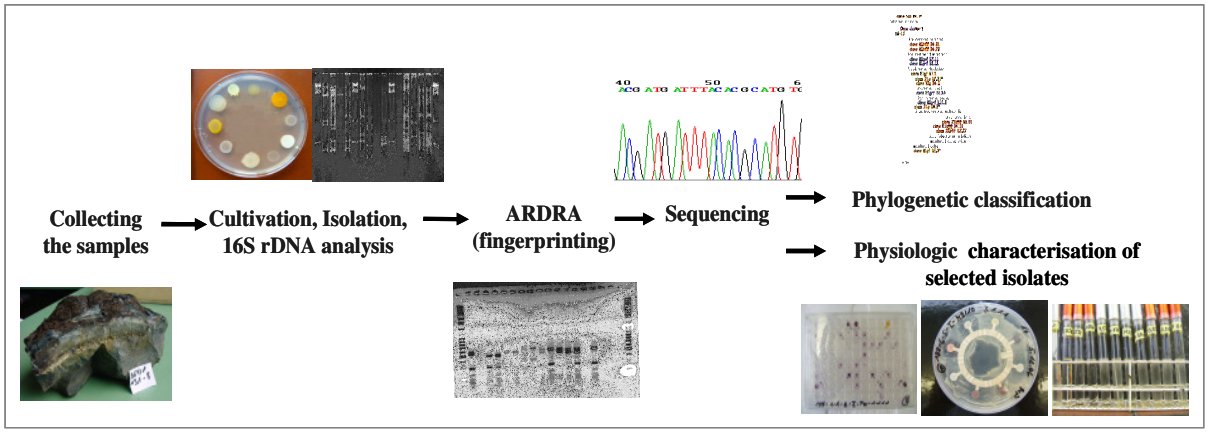

Figure 1.3
Experimental design: starting with collecting samples of basaltic rocks, glass, weathered basaltic surface, sediment and deep ambient seawater along the Acsension-Fracture-Zone during the cruise M64/1 in 2005. Afterwards cultivation of microorganisms, isolation and 16S- rDNA PCR analysis. Followed by fingerprinting analysis (ARDRA, amplified rDNA restriction analysis) for rapid genotypic identification, sequencing for identification, then phylogenetic classification and physiologic characterisation (for details see chapter 3).

Chapter 2 gives an introduction to the deep marine biosphere beginning with the history of it´s exploration and further details of nutrient distribution and types of sediments. The following paragraphs deal with the microbial diversity in sediments and hydrothermal based ecosystems (hot vent, cold seeps, mud volcanoes). The chapter closes with a description of the microbial colonisation in the oceanic crust. In the following chapters (chapter 3 and chapter 4) the investigation of the rock samples obtained from low-temperatures influenced habitat is presented. For the first time pure cultures have been obtained from environmental samples collected in the vicinity of the diffuse venting area of the Acsension-Fracture-Zone (Mid-Atlantic-Ridge). To examine the bacterial diversity, colonising the collected basaltic rocks, glass, sediment samples and deep ambient seawater, nearly full-length sequences were used for the identification of the phylogenetic groups. Additionally, these efforts were combined with growing analysis to investigate physiological characteristics of the prokaryotic community within this habitat. These samples demonstrated the existence of a wide variety of

microbial population in basaltic rocks that are distinguishable from those of the sediment and the overlying deep ambient seawater. A further detailed examination of the polar lipid compositions of selected isolates showed that established phylogenetic relationships of the genera *Aurantimonas* should be rearranged. Furthermore the description of *Fulvimarina* could be emended.

These results of this study underline the need to investigate this unique habitat and to put it in a global context.

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CHAPTER 2

DEEP BIOSPHERE OF THE DEEP SEA/ OCEANIC

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2.1 DEFINITION AND OVERVIEW

Although used in many different ways, the term "biosphere" is principally defined either as zone in which life occurs, thereby overlapping the atmosphere, the hydrosphere, and the lithosphere, or as the entity of living organisms on Planet Earth. Both perceptions commonly focus on the Earth's near-surface environment, with all domains sharing solar energy used in the process of photosynthesis.The deep-sea realm takes a special position in this context, as deep-sea pelagic and the majority of benthic organisms live in the ocean's aphotic zone and inhabit the widespread abyssal plains, respectively. For a long time, their main food source has been considered to be based on particulate organic matter (POM) from the ocean's surface primary production and its sedimentation to abyssal depths (Gage and Tyler, 1991 and references therein, D'Hondt et al., 2002, 2004).With the discovery of "ocean vents" in the late 1970s (Corliss et al., 1979), this general perspective was broadened by the perception of the enormous potential of chemical energy through the reaction of seawater, rock material, and fluids rising from the Earth's interior. According to this concept of energy for life, the term 'surface biosphere' has been opposed to 'subsurface biosphere' (also commonly found in literature as 'deep biosphere'). Following this definition, the deepseafloor with its highly diverse topography from heterotrophic to pure chemotrophic habitats has to be treated as a transition zone between both biospheres. Opposed to the "deep hot biosphere" (Gold, 1992), occurring by definition in oceanic as well as terrestrial subsurface environments, stands the "deep cold biosphere" as defined for permafrost sediments (Vorobyova et al., 1997) and ice cores from the depths of Lake Vostoc (Venter, 2001).

2.2 LIFE IN THE DEEP SEA

Comprising approximately 65% of the Earth's surface, the deep-sea environment is characterized by hyperbaric, aphotic, and low-temperature conditions and highly diverse seascapes. Canyons, seamounts, ridges, fractures, and trenches, but also biogeochemical oases

such as cold seeps, mud volcanoes, carbonate mounds, brine pools, gas hydrates, hot vent systems, and deep-water coral reefs provide ample niches for a highly diverse pelagic and benthic deep-sea community (Tyler, 2003). It was only during the construction of the transoceanic telegraphic communication network that people realized the ocean's topographic alterations and astonishing depths. In 1861, the repair of an overgrown cable from 1,800 m water depth in the Mediterranean finally aroused the scientific community which by then adhered to Edward Forbes' theory on a completely 'azotic' zone below a water depth of 550 m. Though, 11 years had elapsed before the first global, scientific expedition onboard the "Challenger" (1872–1876) finally convinced people that a flourishing life in fact exists in the deep-sea realm. Numerous, further expeditions and a rushing development of technical facilities allowed deep-sea researches in 1960 to reach even the ocean's deepest surveyed point, the Challenger Deep at 10.911 meters below sea level (mbsl), located at the southern end of the Mariana Trench within the western Pacific Ocean (Piccard and Dietz, 1961). Since then, several studies on large-scale patterns and the zoogeographical origins of deep-sea organisms evidenced a high macrobenthic diversity (Gage and Tyler, 1991 and references therein). These organisms display a depthdependent zonation as a result of basin age, deep currents (as barriers or dispersal), topographic boundaries, disturbance processes, and sedimentation in connection with depth-related environmental patterns (for review see Levin et al., 2001; Stuart et al., 2003). Macro- and meiofauna are loosing importance with increasing water and sediment depth, whereas microorganisms like bacteria, archaea, and fungi account for up to 90% of the deep-sea benthic biomass (Pfannkuche, 1992). Sinking particles may carry large numbers of microorganisms from upper zones (10^8–10^{10} cells m^{-2} d^{-1}, inoculating deep marine surface sediments with an autotrophic and heterotrophic microbial community, as demonstrated by results from sediment traps (Turley and Mackie, 1995; Danovaro et al., 2000; Vanucci et al., 2001) or the deepseafloor (Lochte and Turley, 1988).

2.3 Particulate organic matter (POM)

Due to the fact that most deep-sea benthic species are deposit feeders (Sanders and Hessler, 1969), the locally qualitatively and quantitatively, variable import of POM from the ocean's surface waters plays a crucial role for macro-, meio-, and microorganisms living in deep surface sediments (Gooday and Turley, 1990). Mainly consisting of phytoplankton, marine snow, fecal pellets, (dead) zooplankton and molts, this material undergoes different steps of degradation during its passage from the photic, epipelagic (0–200 mbsl), through the mesopelagic (200–1,000 mbsl) to the actual deep-sea zones, in particular the bathypelagic (1,000–4,000 mbsl), the abyssal (4,000–6,000 mbsl), and the hadal zone (6,000–11,000 mbsl). Depending on the residence time in the water column, the bioavailable part of POM finally reaching the deep-seafloor may be small (De La Rocha and Passow, 2007 and references therein). The refractory remainders such as animal skeletons are continuously accumulating at the seafloor and turn into deeply buried sediment over time, thereby representing the largest global reservoir organic carbon (Parkes et al., 2000 and references therein).

2.4 DEEP-SEA SEDIMENT TYPES

Grain size (Gray, 1974) and sediment heterogeneity (Etter and Grassle, 1992) may additionally govern community composition and distribution of macro-, meio-, and microorganisms in deep-sea benthic environments. In relation to their basic sources, deep-sea sediments may be biogenic (POM from pelagic primary production, benthic in-situ production), lithogenous (terrestrial weathering and transport by wind and rivers), hydrogenous (precipitation from seawater or pore water), volcanic, or cosmic (Seibold and Berger, 1996). According to grain size and settling velocity, lithogenous gravel and sandy fractions usually are deposited along the coast, while silt and clay are transported farther offshore through waves and currents, hence dominating the basically biogenous deep-sea

sediments. Regional deviations may be linked to currents, downslope slides, submarine canyon dynamics, or to a release of ice-trapped rock material in polar waters (e.g., Ramseier et al., 2001). Covering almost one-half of the shelves and more than half of the deep ocean bottom, biogenous sediments mainly consist of calcite, aragonite, opal, and calcium phosphate, originating from foraminifera, diatoms, and radiolarians (Hay et al., 1988).

2.5 DEEP BIOSPHERE OF DEEP-SEA SEDIMENTS

Microbiological studies on sediment cores collected during several cruises of the Deep Sea Drilling Project (DSDP), the Ocean Drilling Program (ODP), and the Integrated Ocean Drilling Program (IODP) gave evidence for the presence of complex microbial communities in deeply buried marine sediments down to several hundred meters below seafloor (e.g., Whelan et al., 1986; Parkes et al., 1994; Roussel et al., 2008). Most striking, new insights into subsurface microbiology were gained during the ODP cruise Leg 201 to the equatorial Pacific Ocean and the continental margin of Peru, including sites recognized as most typical for oceanic subsurface environments (D'Hondt et al., 2002). A large fraction of the sub-seafloor bacteria has been proven to be alive and culturable, displaying turnover rates (based on sulfate reduction as dominating mineral process at these sites) comparable to surface sediment communities (D'Hondt et al., 2004; Schippers et al., 2005).

After a logarithmic decline within the uppermost 6 meters below seafloor (mbsf) (Parkes et al., 1994) to about 40 mbsf (Schippers et al., 2005), bacterial cells have proven to be more or less evenly distributed down to several hundred mbsf. Local peaks within these deeply buried sediments seem to mirror sulfate (diffusing from crustal fluids) and methane (from in-situ production) concentration shifts (Engelen et al., 2008). However, published variations of absolute cell numbers (by a factor of up to 3) have to be treated with caution: varying estimations not only depend on the geochemical conditions at the respective sampling sites,

but also on the enumeration techniques applied. Calculations based on early results revealed that sub-seafloor sediments comprise –at least – half of all prokaryotic cells and up to one-third of the living biomass on Earth pointing to a slow-growing strategy of high biomass in areas of low-energy flux (Whitman et al., 1998).

The prokaryotic community in deeply buried sediments can not exclusively be traced back to contaminations from biologically active surface layers or reactivation of spores and dormant cells (Parkes et al., 2000 and references therein).

Porewater chemistry data obtained from sites throughout the world's oceans (ODP, DSDP) showed that sulfate reduction, methanogenesis, and fermentation are the principal degradative metabolic processes in subsurface sediments. These results give evidence for significant lower metabolic rates for the subsurface compared to the surface biosphere and for methanogenesis becoming more important the more sulfate gets depleted with increasing sediment depth (D'Hondt et al., 2002 and references therein).

2.6 WINDOWS TO THE SUBSURFACE BIOSPHERE

2.6.1 Hydrothermal vents

The discovery of the "ocean vents" near Galapagos Island (Corliss et al., 1979) was the first proof for the active movement of the gigantic oceanic plates of the Earth's crust creating series of cracks in the ocean floor, teeming with life. At these discharge areas, hydrothermal fluids with temperatures of more than 400°C (Haase et al., 2007) mix up with the cold ocean seawater, resulting in a precipitation of dissolved metals and in the formation of characteristic chimneys over time. Iron and sulfide precipitates turn the smokers black ("black smokers," Figure 1), while barium, calcium, and silicon minerals result in "white smokers." Thermal precipitation and/or direct magma degassing of H_2, H_2S, CH_4, CO, and CO_2 in combination with oxygen as electron acceptor provide enough energy to support a highly productive and

physiologically diverse chemoautotrophic microbial community (Reysenbach and Shock, 2002).

As the highly diverse and dense hot vent macrofauna (e.g., vestimentiferan tubeworms, bivalve mollusks, provannid gastropods, alvinellid polychaete, and bresiliid shrimps) cannot feed on the released chemicals themselves, they either feed on chemoautotrophic microbes or host them as symbionts. The predominant endosymbionts are mesophilic to moderately thermophilic chemoautotrophs (mostly Gammaproteobacteria), whereas most episymbionts belong to the Epsilonproteobacteria, which can oxidize H2 and sulfur compounds while reducing oxygen, nitrate, and sulfur compounds (for review, see Nakagawa and Takai, 2008). The vent habitat proved to harbor methanogens (Methanococcus), sulfate-reducers (Archaeoglobus), and facultative autotrophs and heterotrophs such as the thermophilic aerobic Thermus and Bacillus (Harmsen et al., 1997) or, for example, Thermococcus, Phyrococcus, Desulfurococcus (Prieur et al., 1995; Teske et al., 2000; Nercessian et al., 2003; Schrenk et al., 2003). Generally detected archaeal phylotypes were affiliated with hyperthermophilicCrenarchaeota, Euryarchaeota Group I, II, III (Takai and Horikoshi, 1999) and the “Deep-sea Hydrothermal Vent Euryarchaeotal Group” (Hoek et al., 2003).

2.6.2 Cold seeps and mud volcanoes

Just a few years after the discovery of the hydrothermal vent systems, cold seep ecosystems were reported from active and passive continental margins and subduction zones all over the world (Aharon, 1994 and references therein).

High-pressure, low oxygen and low-temperature conditions favour the formation of marine gas hydrates. In the subsurface realm, such gas reservoirs are stored in a crystalline form, whereas they get, dissolved in pore waters and finally leave the sediment surface in gaseous form. High fluxes of methane, sulfide, and other reduced elements characterize these

ecosystems such as cold seeps, hydrocarbon vents and mud volcanoes, often leaving mineral precipitation in their immediate surroundings. Coupled to sulfate reduction, rich bacterial and archaeal communities perform anaerobic oxidation of hydrocarbons, but predominately of methane (Boetius et al., 2000; Borowski et al., 2000; Treude et al., 2005). Conversion of methane is mainly mediated by two different groups of anaerobic methanotrophic archaea (ANME-I and ANME-II) (Nauhaus et al., 2005), forming syntrophic consortia with the sulfate-reducing bacteria (SRB) Desulfosarcina and Desulfococcus (Hinrichs et al., 1999; Boetius et al., 2000; Michaelis et al., 2002; Knittel et al., 2003).

The methane-emitting Haakon Mosby Mud Volcano (HMMV, Barents Sea) has shown to harbor three key communities in methane conversion such as aerobic, methanotrophic bacteria (Methylococcales), anaerobic methanotrophic archaea (ANME-2) thriving below siboglinid tubeworms, and a previously undescribed clade of archaea (ANME-3) associated with bacterial mats (Niemann et al., 2006). Similarly, some cold seeps on the deeper Black Sea shelf are characterized by intense methane bubble discharge, mainly related to microbial methanogenesis (Pape et al., 2008 and references therein). Diffuse gas seeps in more shallow, oxic Black Sea waters often exhibit a netlike coverage of microbial mats similar to Beggiatoa-mats observed at HMMV (Figure 2). Beggiatoa spp. are discussed as keystone members of seep communities owing to their ability to (directly and indirectly) influence the metabolic activity of d-Proteobacteria, Planctomycetales, and ANME archaea by providing sulfate and ammonia as reactants (Mills et al., 2004).

The question remains, to which extent such seep systems influence the global methane cycle, as the quantification of bubble dissolution and/or the release of methane- rich pore fluids from the sediment into the hydrosphere is difficult to achieve (Vogt et al., 1999; Reeburgh, 2007). Niemann et al. (2006) estimated that methanotrophy at active marine mud volcanoes

consumes less than 40% of the total methane flux, due to limitations of the relevant electron acceptors in the upward flowing, sulphate- and oxygen-free fluids.

2.7 DEEP BIOSPHERE OF THE OCEANIC CRUST

The fact that microorganisms are present in the subsurface realm had been reported decades ago in terrestrial subsurface environments (Farrell and Turner, 1931; Lipman, 1931). Early drilling operations performed for commercial purposes such as mining, oil and hot water recovery, and the search for underground waste repositories reported on the existence of a large community of microorganisms obviously involved in geochemical processes in the deep biosphere (Gold, 1992; Pedersen, 1993 and references therein). Hence, it was only in the early 1990s that scientists started to focus on the investigation of prospering life beneath the Earth's crust, thanks to a chance encounter of a deep oceanic, volcanic eruption during a dive onboard the submersible Alvin, releasing white microbial bulk mats (Haymon et al., 1993). The upper layers of the oceanic crust are characterized by high basaltic porosity, hosting a vast hydrothermal reservoir (Johnson and Pruis, 2003) inhabited by a microbial community composed of species that are also found in deep-sea waters, sediments, and the deep oceanic crust (Thorseth et al., 2001; Huber et al., 2006). Among the most prominent anaerobic thermophiles indigenous for the oceanic crust, the Ammonifex group of bacteria (Nakagawa et al., 2006) or groups within Crenarchaeota, Euryarchaeota, and Korarchaeota (Ehrhardt et al., 2007). Since 3.5 billion years, basaltassociated glass textures and vesicular cavities within the basaltic matrix provide niches for microbial colonization (Furnes et al., 2004; Peckmann et al., 2008). For instance, the fossil record of the oceanic crust even gives evidence for a previous fungal life in deep ocean basaltic rocks (Schumann et al., 2004).

Much effort has been put into the investigation of the deep biosphere of the deep sea during the past 20 years. However, we still are neither aware of the final composition of the living

subsurface community, nor of its interrelationship to, for example, crustal fluid-derived compounds, nor of its global impact.

2.8 FIGURES

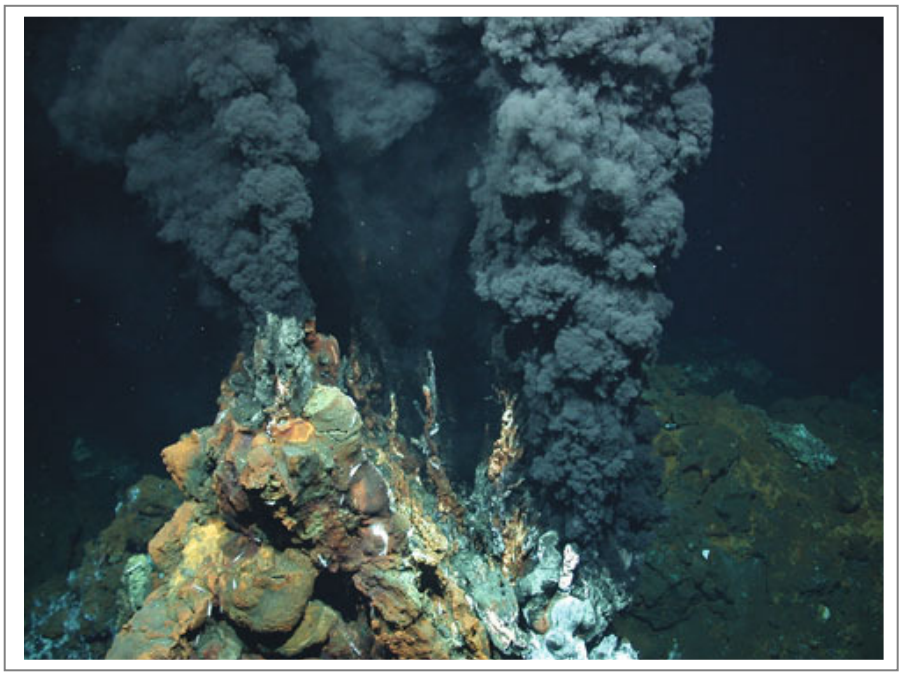

Figure 1
Black smoker "Candelabra," Logachev hydrothermal field, Mid-Atlantic-Ridge (3,000 m water depth). (By courtesy of MARUM, Center for Marine Environmental Sciences, Bremen, Germany.)

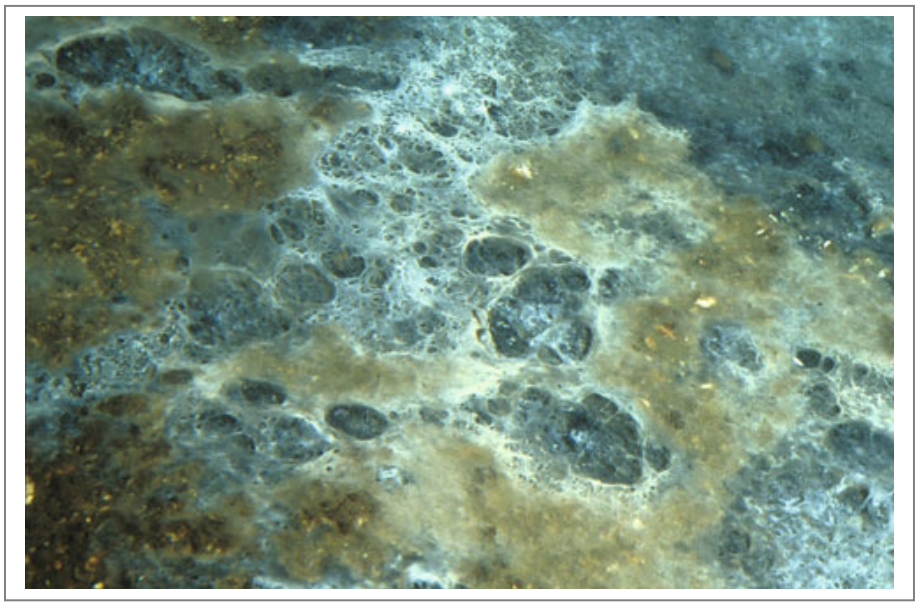

Figure 2

Shallowwater seep area "GHOSTDABS-field," Ukrainian shelf, Black Sea. White nets are constructed of sulfide oxidizing bacteria ("Beggiatoa"). (By courtesy of Karin Hissmann and Jürgen Schauer, Jago Team, Leibniz-Institut für Meereswissenschaften (IFM-GEOMAR) Kiel, Germany.)

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CHAPTER 3

MICROORGANISMS ISOLATED FROM DEEP SEA LOW-TEMPERATURE INFLUENCED OCEANIC CRUST BASALTS AND SEDIMENT SAMPLES COLLECTED ALONG THE MID-ATLANTIC-RIDGE

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3.1 ABSTRACT

Basalt and sediment samples were collected (at 1,460 - 2,996 m of water depth) along the Mid-Atlantic-Ridge. The microbial diversity was analysed by aerobic cultivation on three different media enriched for manganese- and sulphur-oxidising bacteria. Phylogenetic analyses of bacterial isolates revealed affiliations to the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and *Bacilli*. Our data revealed significant differences in the occurrence and diversity of these communities between the respective deep seafloor biosphere sources. Among 138 genotypes, 12 putatively unknown isolates were detected. Characterisation of selected isolates revealed high tolerances against a broad range of temperatures, pH-values and salt concentrations.

Keywords: basalt, deep biosphere, bacteria, diversity, Ascension Fracture Zone, Mid-Atlantic-Ridge (MAR)

3.2 INTRODUCTION

Microorganisms are centrally involved in fluxes of energy and matter in the earth's crust, yet their vertical distribution and functional variability are still poorly known. It is calculated that the largest portion of prokaryotes on earth occur in the deep marine biosphere, due to the volume of the deep subsurface (Whitman et al. 1998; Parkes et al. 2000) and microbial life has hitherto been detected as low as 1,626 m below sea floor (mbsf) (D'Hondt et al. 2004; Lysnes et al. 2004b; Schumann et al. 2004; Schippers et al. 2005; Parkes et al. 2005; Roussel et al. 2008). In the last decade, numerous studies about the microbial activity within the basaltic crust biosphere have been published (e.g., Giovannoni et al. 1996; Torsvik et al. 1998; Thorseth et al. 2001; Edwards et al. 2003), but endemic microorganisms have been described only recently (Mason et al. 2007; Santelli et al. 2008). Beyond doubt, the microbial community displays the core of the complex ecosystem within the deep seafloor and therefore in the hydrothermal influenced environment, as well. Accordingly, the essential question is: who inhabits this environment?

During a cruise in April 2005 (SPP 1144, M64/1), basalt and sediment samples were collected along the low spreading crust of the Mid-Atlantic-Ridge. In the current study, the cultivated aerobe part of the deep seafloor microbial community was determined in the scope of low-temperature influenced vent systems. In contrast to hot hydrothermal-influenced systems, the temperature does not increase above 50 °C at this site. We chose a recently discovered cluster of low-temperature vent systems along the Ascension-Fracture-Zone for our investigation (German et al. 2005; Haase et al. 2005). By using a remotely operated vehicle (ROV QUEST, MARUM, Bremen) and TV-grab, samples of basaltic rocks and sediment were collected from 1,460 to 2,996 mbsl (m below sea level) in the proximity of thermal vent systems, precisely from the diffuse area. Additionally, samples of deep ambient seawater were also taken to determine the microbial background. The phylogenetic diversity of the cultivated

microorganisms from rock and sediment samples was investigated by 16S rRNA-based fingerprinting and subsequent sequencing of the ribosomal 16S rRNA genes. Selected isolates were characterised by different growth parameters like pH, temperature and salinity.

Our goal was to enhance the currently existing information about the occurrence and abundance of microbial life within the deep marine ecosystem of low-temperature influenced venting systems along the Mid-Atlantic-Ridge. This was achieved by evaluating the cultivable part of the microbial diversity within this unique habitat and comparing the data with the findings of previous studies.

3.3 MATERIALS AND METHODS

3.3.1 Sample site and sample collection

All samples were collected during the HYDROMAR II cruise with the research vessel Meteor (M64/1) in April 2005. The sampling site, the Ascension-Fracture-Zone (7-12°S, 13°W) is situated south of the Equator roughly between South America and West Africa along the slow spreading ridge of the Mid-Atlantic (Fig. 1). Samples were collected at the seafloor with a TV-grab and the remotely operated vehicle (ROV QUEST, MARUM, Bremen) in the scope of fluid emission vent systems of three sampling sites: (i) Wideawake low-temperature field (4°48´S), (ii) peripheral area of the high-temperature Nibelungen field (8°45`S) and (iii) Lilliput low-temperature field (9°33`S). The mussel field Wideawake is situated at a water depth of 3,000 mbsl (m below sea level) and characterised by a low-temperature diffuse-flow with an average temperature of 19 °C. The diffuse hydrothermal activity is abundant in this area, but extinct hydrothermal mounds suggest more intense hydrothermalism in the past (Haase et al. 2005; Koschinsky et al. 2006). The Nibelungen field is located on a precipitous slope at a depth of 2,900 mbsl. It is characterised by one hot hydrothermal plume in the south, called ‘Dragon Throat’ (8°17.9’S), and numerous inactive vents nearby where the

investigated rock samples derive from (8°48.98S and 8°47.46S). The Lilliput field forms an 11 km thick crust in a shallower water depth of around 1,500 mbsl and is characterised by the absence of high-temperature venting systems (Perner et al. 2007b). At this site, as well as at the Wideawake field, hydrothermal activity was formed by several low-temperature vents with an average temperature of 20 °C. Sampling was done either by TV grab or using the remotely operated vehicle QUEST.

Furthermore, deep ambient seawater was taken using Niskin bottles (Gibbs & Konwar, 1983) to determine the microbial background outside the collected rock samples and sediments. The samples taken from 1,460-2,996 mbsl were immediately processed on board and handled aseptically. Briefly, the surface of the collected rock samples was treated with ethanol, split with a hammer and chisel and the interior fractions were ground in a sterilised mortar. The ground basalt and sediment were suspended in sterile seawater, intensively shaken and after sedimentation, the supernatant was used for cultivation. All tools for crushing were autoclaved and disinfected by ethanol before use. Basaltic and sediment samples, as well as 50 ml seawater samples, were directly frozen on board at -20 °C for later onshore cultivation in the laboratory.

3.3.2 Cultivation, 16S rDNA gene amplification, fingerprinting, sequencing and growth parameters

Five different culture media were used, from 2005 on, to enrich microbial populations gained from basalt, sediment and deep ambient samples. To address different trophic levels, ZoBell´s Medium (Zobell, 1946) was used as described and in a nutrient reduced form (1:10, 1:100). To enrich for manganese oxidisers, PYGV medium (Staley et al. 1968) was used, while SOX medium was used for sulphur-oxidising bacteria (Teske et al. 2000). All enrichment cultures were incubated at 20 °C on agar plates under aerobic conditions. Anaerobic growth was tested

by sparging inoculated stab tubes with nitrogen. However, the medium contained no resazurin (oxidation reduction indicator) for displaying colour change. The only indicator for anaerobic growth was the observation of colonies.

Single colony DNA was extracted from pure cultures (yielded by several inoculation steps). PCR was performed using the universal bacterial primers 616V (5'-AGA GTT TGA TYM TGG CTC AG-3´) (*Escherichia coli* 16S rDNA positions 8 to 27) (Juretschko et al. 1998) and 1492R (5`-CGG YTA CCT TGT TAC GAC-3´) (modified after Weisburg et al. 1991) or 1525R (5´-AGA AAG GAG GTG ATC CAG -3´) (Lane et al. 1991). Primers, $MgCl_2$ and dNTPs were used as recommended by the manufacturers. The PCR program started with an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. A final extension at 72 °C for 5 min was also included. A negative control was always used to exclude contamination. Amplification products were confirmed by agarose gel electrophoresis (1%) stained with ethidium bromide (5%). For genotype screening, PCR products were investigated by ARDRA (amplified rDNA restriction analysis) (Promega, Mannheim, Germany). Purified PCR product (1 µg, Mi-PCR-Purification kit, Martinsried, Germany) was separately digested with 10 U of the restriction endonuclease *Msp* I (Promega) in buffer and 0.1 mg/ml BSA and made up to 20 µl with the amplicons. The digestion mixture was incubated at 37 ^{o}C for 6 h in order to ensure complete digestion of the amplicons. The reaction was stopped by heating the mixture at 65 ^{o}C for 20 min. Restricted DNAs were analysed by 1.8% agarose gel electrophoresis [wt:v] with TAE buffer containing 0.5 µg/ml of ethidium bromide and restriction patterns were compared visually (Sambrook & Russell, 2001). Isolates which displayed different restriction patterns were used for subsequent phylogenetic analysis.

Additionally, selected strains were characterised by growth parameters like pH, salinity and temperature. To determine the growth under different temperatures and pH-values, strains

were inoculated in liquid ZoBell´s Medium and cultured for a period of 14 days with temperatures ranging from 3 °C to 50 °C. The pH (from 3-13) was adjusted with HCl (10%) and NaOH (1M). Salinity gradients beneath 33‰ were established by diluting ZoBell´s-Medium and the different pH-values were adjusted with NaOH and HCl. For obtaining salinity concentrations above 33‰, NaCl was added.

Sequencing was performed on an ABI 3100 automated sequencer with the Big Dye Terminator chemistry for direct sequencing (Applied Biosystems, Darmstadt, Germany) with the 16S rDNA-specific primers 50F, 614V, 616V, 610R, 699R, 1392R, 1492R and 1525R. Sequences were subjected to a BLAST search (Altschul et al. 1997) to approach phylogenetic affiliation. Strains showing similarities lower than 99-100% were sequenced to complete 16S rDNA sequences. Phylogenetic trees were constructed using the ARB software package (http://www.arb-home.de) (Ludwig et al. 2004). Sequences were aligned using the automatic alignment tool implemented in the ARB program package and corrected manually for alignment errors. The new sequences were initially analysed against the phylogenetic tree containing all sequences in the updated ARB database (ssu_jan04_corr_opt.arb) using the maximum parsimony tool. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single positions by the method of Jukes and Cantor (1969). Phylogenetic trees were reconstructed by the neighbour-joining method of Saitou and Nei (1987). Partial sequences were added subsequently to the respective trees without changing their topology by use of the ARB parsimony interactive method. A selection of 138 nearly full-length 16S rRNA sequences representing all bacterial phyla was used as the out group in the tree calculation. Taxonomic nomenclature was used according to Bergey's Manual of Systematic Bacteriology (Garrity et al. 2001).

Nucleotide sequence accession numbers

The nucleotide sequence accession numbers determined in this study have been deposited in the GenBank database under accession numbers EU935222 to EU935325.

3.4 RESULTS

3.4.1 Fluid chemistry

The emission analysis of the Wideawake field revealed an average temperature of 19.15 °C and a pH of 7.58. For the Lilliput vent system an average temperature of 20.6 °C and a pH 6.5 was determined. No fluid data are available for the Nibelungen field (Tab. 1). As compared to the ambient seawater temperature, rock and sediment sampling sites were increased while the pH was lowered. Furthermore, both sampling sites displayed high values of iron, silicon and manganese, whereas the chloride concentrations were lower compared to the seawater samples.

3.4.2 Cultivation and 16S rDNA amplification

Overall, 1,011 pure cultures representing 138 different genotypes (ARDRA (amplified rDNA restriction analysis) data not shown), were obtained from basalt, glass, WBS (weathered basaltic surface), sediment and deep ambient seawater (for comparison) (Tab. 2). We obtained 798 CFUs from basalt, glass, WBS and sediment, from which 387 pure cultures were isolated. 138 different genotypes were identified by ARDRA and the 16S rDNA gene was sequences nearly full-length. The highest number of genotypes was obtained from the nutrient reduced ZoBell´s-Medium (1:100).

3.4.3 Phylogenetic affiliation of isolates

Percent affiliation of genotypes to the revealed 20 genera is shown in Table 3. The predominant gammaproteobacterial phylotypes could be affiliated to cultured representatives of the genera *Idiomarina*, *Pseudidiomarina*, *Alteromonas*, *Halomonas*, *Marinobacter*, *Acinetobacter* and *Pseudomonas* (Fig. 2a). The highest diversity was observed within glass textures. Moreover, four hitherto undescribed strains revealed from glass samples of the Nibelungen field grouped within the family *Idiomarinaceae*. Strain N159G.615 and strain N159G.82 shared a similarity of 98.6% and clustered next to *Idiomarina salinarum* (96.14%-96.52%). Strain N159G.106 and strain N159G.618 displayed a close relationship amongst each other (97.7%). No other close representative was detected (92.4% and 94.4% difference to the next related *Pseudidiomarina taiwanensis*). There was a close relationship between strain N159G.62 and some uncultured clones obtained from ocean seafloor basalt (Lysnes et al. 2004b).

Phylogenetic analysis also indicated a high diversity of genotypes affiliated to alphaproteobacterial genera: *Fulvimarina*, *Nitratireductor*, *Martelella*, *Sulfitobacter*, *Oceaniciola* and *Erythrobacter* (Fig. 2b). Whereas the majority of strains obtained from deep ambient seawater samples were related to *Erythrobacter*, i.e., *Erythrobacter flavus* and *Erythrobacter citreus*, three isolates (daSW.67, daSW.82, daSW.85) grouping with *Sulfitobacter* were highly similar to recently identified isolates from submarine basalt (Templeton at al. 2005) (99.9%- 98.8%). Another five strains isolated from basaltic textures showed less than 97.9% similarity to described species (strains L200B.634, N159B.622, L200B.91, 200B.336, L200B.637).

Of the 20 gram-positive strains, 2 were phylogenetically affiliated to the class *Actinobacteria* (Fig. 2c). Strain N159G.617, which was obtained from a glass sample of the Nibelungen field,

was most closely related to *Microbacterium schleiferi* (99.3%), while strain L200B.327 showed a similarity of 98.6% to *Dietzia natronolimnaea*.

Genotypes affiliated to Firmicutes fall into four genera: *Staphylococcus*, *Planococcus*, *Geobacillus* and *Bacillus* (Fig. 2c). All of them were absent in sediment and deep ambient seawater samples, with the exception of three strains assigned to *Staphylococcus succinus* that were recovered from deep ambient seawater. All other strains were isolated from basalt and the basalt associated textures glass and WBS. Strain N155G.103, isolated from glass sample of the Nibelungen field, was related to *Planococcus maitriensis* (93.2%). The majority of isolates were closely related (99.9%- 98.2% similarity) to species of the genus *Bacillus* (Fig. 2c). One strain from a basalt sample of the Nibelungen field was only distantly related (91.1%) to *Bacillus niabensis*.

Some strains were cultivated exclusively from a single habitat, which strongly indicated that no cross-contamination took place. Examples include *Alteromonads* from sediment samples, *Martellela, Fulvimarina*, *Nitratireductor*, *Geobacillus* and *Dietzia* from basalt, members of *Idiomarina, Pseudidiomarina*, *Marinobacter*, *Planococcus* and *Microbacterium* from glass and *Oceanicola* as well as *Staphylococcus* from deep ambient seawater.

3.4.4 Determination of growth parameters

An advantage of cultivation is that in addition to the phylogenetic position, physiological growth parameters can also be determined. A random selection of strains (35 isolates), which covered 34.6% of all isolates of the three samplings sites, were chosen for the determination of their growth at various pH-values (3-13), salinities (1‰-200‰) and temperatures (3 °-60 °C) (Table 1). As little is known about basalt-inhabiting bacteria and their *in situ* conditions, more isolates of basalt samples (15 strains) than of glass (6 strains), WBS (3 strains) and sediment (8 strains) were selected. All but three strains displayed similar growth

characteristics, independent of their respective sampling source. The exceptions originated from the stations 132-Basalt and 200-Basalt, showing growth at 50 °C, and from station 194-WBS, growing at 4 °C. Strains from station 155-Glass displayed a restricted range of salt concentrations (1‰-33‰).

Although all isolates were cultivated under aerobic conditions, apparent growth under anaerobic conditions indicates that the isolates were capable of growing as facultative anaerobes. Further studies are in progress to verify this.

3.5 DISCUSSION

In this study, the culturable microbial diversity of aerobes was studied in the vicinity of vent systems of three sites along the Mid-Atlantic-Ridge. The venting systems Wideawake field and Lilliput field were characterised by diffuse low-temperature fluid emissions with average temperatures of about 20 °C, while the north of the Nibelungen field contained numerous inactive black smoker sites. The cultivation approach revealed significant differences in composition and occurrence of isolates between the particular vent systems, as well as between the different sample sources (basalt, glass, WBS (weathered basaltic surface), sediment and deep ambient seawater). The majority of isolates were obtained from the Nibelungen and Lilliput field. Only a small number of isolates could be grown from the diffuse venting region of the Wideawake field. Overall, the isolates clustered into 138 different genotypes, affiliated to 20 genera on the basis of 16S rRNA gene sequence analysis. The highest diversities in the microbial communities were observed within glass (10 genera) and basalt (11 genera) samples, whereas a significant reduction was observed within WBS (2 genera), as compared to basalt and glass. Also, fewer genera were obtained within sediment (6 genera) and deep ambient seawater (7 genera) samples, compared to glass and basalt. Likewise, Santelli and colleagues (2008) detected an enhanced microbial richness within deep

sea basalts, versus other investigated habitats. Results of further studies demonstrated that surface basalt harbour a diverse microbial community (Bach & Edwards, 2003; Templeton et al. 2005; Mason et al. 2007; Staudigel et al. 2008). Assumingly, part of the alteration in the basaltic rock is most likely mediated by microorganisms accessing metabolic energy through chemical exchange with oceanic seawater. However, further studies are needed to provide detailed insight of their special functional roles regarding geochemical processes.

None of the isolates assigned to *Bacillus* originated from sediment samples or deep ambient seawater; instead, they were exclusively cultivated from basalt, glass and WBS. Half of these isolates grew on a medium specific for manganese oxidisers (PYGV). Francis and Tebo (2002) demonstrated that members of the genus *Bacillus* were able to oxidise manganese at different temperatures and salt concentrations. Templeton and colleagues (2005) showed that Mn-oxidising bacteria occur in cold, deep-sea basalt. Dick et al. (2006) investigated high- and low-temperature influenced sites in the deep sea and found that the most abundant organisms involved in manganese oxidation could be affiliated to *Bacillus*. Therefore they postulate that these bacteria play an important role in oxidising the manganese of the deep ocean crust. This might indicate that strains isolated on PYGV in this study are capable of oxidising the manganese of the basalt and thus take part in the weathering of the basalt. Further analyses are needed to verify this first observation.

Previous studies demonstrated that the marine environment is dominated by proteobacterial sequences, in which the *Gammaproteobacteria* and *Alphaproteobacteria* are the dominant bacterial classes (Gray and Herwig, 1996; Bowman et al. 2000; Thorseth et al. 2001; Lysnes et al. 2004 a,b; Templeton et al. 2005; Webster et al. 2006; Lauro and Bartlett, 2006; Zaballos et al. 2006; Mason et al. 2007). Our data are in accord with those of Templeton and colleagues (2005) who also applied cultivation methods. Likewise they affiliated isolates to the classes *Gammaproteobacteria* and *Alphaproteobacteria* including the genera

Sulfitobacter, *Alteromonas*, *Marinobacter, Pseudomonas, Acinetobacter* and *Halomonas*. Also, Lysnes and colleagues (2004a,b) reported the presence of cultured representatives of the five classes found in this study within seafloor basalt. Besides members of *Pseudomonas, Alteromonas*, *Acinetobacter* and *Marinobacter,* several additional genera were identified. Lysnes et al. also recovered members of *Chloroflexi* and *Epsilonproteobacteria*. Comparison with previous data based on culture-independent techniques (DGGE) (Thorseth et al. 2001; Lysnes et al. 2004 a,b; Mason et al. 2007), revealed more differences versus findings of the present study. *Gammaproteobacteria* were dominant, but numerous additional phylotaxa (e.g., *Chloroflexi*, *Acidobacteria, Deltaproteobacteria*, *Green nonsulfur bacteria*, *Cytophaga/ Flavobacterium/ Bacteroides* group and *Chlamydiae/ Verrucomicrobia* group) were also detected. These studies demonstrate that the affiliation of isolates or phylotypes from basaltic samples depends upon the applied methods and confirm that the enriched microorganisms comprise only a restricted part of the total in situ microbial community (Templeton et al. 2005; Lysnes et al. 2004 a,b).

Notably, within the present study, representatives of some genera (e.g., *Alteromonas*, *Idiomarina, Pseudidiomarina*, *Nitratireductor)* were detected exclusively in certain samples. Organisms assigned to the genus *Alteromonas* were only isolated from sediment samples of the Lilliput field. The microbial community of the fluid from this venting system was determined by Perner and co-workers (Perner et al. 2007b), who found 6.5% of their clones to be affiliated with *Gammaproteobacteria*. In contrast to the affiliation of the cultured isolates from this site, the clones showed a close relationship to the genera *Oceanobacter*, *Pseudoalteromonas* and *Thiomicrospira*. Obviously, the microbial communities derived from fluid and sediment differed completely as the composition of isolates and phylotaxa did not overlap.

Interestingly, most of the isolates from sediment and deep ambient seawater samples were affiliated to *Halomonas* and *Erythrobacter*. Several previous investigations have demonstrated that members of *Halomonas* are commonly distributed in deep low-temperature vent systems (Brettar et al. 2001; Radjasa et al. 2001; Kaye et al. 2004; Kaye and Baross, 2004). Kaye and Baross (2004) showed that representatives of *Halomonas* are perfectly adapted to the conditions of deep low-temperature influenced vent systems. Thus, the clear predominance of *Halomonas* in marine seawater and its success may be attributed to the physiological versatility of its members. Likewise, the spatial distribution of *Erythrobacter* from the upper ocean to the lower depths, confirmed by numerous studies with culture dependent and independent approaches (Kolber et al. 2001; Denner et al. 2002; Huber et al. 2003; Koblížek et al. 2003; Du et al,. 2006), could be supported by this study.

Although numerous basalt-associated sequences were analysed by Mason and co-workers (2007), no organism could be assigned to *Idiomarinaceae*. In a study by Santelli et al. (2009), only one basalt-associated uncultured organism could be assigned to one representative of the *Idiomarinaceae*, originating from submarine hydrothermal fluids (Donachie et al. 2003). The other members of the family were isolated from shallow coastal water and oceanic waters, coastal sediments and solar salterns as well as inland hypersaline wetlands. However, no described strain was isolated from deep marine basaltic rock sources. Apparently, members of this family do not constitute a significant part of the microbial population in the deep marine habitat, but could probably be considered particularly as inhabitants of the marine environment. In the present study, four isolates were assigned to this family. The closest known relatives of the isolates, *Idiomarina salinarum* (Yoon et al. 2007) and *Pseudidiomarina taiwanensis* (Jean et al. 2006), were both described from marine seawater sources. Our isolates, however, originated from glass samples, not from seawater. As the sequences show only 96.8%- 97.7% similarity to the nearest type strains, the four isolates

constitute putative novel taxa, the rank of which need to be decided by thorough taxonomic evaluation (Stackebrandt & Goebel, 1994). The same is true for other strains that are not affiliated with known taxa (e.g., strain L200B.336, L200B.637, 200B.91). Mason et al. (2007) analysed ocean crust samples (sediment, basalt and gabbro) and postulated ocean crust clades (OCCI- OCCVII) of bacteria consisting of monophyletic clades. Two of our *Sulfitobacter* isolates (obtained from sediment samples) had close relationships with members of the OCCI clade, which consisted of uncultured Mn(II)-oxidising organisms (KBB-2 and SPB: 1–4) obtained from basalts and sediments (Templeton et al. 2005; Zeng et al. 2005). These two isolates, which were also retrieved from ocean crust, would properly extend the OCCI. However, three deep ambient seawater isolates assigned to *Sulfitobacter* are also closely related to sequences of the OCCI clade (99.4%-99.9% similarity). As the postulated ocean crust clades did not include a seawater source, the definition of the OCCI clade should be expanded to include organisms from other habitats.

In the last decades, it has become clear that the diversity of microbial life is vast within the deep marine biosphere in general and within temperature influenced venting systems. This study confirms the results of other studies (e.g., Reysenbach et al. 2000; Huber et al. 2002; Mason et al. 2007; Perner et al. 2007a,b) and increases the knowledge about the abundance and distribution of microbial diversity within the slow spreading area along the Mid-Atlantic-Ridge. The cultivation approach of the current study revealed the predominance of *Proteobacteria* within the investigated samples. Furthermore, particular genera occurred only in a source from one defined sampling site. For instance, the putatively new species assigned to *Idiomarinaceae* were exclusively found in glass samples of the Nibelungen field and putatively new species within the *Alphaproteobacteria* (i.e., *Martelella*, *Fulvimarina* and *Nitratireductor*) were solely isolated from basaltic samples. Additionally, a considerable number of assumedly new, undescribed species could be observed within the basalt and

basalt-associated textures. Thus, it became apparent that differences exist in the phylogenetic distribution of microorganisms between the different sampling sources as well as in comparison with findings of culture-independent approaches. Furthermore, it emerged that the postulated ocean crust clade I (OCCI) should be reconsidered.

However, further studies are needed to examine and characterise newly isolated microorganisms, with the purpose of getting more detailed insight into the living conditions along these moderate influenced vent systems. In the respect of a comprehensive overview about prevalent microbial communities, we believe that archea and fungi should be considered in additional investigations.

3.6 ACKNOWLEDGEMENTS

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3.7 TABLES

Table 3.7.1.

Sampling site characteristics of the fluid from the investigated vent systems with the growth characteristics of 35 isolates of different sampling stations and sources.

Sampling site	Wideawake field	Nibelungen field		Lilliput field		Deep ambient seawater
T [°C]	19.1	n.d.		20.6		2
pH	7.6	n.d.		6.5		7.95
Mn [µg/l]	1.7	n.d.		0.6		0.2
Fe [mg/l]	0.12	n.d.		0.432		0.0002
Cl [mg/l]	16.4	n.d.		19.6		20.5
Si [mg/l]	2.8	n.d.		7.3		1.0
Station Parameter	132-Basalt	155-Glass	159-Glass	188-Sediment	194-WBS	200-Basalt
Depth [mbsl]	2,996	2,161	2,219	1,866	1,460	1,495
pH	5-10	5-10	5-11	5-12	5-9	5-11
Salt con. [‰]	1-150	1-33	1-150	1-200	1-100	1-100
T [°C]	8-50	8-40	8-40	8-40	4-40	8-50

Table 3.7.2.

CFUs, pure cultures, genotypes and sequences obtained from basaltic, sediment and deep seawater samples. Sequences that resulted in a similarity of less than 99% were sequenced to determine the complete 16S rDNA sequences.

Sources of samples	CFUs	Pure cultures	Genotypes[1]	16S rDNA sequences	
				Partial	Complete
Basalt	235	108	38	1	37
Glass	130	54	24	2	22
WBS (weathered basaltic surface)	157	40	6	1	5
Sediment	276	185	33	5	28
Deep ambient seawater	846	624	37	25	12

1, based on ARDRA analyses

Table 3.7.3.

Phylogenetic distribution of genotypes in correlation to the appropriate sample source

Phylogenetic class	Genus	Basalt	Glass	WBS	Sediment	Deep ambient seawater
Gammaproteobacteria	Idiomarina	-	2	-	-	-
	Pseudidiomarina	-	2	-	-	-
	Alteromonas	-	-	-	11	-
	Halomonas	1	7	-	13	14
	Acinetobacter	5	3	-	1	1
	Marinobacter	-	1	-	-	-
	Pseudomonas	9	2	1	5	-
Percentage of total		40%	68%	17%	91%	41%
Betaproteobacteria	Ralstonia	4	4	-	1	2
Percentage of total		11%	16%	-	3%	5%
Alphaproteobacteria	Martelella	1	-	-	-	-
	Fulvimarina	1	-	-	-	-
	Nitratireductor	3	-	-	-	-
	Oceanicola	-	-	-	-	2
	Sulfitobacter	-	-	-	2	3
	Erythrobacter	5	-	-	-	12
Percentage of total		27%	-	-	6%	46%
Actinobacter	Dietzia	1	-	-	-	-
	Microbacterium	-	1	-	-	-
Percentage of total		3%	4%	-	-	-
Bacilli	Staphylococcus	-	-	-	-	3
	Planococcus	-	1	-	-	-
	Geobacillus	1	-	-	-	-
	Bacillus	6	2	5	-	-
Percentage of total		19%	12%	83%	-	8%

3.8 FIGURES

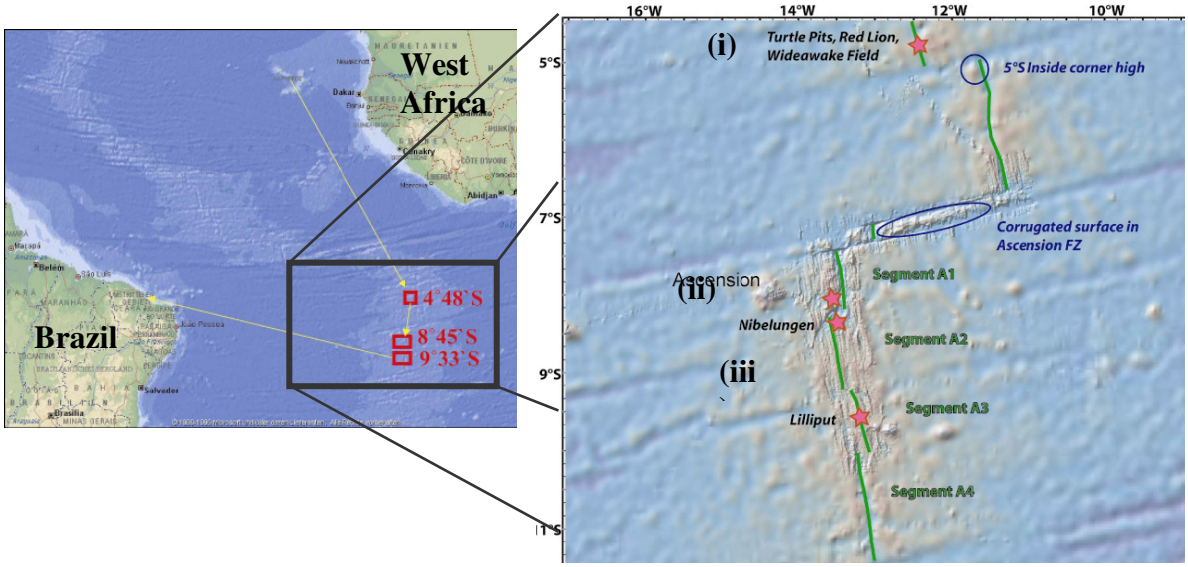

Figure 3.8.1

Sampling sites of the HYDROMAR II (M64/1)

The left picture shows an overview of the sampling sites and the close-up shows the sites in more detail: (i) Wideawake field (4°48´S), the area of the (ii) Nibelungen field (8°45`S) in segment A2 and the (iii) Lilliput field in segment A3 (9°33`S).

(*http://www.ifm.uni-hamburg.de/~wwwls/M64/M64-1-SCR.pdf*)

Figure 3.8.2a Phylogenetic tree of 16S rRNA gene sequences from cultivated *Gammaproteobacteria* of basalt, glass, WBS, sediment and deep ambient seawater and their closest relatives from the GenBank database. B, basaltic origin; S, sediment; G, glass; W, weathered basaltic surface and daSW, deep ambient seawater. Putative new species are marked with an asterisk. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Figure 3.8.2b Phylogenetic tree of 16S rRNA gene sequences from cultivated *Betaproteobacteria* and *Alphaproteobacteria* of basalt, glass, WBS, sediment and deep ambient seawater and their closest relatives from the GenBank database. B, basaltic origin; S, sediment; G, glass; W, weathered basaltic surface and daSW, deep ambient seawater. Putative new species are marked with an asterisk. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

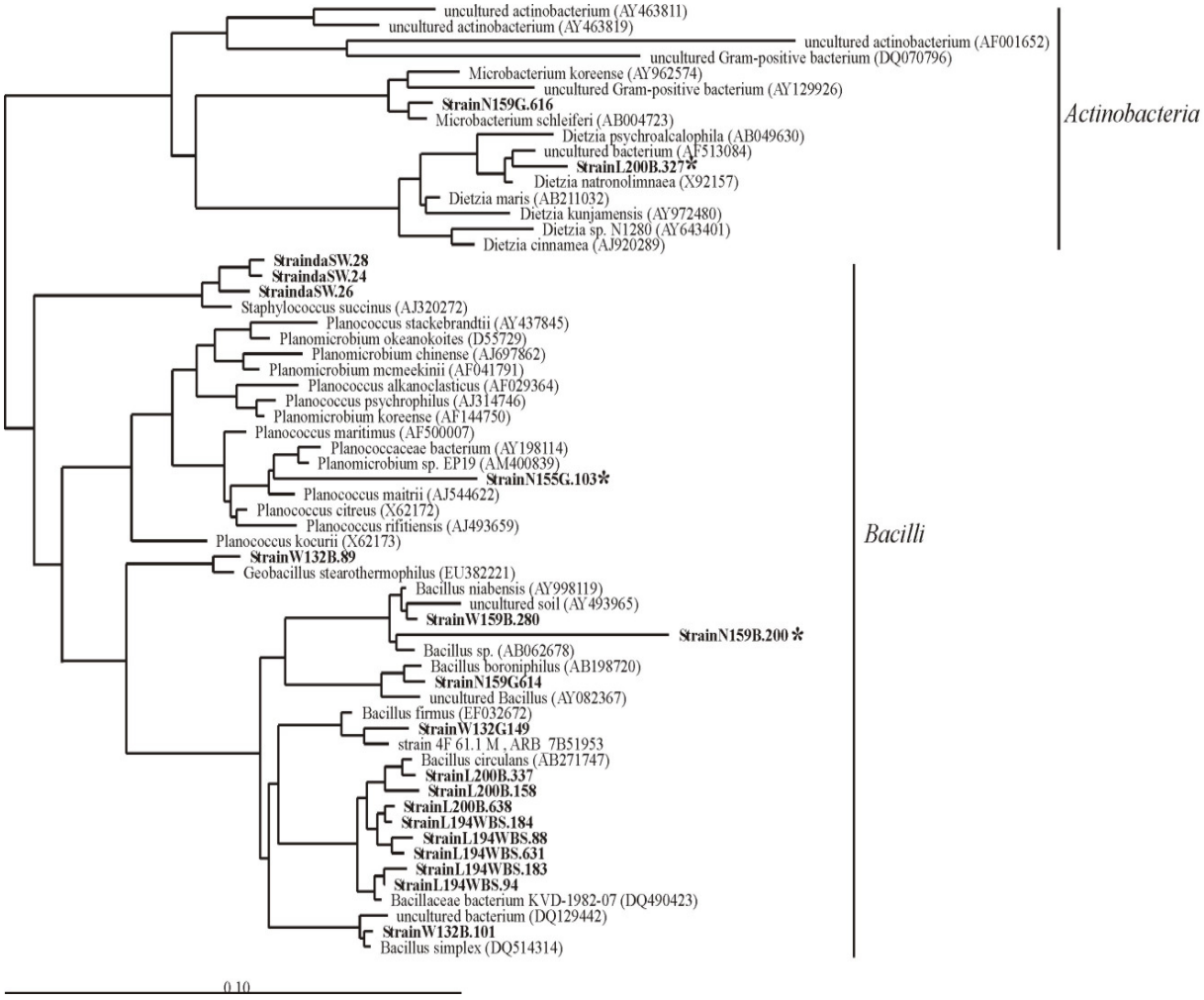

Figure 3.8.2c

Phylogenetic tree of 16S rRNA gene sequences from cultivated Actinobacteria and Bacilli of basalt, glass, WBS, sediment and deep ambient seawater and the type strains their most closely related relatives from the GenBank database. B, basaltic origin; S, sediment; G, glass; W, weathered basaltic surface and daSW, deep ambient seawater. Putative new species are marked with an asterisk. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

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CHAPTER 4

Reclassification of *Aurantimonas altamirensis* (Jurado *et al.* 2006), *Aurantimonas ureilytica* (Weon *et al.* 2007) and *Aurantimonas frigidaquae* (Kim *et al.* 2008) as members of the genus *Aureimonas* gen. nov., and emended descriptions of the genera *Aurantimonas* and *Fulvimarina*

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4.1 ABSTRACT

Members of the genera *Aurantimonas* and *Fluvimonas* have been largely described on the basis of 16S rRNA sequence, biochemical tests and limited chemotaxomic data. Examination of the polar lipid compositions of members of these two genera indicate that although the patterns were similar with regards the phospholipid and amino lipid compositions, there were clear differences in the presence/absence of the glycolipid sulfoquinovosyldiacylglycerol (SQDG). This glycolipid was absent in *Aurantimonas coralicida* and *Fluivimarina pelagi*, but present in *Aurantimonas altamirensis*, *Aurantimonas ureilytica* and *Aurantimonas frigidaquae.* This is also consistent with the 16S rRNA sequence based grouping. We therefore propose that the genus *Aurantimonas* be further divided to reflect this additional information and provide emended descriptions of the genera *Aurantimonas* and *Fulvimarina.*

4.2 INTRODUCTION

The genus *Aurantimonas* has been assigned to the *Alphaproteobacteria* within the order *Rhizobiales* (Kuykendall et al. 2005). The genus was originally described by Denner et al. (2003) with *Aurantimonas coralicida* as the type and only species that was isolated from the marine environment. At present the genus *Aurantimonas* contains four species. Although the species that were described subsequently were from terrestrial, aquatic or aerial environments such as a subterranean cave (*Aurantimonas altamirensis*, Jurado et al. 2006), air (*Aurantimonas ureilytica*, Weon et al. 2007) and a water-cooling system (*Aurantimonas frigidaquae*, Kim et al. 2008) no attempt has been made to emend the genus description to take these different habitats into account. As currently defined members of the genera *Fulvimonas* and *Aurantimonas* form a monophyletic group based on the evaluation of the 16S rRNA sequences, which can be further divided into two monophyletic groups, the genera *Fulvimonas* and *Aurantimonas.* It should be also noted that representatives of the genus *Aurantimonas* isolated from terrestrial, aquatic or aerial environments sources form a subgroup distinct from those strains from marine sources, based on the 16S rRNA sequence data. A comprehensive study of the polar lipid composition of all type strains of species with validly published names was undertaken in order to elucidate the taxonomic infrastructure within this group of prokaryotes.

4.3 METHODS

Aurantimonas coralicida (DSM 14790), *Aurantimonas ureilytica* (DSM 18598), *Aurantimonas altamirensis* (DSM 21988), *Aurantimonas frigidaquae* (DSM 21987) and *Fulvimarina pelagi* (DSM 15513) were obtained from the DSMZ (Braunschweig). All strains were grown on Bacto marine agar 2216 or Bacto marine broth (Difco). Agar plates or liquid cultures were incubated at 28°C.

Phenotypic characterisation of the following physiological and biochemical properties were performed using methods described in Tindall et al. (2007); cell morphology, Gram-staining (KOH) (method 15.2.34.1), L-alanine-aminopeptidase (method 15.2.34.2), oxidase (tetramethyl-p-phenyldiamine dihydrochloride test) (method 15.2.63), catalase (H_2O_2 test) (method 15.2.16.2), phenylalanine (method 15.2.68) and hydrolysis of tyrosine (Gordon et al. 1973), casein (method 15.2.15), gelatine (method 15.2.32.2), starch (method 15.2.77), DNA (method 15.2.54.1) and Tween-80 (method 15.2.47.2). Furthermore oxidative/ fermentation of glucose (aerobe/ anaerobe) (method 15.2.64), arginine dihydrolase (method 15.2.6.2) were also analysed. All tests were performed with the modification that artificial seawater was used for preparation of the media. The strains tested were incubated under aerobic conditions at 28°C in the dark. API 20NE micro test system (bioMérieux) was used according to the manufacturer's instructions manual with the modification that natural seawater (Biomaris, Bremen) was added to the saline and AUX-medium. To test for the ability to metabolise various carbon substrates BIOLOG GN2 microtitre plates (BIOLOG, Inc) were inoculated with exponential phase cultures (150 µl per well), with a cell density of 50% transmission as determined with the Biolog turbidimeter. After incubation at 28°C for 24 hours and 48 hours the development of a purple colour indicative of substrate oxidation was examined photometrically.

The chemotaxonomic analysis of quinones and polar lipids were performed from 100 mg freeze-dried cell material according to the two-stage method described by Tindall (1990a, b). Respiratory quinones were first separated by TLC and then subject to separation by HPLC using an RP_{18} column as described previously (Tindall 1990a, b). Polar lipids were separated by 2 dimensional chromatography and visualised using a combination of a non-specific spray reagent as well as specific spray reagents, as described previously (Tindall, 1990a, b; Tindall et al. 2007).

16S rRNA gene sequences obtained from the GenBank database were aligned using the automatic alignment tool implemented in the ARB program package (http://www.arb-home.de/). Calculation of the distance matrices for the aligned sequences and reconstruction by the neighbour-joining method (Saitou & Nei, 1987), maximum-likelihood and bootstrap calculations (1.000 resamplings) were performed using programmes provided in the ARB package. Phylogenetic relations of all members of the genera *Aurantimonas* and *Fulvimarina* are given in figure 1.

4.4 RESULTS AND DISCUSSION

4.4.1 Phenotypic characterisation

All strains tested were catalase and oxidase positive. Further phenotypic properties are shown in Table 1. The pattern of metabolism of carbon and energy (BIOLOG results) is given in Table 1 (GN MicroPlate).

API NE20 analysis revealed that in all investigated strains glucose, L-arabinose, D-mannitol and malic acid could be utilised. Nitrate reduction, indole production from tryptophan, fermentation of glucose, arginine dihydrolase activity, hydrolysis of β-glucosidase and gelatine as well as assimilation of capric acid, *N*-acetyl-glucosamine, trisodium citrate and phenylacetic acid were absent in all tested strains (Table 1).

4.4.2 Phylogenetic analysis

The analysis of the 16S rRNA gene sequences of the type strains of the species *A. ureolytica*, *A. frigidaquae*, *A. altamirensis* and *A. coralicida* indicated that *A. coralicida* did not group with the remaining species *A. ureolytica*, *A. frigidaquae* and *A. altamirensis* (Fig. 1).

4.4.3 Chemotaxonomic data (respiratory lipoquinones and polar lipids)

Ubiquinone 10 (Q10) was the dominant respiratory lipoquinone in all examined strains, which is consistent with their placement in the *Alphaproteobacteria*. The analysis of the polar lipid (Fig. 2) gave patterns that were largely consistent with previous reports, although there were differences in the identity of the lipids. The phospholipids present comprised diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanol-amine (PME) and phosphatidylcholine (PC). This differs from the reports of Denner et al. (2003) and Weon et al. (2007), both reporting on the presence of phosphatidyldimethylethanolamine (PDE). However, the lipid running with an R_f value similar to that of PDE in the images of the TLC plates in the supplementary file that accompanies the paper of Weon et al. (2007) was shown in this work to be ninhydrin (primary amines) positive and Zinzadze (phosphate) negative. Denner et al. (2003) did not provide an image of their TLC plate. It should be noted that the lipid labelled PME (phosphatidylmonomethyethanolamine) was both phosphate and ninhydrin positive as was an authentic sample of PME purchased from Sigma and the head group structure checked by mass spectrometry (Tindall, unpublished). Authentic phosphatidyldimethylethanolamine (PDE) is phosphate positive and ninhydrin negative. Similarly, both Denner et al. (2003) and Weon et al. (2007) do not mention aminolipids, although the lipid labelled L3 on the images of the TLC plates in the supplementary file that accompanies the paper of Weon et al. (2007) has an R_f value similar to that of the second amino lipid (AL2) reported here. All lipids labelled AL1 or AL2 reacted with the ninhydrin reagent. The polar lipids of the type strains of *Aurantimonas altamirensis, Aurantimonas ureilytica* and *Aurantimonas frigidaquae* contained a polar lipid that stained positively with the α-naphthol reagent, indicating that it contained a carbohydrate head group. The R_f value is consistent with it being identified as sulfoquinovosyldiacylglycerol (SQDG), a sulfonic acid containing glycolipid derivative.

Significantly this polar lipid was not present in either *Fulvimonas pelagi* or *Aurantimonas coralicida*. Weon et al. (2007) do not mention the presence of this or other glycolipids, although the lipid labelled L2 in the images of the TLC plates in the supplementary file that accompanies that paper has an R_f value similar to that of SQDG. This lipid only appears to be present in *Aurantimonas ureilytica* in those images, which is consistent with the work reported here. It should be noted that the presence/absence of SQDG may be of taxonomic significance as discussed by Biebl et al. (2007). We therefore propose that the genus *Aurantimonas* be split on the basis of the presence/absence of the glycolipid sulfoquinovosyl diacylglycerol (SQDG), which is consistent with both the 16S rRNA groupings and the marine/non-marine habitats.

Previous work has shown that in all strains the predominant fatty acid is $C_{18:1}$ ω7c which is a feature of the vast majority of members of the *Alphaproteobacteria*. However, distinct differences have been observed within *A. ureolytica*, *A. frigidaquae* and *A. altamirensis* and *A. coralicida* regarding the fatty acids $C_{16:0}$ and $C_{20:1}$ ω7c. Although all strains contained $C_{16:0}$ the amount is up to three times higher in *A. ureolytica*, *A. frigidaquae* and *A. altamirensis* compared to *A. coralicida.* Furthermore, only in *A. coralicida* more than traces of the fatty acid $C_{20:1}$ ω7c could be observed.

On the basis of the chemotaxonomic analysis significant differences emerged and that chemotaxonomic features contribute considerably to classify organisms was already discussed in Tindall et al. (1994). Due to the heterogeneity revealed within the genus *Aurantimonas* it is strongly suggested the exclusion of *A. ureolytica*, *A. frigidaquae* and *A. altamirensis* from the genus *Aurantimonas*.

It should be noted that although members of the genera *Fulvimonas* and *Aurantimonas* have been included in the order *Rhizobiales* (Kuykendall, 2005; 2006), the circumscription of that order includes the genus *Hyphomicrobium*. The genus *Hyphomicrobium* is the type of the

order *Hyphomicrobiales* (Douglas, 1957; Approved Lists 1980). Not only does the name *Hyphomicrobiales* (Douglas, 1957; Approved Lists 1980) have priority over the name *Rhizobiales* (Kuykendall, 2006), but according to Rule 51b 1 this name is illegitimate and Rule 51a clearly states that "a name contrary to a Rule is illegitimate and may not be used." While an emended description and circumscription of the order *Hyphomicrobiales* (Douglas, 1957; Approved Lists 1980) is clearly overdue, it is beyond the scope of the present article.

4.4.4 Description of Aureimonas gen. nov.

Aureimonas (Au.re.i.mo'nas. L. adj. *aureus*, golden; Gr. fem. n. monas a unit; N.L. fem. n. *Aurantimonas* orange-coloured unicellular organism).

Cells are gram-negative, short rods (1.0 µm wide and 2.0µm long on average). Colonies are yellow-in colour. Growth occurs between 4– 40°C, optimum 25– 30°C. The salinity range for growth is from 0– 3% NaCl. Cells are catalase-, urease- and oxidase-positive, but negative for L-alanine-aminopeptidase. Positive for hydrolysis of DNA and starch, but negative for tyrosine, Tween 80 and casein. Cells are capable of assimilation of glucose, L-arabinose, D-mannitol and malic acid. Nitrite reduction, production of indole from tryptophan , fermentation of glucose, arginine dihydrolase activity, hydrolysis of β-glucosidase, assimilation of capric acid, N-acetyl-glucosamine, trisodium citrate and phenylacetic acid were absent. Positive for utilisation of D-arabitol, L-arabinose, I-erythritol, D-fructose, L-fucose, D-galactose, α-D-glucose, m-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, xylitol, acetic acid, adonitol, formic acid, D-gluconic acid, β-hydroxybutyric acid, succinic acid, bromosuccinic acid, L-alanyl-glycine, hydroxy-L-proline and L-proline. The major respiratory lipoquinone is Q10. Polar lipids comprise diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, two lipids, two unidentified aminolipids and

sulfoquinovosyl diacylglycerol. The predominant fatty acids is $C_{18:1}\omega7c$ with significant proportions of $C_{16:0}$, $C_{18:1}$ 2OH, $C_{18:1}$ and cyclo-$C_{19:0}\omega8c$. Members of this genus have been isolated predominantly from non-marine habitats. The genus currently comprises three species *Aureimonas altamirensis*, *Aureimonas ureilytica* and *Aureimonas frigidaquae*. The type species is *Aureimonas altamirensis*.

4.4.5 Description of the Aureimonas altamirensis comb. nov.

Basonym: *Aurantimonas altamirensis* (Jurado et al. 2006)

Description of the *Aurantimonas altamirensis* is as given by Jurado et al. (2006), with the following additions. The polar lipids and respiratory lipoquinone compositions are consistent with those listed in the genus description. The type strain is S21B^T (= CECT 7138 = CIP 109525 = KCTC 22106 = LMG 23375 = DSM 21988).

4.4.6 Description of the Aureimonas ureilytica comb. nov.

Basonym: Aurantimonas ureilytica (Weon *et al.* 2007)

Description of the species is as given by Weon et al. (2007), with the following additions. The polar lipids and respiratory lipoquinone compositions are consistent with those listed in the genus description. It should be noted that this differs from the polar lipid patterns discussed by Weon et al. (2007), but they are not mentioned as part of the species description. The type strain is 5715S-12^T (= KACC 11607 = DSM 18598 = CIP 109815).

4.4.7 Description of the Aureimonas frigidaquae comb. nov.

Basonym: *Aurantimonas frigidaquae* (Kim et al. 2008)

Description of the specie *Aurantimonas frigidaquae* is as given by Kim et al. (2008), with the following additions. The polar lipids and respiratory lipoquinone compositions are consistent

with those listed in the genus description. The type strain CW5^T (=KCTC 12893 =JCM 14755 = DSM 21987).

4.4.8 Emended description of the genus Aurantimonas (Denner et al. *2003)*

The genus *Aurantimonas* exhibits the following properties in addition to those given by Denner et al. (2003). The polar lipid phosphatidyldimethylethanolamine (listed in the genus description by Denner et al. (2003) and sulfoquinovosyl diacylglycerol could not be detected. Additionally, two amino lipids are observed. Isolated from marine environments and does not grow in the absence of NaCl. The type species is *Aurantimonas coralicida.* The genus currently consists of one specie *Aurantimonas coralicida*.

4.4.9 Emended description of the species Aurantimonas coralicida (Denner et al. *2003)*

The species has the characteristics listed in the emended genus description. The type strain is strain WP1^T (= CIP 107386 = DSM 14790 = CCUG 53900).

4.4.10 Emended description of the genus Fulvimarina (Cho & Giovannoni, 2003)

Description of the genus *Fulvimarina* is as given by Cho & Giovannoni (2003) with the following additions. The polar lipids comprise diphosphatidylglycerol, phosphatidylmonomethyl-ethanolamine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, two lipids and two unidentified aminolipids. The sole respiratory lipoquinone is Q10. The type, and currently only, species of the genus is *Fulvimarina pelagi* HTCC2506^T (= ATCC BAA-666 = KCTC 12091 = DSM 15513).

4.4.11 Emended description of the species Fulvimarina pelagi (Cho & Giovannoni, 2003)

The species has the characteristics listed in the emended genus description. The type strain is strain HTCC2506 (= ATCC BAA-666 = DSM 15513 = KCTC 12091).

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4.7 TABLES

Table 1

Biochemical characteristics of the examined species. Strains: 1, *Aurantimonas coralicida*^{T} (data from this study); 2, *Aurantimonas ureilytica*^{T} (data from this study); 3, *Aurantimonas altamirensis*^{T} (data from this study); 4, *Aurantimonas frigidaquae*^{T} (data from this study); 5, *Fulvimarina pelagi*^{T} (data from this study/ Cho & Giovannoni, 2003 (Biolog data)).

All strains were positive for catalase, oxidase reaction and for hydrolyse of DNA and negative for hydrolyse of casein and arginindihydrolase (aerobe/anaerobe) activity. All strains were negative for Tween 80, α-keto glutaric acid, malonic acid, L-pyroglutamic acid and 2,3-butanediol utilization (BIOLOG results).

+, positive; +*, colour change restricted at the surface; –, negative; w, weakly positive reaction

Strain **Characteristic**	**1**	**2**	**3**	**4**	**5**
Catalase activity	+	+	+	+	+
Oxidase activity	+	+	+	+	+
L-Alanine-Aminopeptidase	–	–	–	–	–
KOH	–	–	–	–	–
Phenylalanine desaminase	–	–	–	+	–
Hydrolysis of starch	+	+	+	+	–
Hydrolysis of DNA	+	+	+	+	+
Hydrolysis of gelatine	–	–	+	+	–
Hydrolysis of tyrosine	+	–	–	–	+
Hydrolysis of Tween 80	–	–	–	–	–
Hydrolysis of casein	–	–	–	–	–
Oxidation/fermentation test for glucose (aerobe)	–	–	+*	+*	–
Oxidation/fermentation test for glucose (anaerobe)	+*	+*	+*	+*	+*
Arginine dihydrolase (aerobe)	–	–	–	–	–
Arginine dihydrolase (anaerobe)	–	–	–	–	–
API 20NE					
Reduction of nitrates to nitrites	–	–	–	–	–

Strain	1	2	3	4	5
API 20NE					
Fermentation of glucose	–	–	–	–	–
Hydrolysis of arginine dihydrolase	–	–	–	–	–
Hydrolysis of esculin	–	–	–	–	–
Hydrolysis of gelantin	–	–	–	–	–
Hydrolysis with β-Galactosidase	–	+	–	–	–
Assimilation of glucose	+	+	+	+	+
Assimilation of arabinose	+	+	+	+	+
Assimilation of mannose	+	+	+	+	–
Assimilation of mannitol	+	+	+	+	+
Assimilation of N-acetyl-glucosamine	–	–	–	–	–
Assimilation of maltose	–	+	+	–	+
Assimilation of potassium gluconate	+	+	+	+	–
Assimilation of capric acid	–	–	–	–	–
Assimilation of adipic acid	+	–	–	–	–
Assimilation of malic acid	+	+	+	+	+
Assimilation of trisodium citrate	–	–	–	–	–
Assimilation of phenylacetic acid	–	–	–	–	–
GN MicroPlate (utilisation of)					
α-cyclodextrin	–	–	–	–	+
Dextrin	+	w	w	–	+
Glycogen	–	–	+	–	+
Tween 40	–	–	–	–	+
N-acetyl-D-galactosamine	–	–	+	–	–
Adonitol	–	w	+	+	+
D-melibiose	–	–	+	–	+
Succinamic acid mono-methyl-ester	–	–	+	+	+
D-arabitol	–	w	+	+	+
D-cellobiose	–	+	–	–	+
L-arabinose	–	w	+	+	+
I-erythritol	–	w	+	+	–
D-fructose	–	w	+	+	+
L-fucose	–	w	+	+	–
D-galactose	–	w	+	+	+
Gentiobiose	–	–	–	–	+
α-D-glucose	–	w	+	+	+
m-inositol	–	w	+	+	–
Maltose	–	w	+	–	+
D-mannitol	–	w	+	+	+
D-mannose	–	w	+	+	+

Strain	**1**	**2**	**3**	**4**	**5**
GN MicroPlate (utilisation of)					
D-psicose	–	w	+	+	–
β-methyl-D-glucoside	–	–	–	–	+
L-rhamnose	–	w	+	+	–
D-sorbitol	–	w	+	+	+
Sucrose	–	w	+	–	+
Turanose	–	–	+	–	–
Xylitol	–	w	+	+	+
Pyruvic acid methyl ester	–	–	+	+	+
Succinic acid mono-methyl-ester	–	–	+	+	+
Acetic acid	–	w	+	+	+
Formic acid	+	w	+	+	+
γ-hydroxybutyric acid	–	–	+	–	–
D-galactonic acid lactone	–	w	–	+	–
D-gluconic acid	–	w	+	+	–
D-glucosaminic acid	–	–	+	–	–
α-hydroxybutyric acid	–	–	+	+	–
β-hydroxybutyric acid	–	w	+	w	+
γ-hydroxybutyric acid	–	w	+	–	–
Itaconic acid	–	–	+	–	–
α-keto butyric acid	–	–	+	–	+
α-keto valeric acid	–	–	+	–	–
D,L-lactic acid	–	–	+	+	+
Propionic acid	–	–	–	–	+
Quinic acid	–	w	+	–	–
Succinic acid	–	w	+	+	–
D-saccharic acid	–		+	–	+
Bromosuccinic acid	–	w	+	+	–
Succinamic acid	–	–	+	+	+
Glucuronamide	–	–	w	–	+
L-alaninamide	–	–	+	+	+
D-alanine	–	–	+	+	–
L-alanine	–	–	+	+	–
L-alanyl-glycine	–	w	+	+	–
L-asparagine	–	w	+	+	–
L-aspartic acid	–	w	w	–	–
L-glutamic acid	–	–	+	+	+
Glycyl-L-aspartic acid	–	–	+	–	–
Glycyl-L-glutamic acid	–	w	+	–	+
L-histidine	–	–	+	–	–
L-leucine	–	–	+	–	+
L-ornithine	–	–	+	–	–
Hydroxy-L-proline	–	w	+	+	–
L-proline	–	w	+	+	+

Strain	**1**	**2**	**3**	**4**	**5**
GN MicroPlate (utilisation of)					
L-threonine	–	–	+	+	–
D,L-canitine	–	–	w	+	–
γ-amino butyric acid	–	–	w	+	–
Urocanic acid	–	–	+	–	–
Uridine	–	w	–	–	+
Thymidine	–	–	–	–	+
Putrescine	–	–	+	–	–
Glycerol	–	–	–	–	+
D,L-α-glycerol phosphate	–	–	w	w	–

4.8 FIGURES

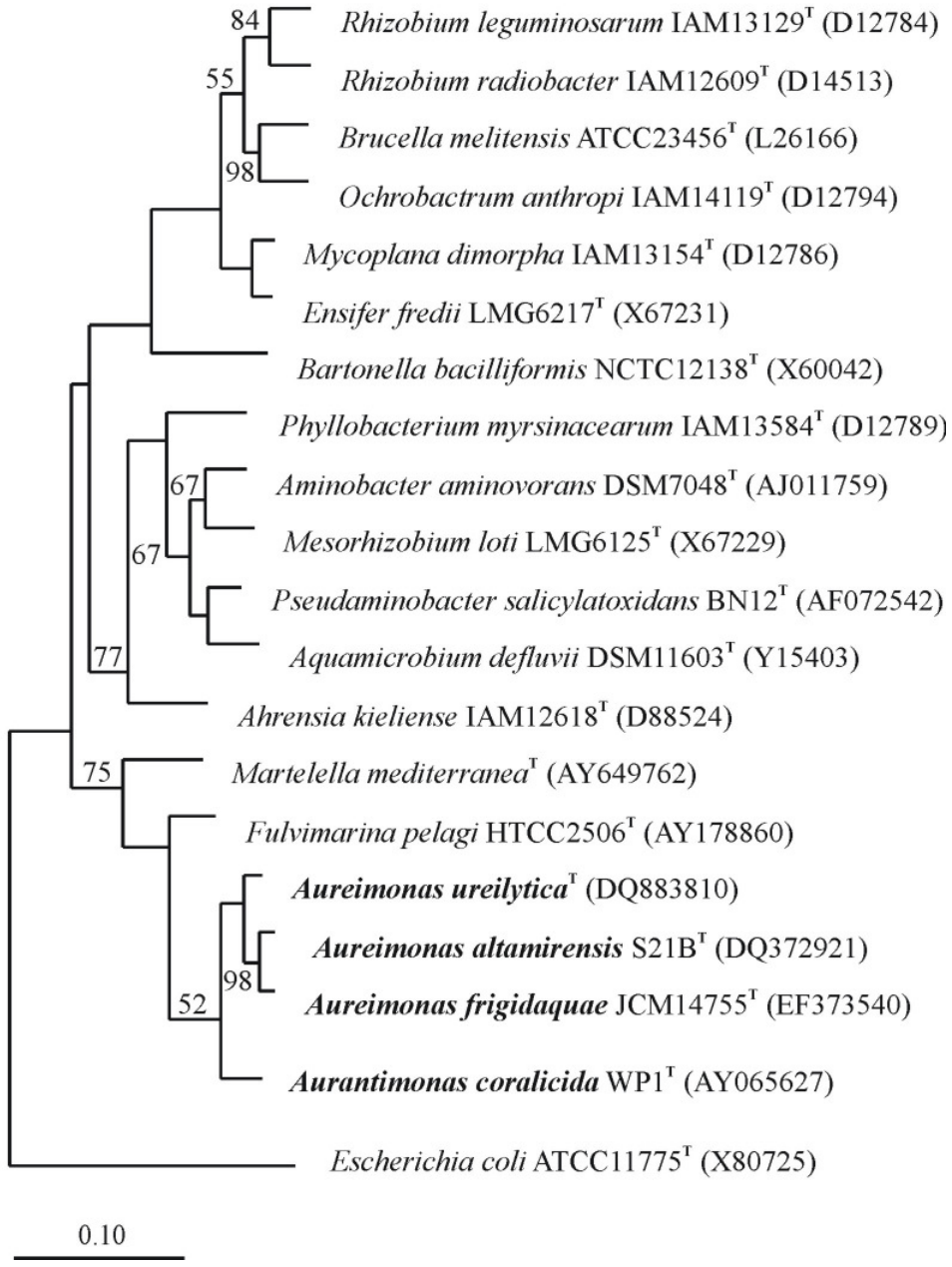

Figure 4.16.1

Neighbour-joining tree showing the phylogenetic position the species of the family *Aurantimonadaceae* with related genera of *Alphaproteobacteria* based on 16S rRNA gene sequences.

Only bootstrap values greater than 50% are shown (based on 1000 replications). Scale bar, 10 nucleotide substitutions per 100 nucleotides.

Figure 4.16.2

Polar lipid composition of the strains. Separation was in two dimensions: first dimension, chloroform/methanol/water (65:25:4, by vol.); second dimension, chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Strains: 1, *Aurantimonas coralicida*^{T} (DSM14790, data from this study); 2, *Aurantimonas ureilytica*^{T} (DSM18598, data from this study); 3, *Aurantimonas altamirensis*^{T} (DSM21988, data from this study); 4, *Aurantimonas frigidaquae*^{T} (DSM21987, data from this study); 5, *Fulvimarina pelagi*^{T} (DSM15513, data from this study). Plates were stained with 5% molybdophosphoric acid in ethanol and heated for 15 minutes at 150°C.

DPG= Diphosphatidylglycerol, PG= Phosphatidylglycerol, PE= Phosphatidylethanolamine, PME= Phosphatidylmonomethylethanolamine, PC= Phosphatidylcholine, AL1, AL2= Unidentified aminolipids, L1 – L2= Lipids, SQDG= Sulfoquinovosyl diacylglycerol

CHAPTER 5

SUMMARY

5.1 SUMMARY

Since the discovery of a flourishing ecosystem in the depth of the sea (Lonsdale, 1977) the investigation of the deep biosphere was brought into scientific focus. Numerous studies confirmed that life is capable to exist everywhere, even in such inhospitable realms like this deep, dark and almost freezing biosphere (Jørgensen & Boetius, 2007 and references therein). However, due to the tremendous dimensions of the habitat with it´s diverse ecological niches (e.g. mud volcanoes, cold seeps, high-temperature vents, low-temperature vents) merely 1% of the deep sea is explored, yet. Thus, we still are neither aware of the final composition of the inhabiting microbial community (e.g. basaltic rocks at low-temperature influenced venting sites), nor of it's influence on e.g. basalt alteration process, nor of it's global impact. Studies have demonstrated that a wide diversity of microorganisms occupy the basaltic deep marine ocean floor (Thorseth et al. 2001; Lysnes et al. 2004 a; Lysnes et al. 2004 b) but only a small fraction of microorganisms have been identified or even cultivated.

The objectives of this thesis were to give a general survey about deep biosphere with it´s varied morphology. Chapter 2 summarises the resident life, nutrient in come, microbial colonisation of sediments and gives insights to the oasis of the deep sea ecosystem: hydrothermal vents, cold seeps and mudvolcanoes and oceanic crust. Subsequently, different rocky textures sampled at that low-temperature venting sites were examined in detail. Chapter 3 reports of the cultivable part of the microbial consortia colonising different basaltic rocks, sediment and deep ambient sea water samples, collected in the vicinity of diffuses venting areas along the Mid-Atlantic Ridge, precisely at the Ascension-Fracture-Zone (7-12°S, 13°W). By using molecular, microbiological and phylogenetic tools the cultivable part of the rock inhabiting microorganisms were investigated. It was established that the basalt and basalt associated glass textures harbour a vast bacterial diversity with a predominance of *Gammaproteobacteria*. In addition, difference in diversity and abundance of microorganisms

colonising the examined textures was found. Particular genera occurred only in a source from one defined sampling site. Furthermore, the characterisation of selected isolates revealed high tolerances against a broad range of temperatures, pH-values and salt concentrations.

The analysis of selected organisms isolated in chapter 3 resulted in a rearrangement of a part of the microbial phylogenetic tree. By chemotaxic examination it turned out that members of the genus *Aurantimonas* had to be reclassified as members of the new genus *Aureimonas*. Furthermore the descriptions of the genera *Aurantimonas* and *Fulvimarina* were emended (see chapter 4).

However, up to now little is known about the metabolism of microorganisms under such limiting conditions and their significant role in the global cycle of elements (e.g. carbon, nitrogen, sulphur and manganese). Nonetheless, studies on the deep biosphere have broadened our perspectives regarding to the oligotrophic, cold, dark and high-pressure environment spotted with oases of hydrothermal vent systems and cold seeps. In future by further expeditions, better equipment and new approaches will contribute to expand and specify our knowledge about deep marine biosphere.

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