Isolation and characterization of bacteria from the deep sea and their potential to produce bioactive natural products

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Summary

Due to the high re-discovery rate of already known active compounds in recent drug research it appears reasonable to expand the search on unexplored environments with unique living conditions and yet undiscovered organisms. The deep sea demonstrates such a marginally investigated environment harboring presumably undiscovered bacterial taxa which are adapted to the extreme living conditions in the deep sea environment and which might possess unknown metabolites. The aim of the present thesis was therefore the characterization of deep-sea bacteria with the purpose to find novel and bioactive substances produced by these bacteria. Heterotrophic mesophilic bacterial strains were recovered and phylogenetically characterized from two totally different deep-sea habitats: the extremely oligotrophic Eastern Mediterranean and the Logatchev hydrothermal vent field (LHF) located at 15°N along the Mid Atlantic Ridge.

The antimicrobially active bacteria isolated from the hydrothermal environments were mainly assigned to the *Gammaproteobacteria*. One bioactive strain, M41^T, revealed to be a representative of a novel genus and species, *Amphritea atlantica*^{T}. This strain was isolated from a mussel field at LHF and physiological analysis showed that it is well adapted to the mesophilic temperatures of hydrothermally influenced environments. In addition, the bacterial community of diffusive fluids emanating out of a mussel field at LHF was investigated. A 16S rRNA gene library was analyzed in combination with functional genes involved in biochemical pathways of CO_2 fixation (aclB, cbbM, cbbL) and sulfur oxidation/reduction (soxB, aprA). It turned out, that *Epsilonproteobacteria* and *Gammaproteobacteria* comprise a considerable part of the microbial community in diffuse fluids and have the genetic potential to use different pathways for carbon fixation and sulfur oxidation.

The bacterial strains obtained from the Eastern Mediterranean deep sea were also analyzed for specifically adapted bacterial strains and antimicrobial activities. Predominantly Gram-positive strains were isolated from the untreated sediment, while incubation of sediment at *in situ* pressure revealed that *Gammaproteobacteria* were enriched at the simulated deep-sea conditions. Bacterial strains affiliating to the genus *Micromonospora* were selected for further analysis in order to investigate their potential to produce bioactive substances. This led to the discovery and structure elucidation of the novel cytotoxic macrolide levantilide A and a derivative thereof,

levantilide B, both of which are produced by a deep-sea *Micromonospora* strain, strain A77.
Thus, the results of the present study demonstrate that deep-sea habitats are a promising source for novel bacterial taxa and for the discovery of new natural products as well.

Zusammenfassung

Im Zuge der hohen Wiederentdeckungsrate bekannter Verbindungen in der heutigen Wirkstoffforschung erscheint es sinnvoll, die Suche auf neue Habitate mit einzigartigen Lebensbedingungen und bislang unbekannten Organismen auszudehnen. Ein solches bislang nur marginal untersuchtes Gebiet stellt die Tiefsee dar. Es ist zu erwarten, dass in der Tiefsee noch viele neue Bakterien-Taxa auf ihre Entdeckung warten, die physiologisch an die extremen Lebensbedingungen angepasst sind und über neue Metabolite verfügen, die wiederum für die biotechnologische oder medizinische Forschung von Interesse sein können. Ziel der vorliegenden Arbeit war es daher, Tiefseebakterien näher zu charakterisieren und sie hinsichtlich ihres Potenzials zur Wirkstoffproduktion zu untersuchen. Mesophile Bakterien wurden von zwei sehr unterschiedlichen Tiefseestandorten isoliert und charakterisiert: die extrem nährstoffarmen Tiefseesedimente des östlichen Mittelmeeres sowie das Logatchev-Hydrothermalfeld (LHF) am Mittelatlantischen Rücken.
Die vom LHF isolierten Stämme mit antimikrobieller Aktivität waren hauptsächlich den *Gammaproteobakterien* zuzuordnen. Unter diesen befand sich auch der Stamm M41^T, welcher im Zuge dieser Studien als Repräsentant einer neuen Gattung, *Ampritea atlantica*^{T}, beschrieben wurde. Physiologische Untersuchungen dieses von einem Muschelfeld isolierten Stammes zeigten, dass dieser gut an die mesophilen Temperaturen von hydrothermal beeinflussten Gebieten angepasst ist. Des Weiteren wurde die Bakteriengemeinschaft an einer diffusen Fluidaustrittstelle über einem Muschelfeld des LHF untersucht. Die Ergebnisse aus einer 16S rRNA Genbank und die kombinierte Untersuchung von Schlüsselenzymen für verschiedene CO_2-Fixierungswege und den Schwefelkreislauf ergaben, dass *Epsilonproteobakterien* und *Gammaproteobakterien* einen beachtlichen Teil der Bakteriengemeinschaften auszumachen scheinen und darüber hinaus das genetische Potenzial haben,

unterschiedliche Biosynthesewege für die CO_2-Fixierung und die Schwefeloxidation zu verwenden.

Auch die Isolate aus dem östlichen Mittelmeer wurden hinsichtlich des Vorkommens neuer Arten, spezieller Anpassungen sowie antimikrobieller Aktivitäten untersucht. Während aus den unbehandelten Sedimenten in erster Linie Gram-positive Isolate gewonnen wurden, hatte eine Inkubation des Sedimentes bei *in situ* Druck eine Verschiebung der Bakteriengemeinschaft, hauptsächlich hin zu Vertretern der *Gammaproteobakterien,* zur Folge. Von den gewonnenen Isolaten wurden die Vertreter der Gattung *Micromonospora* für die Suche nach bioaktiven Wirkstoffen ausgewählt. So konnte ein neues zytotoxisch wirksames Makrolid, levantilide A, und ein Derivat, levantilide B, aus einem der *Micromonospora*-Stämme, Isolat A77, isoliert und beschrieben werden.

Die Ergebnisse der vorliegenden Arbeit zeigen, dass aus Tiefsee-Standorten eine Vielzahl unbekannter Bakterien-Taxa kultiviert werden können und dass Tiefseebakterien eine vielversprechende Quelle für neue Wirkstoffe darstellen.

Introduction

1. Prokaryotic life in the deep sea

The historical beginning of deep-sea research is connected to the Challenger Expedition in 1872-1876, which included all major oceans except the Arctic. Only ten years later, first evidence of bacterial life in the deep-sea environment was provided by Certes during the Travaillier and Talisman Expeditions (1883- 1886). Certes found few bacteria in the bottom debris at 5100 m depth and demonstrated growth of these bacteria under hydrostatic pressure.

More than 75 % of all ocean water is deep-sea water, being located primarily at depths between 1000 m - 6000 m (Figure 1). Thus, the deep sea can be regarded as the largest habitat on earth (Warrant and Locket, 2004). Unfortunately, there is no strict definition about the minimum depth defining the deep sea and thus the literature data about the deep sea refer to arbitrary water depths from 50 m (the maximum depth for scuba diving), 200 - 300 m (the maximum depth for the penetration of light into the water column) to depths of 1000 m – 6000 m (referring to the various definitions of bathyal, abyssal and hadal). Depths greater than 200 – 300 m are characterized by complete darkness and life in these depths depends on the primary biomass production from the photic zone. Generally, from the continental shelf break at 200 m the temperature rapidly decreases down to 1000 m and does not exceed 5°C in depths greater than 1000 m. Within this thesis, the term “deep sea” refers to water depths of ≥ 1000 m, where hydrostatic pressure of 100 bar persists and raises up to 1100 bar at the world´s deepest point, the Mariana Trench.

Apart from several hot spots like cold seeps and hydrothermal vent fields, the majority of the deep-sea bottom surface is covered by soft and muddy sediment. Historically, the deep-sea floor was believed to be an “azoic” zone by Forbes (1844). This “azoic hypothesis” based on Forbes´ dredging work in the Aegean Sea inspired further investigations and today, the deep sea is known to be a habitat colonized by a variety of eukaryotic and prokaryotic organisms. In any square meter of deep-sea sediment hundreds of different species of worms, crustaceans and molluscs can be found (Van Dover, 2000). Also the prokaryotic communities were shown to be of comparably high species diversity. Missing sunlight, generally low temperatures, limited nutrient supply and elevated hydrostatic pressure require specific physiological adaptations of its inhabitants.

In 1949, ZoBell and Johnson defined the term “barophilic” for microorganisms that grow preferentially or exclusively at elevated hydrostatic pressure (ZoBell and Johnson, 1949). Meanwhile this definition shifted to distinguish between barotolerant growth and barophilism with optimal growth at > 1 bar. The term “obligate barophilic” today is subjected to those organisms that lack growth at 1 bar. Bacteria isolated down to 3000 m depth are typically barotolerant. These organisms grow optimally at 300 - 400 bar, but can also grow at 1 bar (Madigan et al., 2009). The first barophilic bacteria were isolated in 1979 by Yayanos from 5800 m depth. The spirillum-like strain revealed a pressure optimum for growth at 1000 bar and grew worse at atmospheric pressure (Yayanos et al., 1979). *Photobacterium profundum* SS9 was isolated from 2551 m depth in the Sulu Sea and became a model organism for studies of barophilic bacteria. SS9 grows over a broad pressure range (900 bar) with an optimum growth at 280 bar and 15°C (Delong, 1986). Genome and gene expression analysis of SS9 showed that the strain passes through a heavy change in gene expression between atmospheric pressure and 280 bar (Bartlett, 1999; Vezzi et al., 2005; Campanaro et al., 2005). Metabolic pathways for the degradation of relatively recalcitrant carbon sources such as chitin, pullulan and cellulose revealed to be up-regulated at elevated hydrostatic pressure (Vezzi et al., 2005). Moreover, it was shown that genes responsible for membrane fatty acid unsaturation such as a putative delta-9 fatty acid desaturase were up-regulated at high pressure. Furthermore, elevated pressure had an effect on the composition of membrane proteins. Elevated pressure resulted in the production of the outer membrane protein OmpH. OmpH is a porine that enables the diffusion of organic molecules through the outer membrane. Presumably, the porines of the outer membrane present at 1 bar do not function at elevated pressure, which leads to the production of OmpH. Strain SS9 revealed a comparably large number of ribosomal RNA operons which may reflect the ability to respond rapidly to favorable changes in growth conditions. Phylogenetically, most barophilic strains isolated up to now affiliated to the *Gammaproteobacteria* and, quite interestingly, all of these were recovered under nutrient-rich cultivation conditions (Delong et al., 1997).

In general, the bacterial community of the deep sea can be considered to be exposed to extreme oligotrophy. Less than 1% of the photosynthetically produced biomass reaches the deep sea. As shown by Wirsen and Molyneaux (1999), the indigenous microbial population has extremely slow generation times (> 600 hr) under *in situ*

pressure and nutrient conditions but already low concentrations of supplemented carbon can drastically increase growth rates. The inter-annual variability in surface water production is therefore proposed to be reflected by the deep-sea community (Pfannkuche et al., 1999). Besides hydrostatic pressure and nutrient availability, the temperature affects the growth rate of bacteria. Especially the rate of biochemical reactions is drastically reduced in speed at low temperatures and thus, psychrophilic bacteria (growing optimally below 15°C) need to have cold-adapted enzymes that optimally function at low temperature (Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006). Furthermore, similar to elevated pressure, low temperatures reduce membrane fluidity and thus psychrophilic bacteria retain their membrane fluidity e.g. by an increased content of polyunsaturated and methyl-branched fatty acids (Russell, 1997). Hence, the microbial community of the permanently cold deep sea (< 5°C) is adapted to elevated hydrostatic pressure and temperatures around the freezing point.

The present thesis presents data obtained from two strikingly different habitats, each of which can be assumed to reflect a characteristic deep-sea ecosystem with specific requirements for their prokaryotic communities: (1) the Eastern Mediterranean Sea, as an example of an oligotrophic deep-sea habitat, and (2) the Logatchev hydrothermal vent field on the Mid-Atlanic Ridge (MAR), representing deep-sea hydrothermal vent regions (Figure 1).

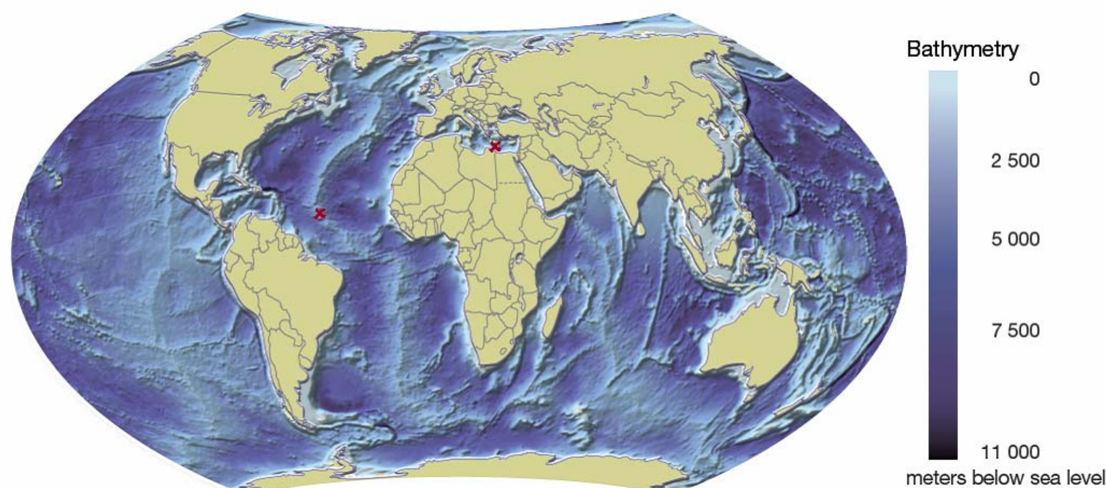

Figure 1: Bathymetric map of the world (http://maps.grida.no/go/graphic/world-ocean-bathymetric-map). The two deep-sea sites which served as basis for this thesis (Eastern Mediterranean Sea and Logatchev hydrothermal vent field at the Mid-Atlantic Ridge) are marked by red crosses.

In the following, the major characteristics and requirements for microbial life in these environments as well as substantial differences between the investigated habitats are specified. A summary of the characteristic abiotic and biotic features of these different deep-sea environments is provided in Table 1.

Table 1: Summary of characteristic features of the contrasting deep-sea environments (oligotrophic deep sea and deep-sea hydrothermal vent fields). Data given in brackets [ ] refer to the special features of the studied Eastern Mediterranean Sea.

	Oligotrophic deep sea	Deep sea hydrothermal vent fields
Temperature	1- 5°C [13.5 °C]	4°C - 400 °C
Temperature gradient	none	steep
Nutrient availability	low	high
Variability of nutrient availability	high	high
Heavy metal concentrations	low	high
Primary productivity	none	high
Dominating bacterial group in 16S rRNA gene libraries*	*Gammaproteobacteria*	*Epsilonproteobacteria*
Macroorganism abundance	low	high
Dominating bacterial "lifestyle"	heterotrophic	autotrophic and heterotrophic
assumed temperature preference of bacteria	psychrophilic [mesophilic][1]	psychrophilic to hyperthermophilic[2]

* according to the environmental 16S rRNA gene clone library of the South Ionian Basin by Polymenakou et al (2005) and the roundup by Thornburg et al (2010); [1] according to Yayanos (1995)
[2] according to Van Dover (2000)

1. 1. Microbial life in the oligotrophic deep sea

In general, the deep sea is regarded as a stable environment which is characterized by an overall depletion of nutrients with constant bottom temperature and elevated hydrostatic pressure (Gage and Tyler, 1992). The input of nutrients into the deep-sea biosphere is in general punctual and not predictable. Therefore, deep-sea communities have to cope with long times of starvation and were shown to respond quite rapidly to nutrient input. The fluctuation in the availability of nutrients and the presence of micro-niches allows the presence of oligotrophic as well as copiotrophic microorganisms in the oligotrophic deep sea (Schut et al., 1997). While copiotrophic bacteria are commonly isolated by the use of diverse complex media, oligotrophic bacteria are difficult to cultivate because their exact nutrient requirements are not known. Most proposed oligotrophic bacterial strains could also grow with elevated nutrient conditions (Koch, 2001). For instance, *Caulobacter crescentus,* the prime example of an oligotrophic bacterium turned out to grow fairly well under high nutrient concentrations (Poindexter, 1964). Nevertheless, genome sequencing of this strain demonstrated that it is well adapted to grow in nutrient-limited environments. This is reflected for example by the large number of genes related to chemotaxis and flagella (2.8% of the genome) (Nierman et al., 2001), which might be of special importance for detection of and rapid movement towards the rare nutrients. Microbial community studies of oligotrophic deep-sea environments are scarce. Studies of prokaryotic diversity in North Atlantic deep-sea waters revealed a considerable bacterial diversity dominated by *Alpha*-, *Gamma* – and *Deltaproteobacteria* (Sogin et al., 2006). These phylogenetic lineages appear to be common in deep-sea habitats (Delong et al., 1997; Li et al., 1999) and also dominate 16S rRNA gene libraries of Mediterranean deep-sea sediments (Polymenakou et al., 2005; Zaballos et al., 2006; Polymenakou et al., 2009). Results of a metagenomic study of Mediterranean deep-sea water reflected a mainly heterotrophic lifestyle of the deep-sea community (Martin-Cuadrado et al., 2007). Interestingly, a variety of genes involved in the degradation of different kinds of compounds, including complex biopolymers, were detected in the metagenomes. Results from this study underline the specific requirements for bacteria thriving in deep-sea ecosystems. The variety of genes associated with nutrient mobilization can be explained by the necessity to quickly react to input of the rare nutrients. Furthermore, nutrients can be assumed to reach

the deep sea in different states. While labile organic matter such as sugars, fatty acids and proteins is already degraded during the sinking process of particles, mainly refractory compounds (such as cellulose and chitin) reach the seafloor without significant degradation. Therefore, indigenous bacterial communities have to treat the supplied nutrients in various ways. Thus, persisting long times of starvation, rapid and heterogenous response to spontaneous nutrient supply as well as the degradation of complex biopolymers and refractory compounds to gain valuable nutrients are important prerequisites for bacteria living in the oligotrophic deep sea. Because the Eastern Mediterranean deep-sea provides the above-mentioned premises in a specific way due to its comparably high deep-water temperatures, making it especially interesting for microbiological work, it served as example for an oligotrophic deep-sea habitat for this thesis.

1.1.1 The oligotrophic deep-sea environment: Eastern Mediterranean Sea

The Mediterranean Sea is the largest (2,969,000 km^2) and deepest (average 1,460 m, maximum 5,263 m) enclosed sea on earth. The Mediterranean Sea is connected to the Atlantic Ocean via the 300 m deep Strait of Gibraltar and is subdivided into two basins, the Eastern and the Western Basin, which are interconnected through the shallow Strait of Sicily. The Eastern Mediterranean, which was subject of this thesis, is further divided in two major basins, the Levantine and Ionian Basins which are connected to the Aegean and the Adriatic Sea. A considerable part of the Mediterranean Sea in general and especially of the Eastern Mediterranean can be classified as deep sea. Characteristic features of the Mediterranean deep sea are the exceptionally high stable bottom water temperatures of around 13 – 15 °C and comparably high salinity values from about 38 to 39.5. These comparably high deep-water temperatures are supposed to account for mesophilic bacteria whereas in general, psychrophilic bacteria dominate deep-sea environments (Yayanos, 1995). Because the present thesis mainly relied on cultivation of bacteria, the comparably high deep-sea temperatures of the Eastern Mediterranean facilitated laboratory cultivation conditions. Furthermore the Mediterranean Sea is characterized by an overall depletion of nutrients with the eastern basin as one of the most oligotrophic regions of the world (Polymenakou

2009). This oligotrophy can be attributed to the semi-enclosed circulation in the Mediterranean Basin. The only connection of the Mediterranean to the Atlantic Ocean is provided via the shallow Strait of Gibraltar, where nutrient-enriched surface water flows into the Western Basin. Mainly due to ongoing evaporation combined with consumption of nutrients on its way eastward, the surface waters are characterized by a west-to-east increase in temperature, salinity, density and of course, oligotrophy. Furthermore, the deep water in the Levantine and Ionian Basins is generated by winter convection in the warm and oligotrophic Aegean and Adriatic Seas. A nutrient outflow from the Eastern Mediterranean can be attributed to the westward flow of nutrient-enriched water at intermediate depths (200 – 1000 m) through the Straits of Sicily and Gibraltar. Concentrations close to or even below detection limits of phosphorus and nitrate, which are crucial nutrients for primary production, cause extremely low primary productivity (20 – 25 g C / m^2 y) especially in the surface waters of the Eastern Basin of the Mediterranean Sea. As a result thereof only very small amounts of organic matter reach the sea floor (Danovaro et al., 1999). The seafloor is covered by oxic sediment and macrofauna biomass is extremely scarce.

Samples from the oligotrophic Eastern Mediterranean analyzed in this thesis were recovered during the research cruise M71/2 of the German research vessel METEOR in December 2006/January 2007. Samples were obtained from two different locations southwards of Crete: the Ierapetra Basin with a depth of 4400 m (34°30.296N, 26°11.507E) and the Herodotos Plain reaching a depth of 2500 m (33°42.989N, 26°20.329E) (Fig. 2a, b). Both sites were sampled by a multiple sediment corer and a rosette sampler equipped with 24 Niskin Bottles connected to a CTD probe.

Figure 2a: Sampling locations at the Eastern Mediterranean Sea: IB =Ierapetra Basin (4400m); HP = Herodotos Plain (2800m)

Figure 2b: Sediment samples from the Eastern Mediterranean deep-sea sediment, recovered by a multiple corer.

1.2. Microbial life at deep-sea hydrothermal vent fields

In strong contrast to the scant environment of the cold deep sea covered by soft sediment stands the flourishing biomass present in hydrothermal vent ecosystems. Such systems arise along the boundaries of tectonic plates like spreading centres of the Mid Ocean Ridges, near subduction zones and also at seamounts. Hydrothermal vent ecosystems are characterized by emanating diffusive fluids and / or hot fluids streaming out of black smokers (exhibiting temperatures up to 400°C). These fluids contain reduced elements such as hydrogen, sulfide, methane and reduced metal ions. Mixing processes of cold seawater with hot fluids lead to the establishment of steep gradients of temperature, reduced elements, heavy metals, pH and oxygen. These physical and chemical gradients provide a variety of microniches, and thus an enormous density and diversity of prokaryotes with distinct physiological capabilities can be found in hydrothermal vent ecosystems. Primary biomass production using inorganic carbon dioxide as sole carbon source is based on the supplied reduced elements and forms the fundament of a food-web independent from sunlight.

Hydrogen sulfide represents one of the major energy sources used by chemolithotrophs and is considered to be a key factor for the development of hydrothermal communities (Kelley et al., 2002; Sievert et al., 2007). Hydrothermal metazoans, like the tubeworm *Riftia pachyptila,* gain amazing growth (85 cm /year)

by the help of their sulfur-oxidizing bacterial endosymbionts (Lutz et al., 1994). Thus, these ecosystems arise to hot spots of biomass and biodiversity. Phylogenetic studies of prokaryotic biodiversity revealed that autotrophic *Epsilonproteobacteria* are commonly found and frequently dominate hydrothermal vent habitats (Moyer et al., 1995; Nakagawa et al., 2006; Sievert et al., 2008; Hügler et al., 2010). Deep-sea hydrothermal vent environments can be regarded as one of the largest reservoirs of diverse *Epsilonproteobacteria* found on earth ranging from the deeply-branching *Nautilales* and *Nitratiruptor* groups to the *Sulfurospirillum*, *Acrobacter*, and Marine group I and II of the *Thiovulgaveae* (Campbell et al., 2006). Most of them, either free-living or as endosymbionts, use hydrogen or reduced sulfur compounds as sole electron acceptor and fix inorganic carbon for primary biomass production. Another important taxonomic group are representatives of the *Gammaproteobacteria. Thiomicrospira*, *Halothiobacillus* and *Beggiatoa* are frequently detected free-living sulfur-oxidizing *Gammaproteobacteria* at hydrothermal vent ecosystems. *Beggiatoa* spp., e.g. were found to build extensive several centimetre thick mats at hydrothermal vents at Guaymas Basin (Teske et al., 2000). Furthermore, *Gammaproteobacteria* are known as endosymbionts of vent metazoans, such as the sulfur-oxidizing and methane-oxidizing endosymbionts from *Bathymodiolus* mussels (Van Dover, 2000; Dubilier et al., 2008). Not solely autotrophic but also heterotrophic bacterial metabolism can be found in hydrothermal ecosystems and thus chemo-heterotrophic and organo-heterotrophic bacteria are an important part of the microbial community and mediate the transfer of energy to higher trophic levels. Further taxonomic groups that are part of the complex prokaryotic food-web of hydrothermal vent systems are for example *Deltaproteobacteria*, *Aquificales*, *Chloroflexaceae*, many deeply rooted lineages like OP11 and T7 as well as hyperthermophilic *Archaea*.

1.2.1 The hydrothermal deep-sea environment: Logatchev hydrothermal vent field

Up to now, seven hydrothermal vent fields along the northern hemisphere of the Mid-Atlantic Ridge have been described: Menez Gwen, Lucky Strike, Rainbow, Broken Spur, TAG, Snake Pit and the Logatchev hydrothermal vent field. The latter, which

was subject of this thesis, was discovered in 1993/94 by a Russian research cruise with the vessel R/V Professor Logatchev and termed thereafter (Batuyev et al., 1994). The Logatchev hydrothermal vent field is located on the eastern rim of the Mid-Atlantic Ridge (14°45 N, 45°W) at approximately 3000 m depth. This ultramafic-hosted vent field consists of six active venting sites with very high (> 350°C) and low temperature fluid emissions: Quest, Irina II, site “B”, Irina I, Anna-Louise and site “A”. The venting sites are located with 50 to 200 m distance between them (Figure 3a). The fluid geochemistry is characterized by very high concentrations of hydrogen and methane (up to 3.5 mM and 19 mM) (Schmidt et al., 2007). Environmental 16S rRNA gene libraries of hot temperature fluids at LHF were dominated by sequences of *Epsilonproteobacteria.* The high concentrations of hydrogen and the considerable fraction of epsilonproteobacterial clone sequences led to the assumption that a important part of the microbial community was composed of hydrogen-oxidizers (Perner et al., 2007). Furthermore, the LHF is characterized by a high biomass supply of predominantly *Bathymodiolus* mussels overgrowing the vent chimneys at Irina II. The biomass on the mussel bed at Irina II exceeds 70 kg/ m^2 (wet weight with shells) and is the highest known on the Mid Atlantic Ridge (Gebruk et al., 1997). Further mussel species as well as diverse shrimps, crabs, actinians, hydrozoans and fish also colonize the hydrothermal area at LHF (Gebruk et al., 2000; Kuhn et al., 2004). During research cruises M60/3 and MSM04 of the German research vessels METEOR and MARIA S. MERIAN, several samples of sediment, fluids, water and metazoans were recovered by the use of a remotely operating vehicle (Figure 3a, b).

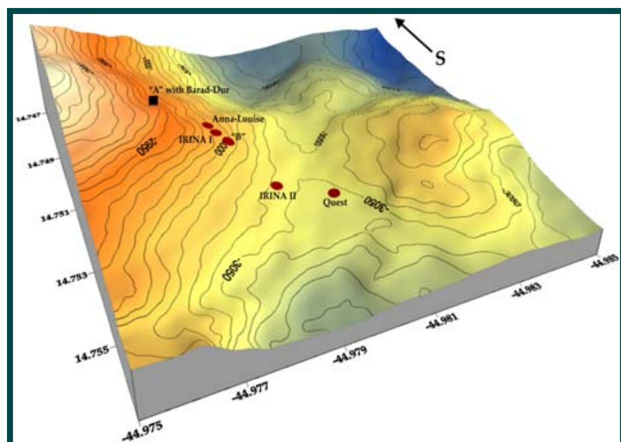

Figure 3a: Bathymetric map of the Logatchev hydrothermal vent field. Black smokers and smoking craters found at the LHF are indicated by red spots. Map: N. Augustin

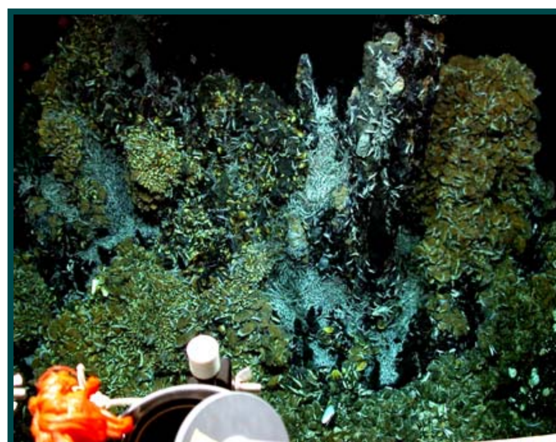

Figure 3b: Irina II smoking complex covered by dense biomass of *Bathymodiolus* mussels. Copyright by MARUM, Bremen.

2. The deep sea as a treasure chest for new natural products

Due to the comparably complicated and cost-intensive accessibility of the deep sea, only few natural products have been recovered from this environment compared to the huge amount of prokaryotes isolated and marine natural products described up to now. In a recent review, Skropeta (2008)summarized all natural products obtained so far from the deep sea (in this review, the threshold depth for “deep sea” was set to 50 m, the maximum depth for scuba diving). According to this review, approximately 20.000 natural products have been reported from marine sources over the past 50 years with less than 2 % derived from deep-water marine organisms. When looking at a depth profile of novel natural products from the sea (Figure 4), it becomes obvious, that most new marine natural products discovered yet originate from coastal areas and the continental shelves where light is still penetrating down to the bottom sediment. In contrast, the amount of new compounds from depths > 500 m rapidly declines. However, this is most likely due to the easier accessibility of coastal areas and shelf regions and does not necessarily imply that there are less new natural products from the deep sea but, more probable, fewer studies on this topic.

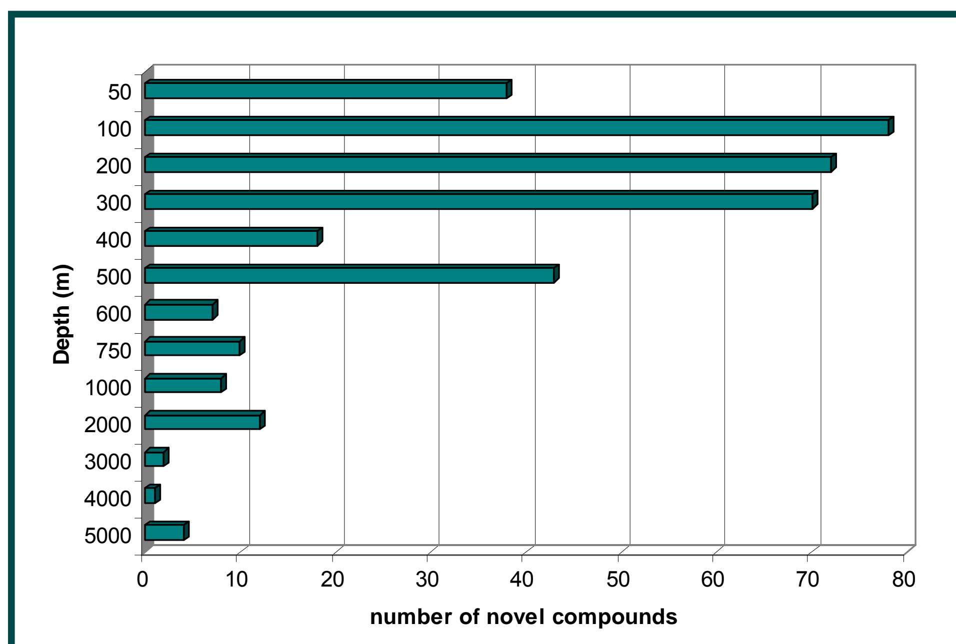

Figure 4: Depth profile of novel natural products form the deep sea (Skropeta 2008).

To date, the bottom of the open ocean and the deep ocean trenches have barely investigated for new natural products. It is proposed that the abiotic conditions characteristic for all deep-sea habitats, e.g. elevated hydrostatic pressure could have an impact on natural product synthesis. Using computer simulations and bioinformatics, Wright et al. (2003) demonstrated that high hydrostatic pressure leads to volume changes of the produced compounds and thus, may influence the biosynthetic polyketide synthase (pks) pathway. The influence e.g. of elevated pressure on metabolic processes in addition to the enormous prokaryote biodiversity of mainly unknown taxa enlarges the potential for the discovery of novel chemical structures of medical or biotechnological relevance.

Novel secondary metabolites with partly promising biological activity that have been recently recovered from deep-sea microorganisms (≥ 1000 m depth) are summarized in Table 2 (chemical structures are depicted in Figure 5). These novel natural products originate from world wide deep-sea sediments as well as from hydrothermal vent fields and even from the world´s deepest point, the Mariana Trench. From a maximum ocean depth of 10.898 m two strains ($MT1.1^T$ and MT1.2) of *Dermacoccus abyssii sp.* nov., have been isolated that produce seven novel phenazine-type pigments, dermacozines A-G (Abdel-Mageed et al., 2010). These compounds exhibited cytotoxic activities against the human chronic myelogenous leukemia cell line K562 with IC_{50} values ranging from 7 µM (dermacozine G) to 220 µM (dermacozine B). Furthermore, dermacozines A-D revealed radical scavenging activities. Dermacozine C displayed even more antioxidant activities against stable DPPH• (2,2-diphenyl-2-picrylhydrazyl) than ascorbic acid with an IC_{50} value of 8.4 µM (IC_{50} of ascorbic acid = 12.1 µM). In addition the production of these novel class of phenazines by two novel strains of the genus *Dermacoccus*, the strain $MT1.1^T$ revealed to be barotolerant as it grows equally well at 400 bar (Pantom-Aree et al., 2006).

Tabel 2: Novel secondary metabolites from the deep sea.
Chemical structures of the listed compounds can be found in Figure 5

Organisms	Compound name or compound class	Depth (m)	Region	Biological activity	References
Gammaproteobacteria					
Alteromonas haloplanktis	bisucaberin **(1)**	3300	Japan	cytotoxic	(Kameyama et al., 1987)
Thermovibrio ammonificans	ammonificins A & B **(2,3)**	~2500	East Pacific Rise	n.s.	(Andrianasolo et al., 2009)
Bacillus					
Bacillus sp.	guaymasol **(4)** epiguaymasol **(5)**	1834	Mexico	n.s.	(Trischman et al., 1998)
Bacillus sp.	phenazine derivative **(6)**	5059	West Pacific Ocean	cytotoxic	(Li et al., 2007)
Actinobacteria					
Nocardiopsis sp.	cyclic tetrapeptide MKN-349A **(7)**	3000	Carion-Clipperton-zone	n.s.	(Shin et al., 2003)
Rhodococcus sp.	rhodofactin	n.s.	Pacific Ocean	biotensidic	(Peng et al., 2008)
Dermacoccus abyssii	dermacozine A-E **(8-12)** dermacozine F-G **(13-14)**	10898	Mariana Trench	radical scavenger cytotoxic	(Abdel-Mageed et al., 2010)
Streptomyces sp.	streptokordine **(15)**	n.s.	Ayu-Trough	cytotoxic	(Jeong et al., 2006)
Streptomyces griseus	streptopyrrolidine **(16)**	n.s.	Ayu-Trough	anti-angionesic	(Shin et al., 2008)
Streptomyces sp.	γ-Indomycinone **(17)**	4680	Marshall Islands	antibiotic	(Schumacher et al., 1995)
Streptomyces sp.	caboxamycin **(18)**	3814	Canary Islands	n.s.	(Hohmann et al., 2009b)
Streptomyces sp.	albidopyrone **(19)**	3814	Canary Islands	n.s.	(Hohmann et al., 2009a)
Streptomyces sp.	ammosamides A & B **(20,21)**	1618	Bahamas Islands	cytotoxic	(Hughes et al., 2009)
unknown					
unidentified Gram-positive	caprolactins A & B **(22,23)**	5065	Central Pacific Basin	cytotoxic, antiviral	(Davidson and Schumacher, 1993)

n.s. = not specified

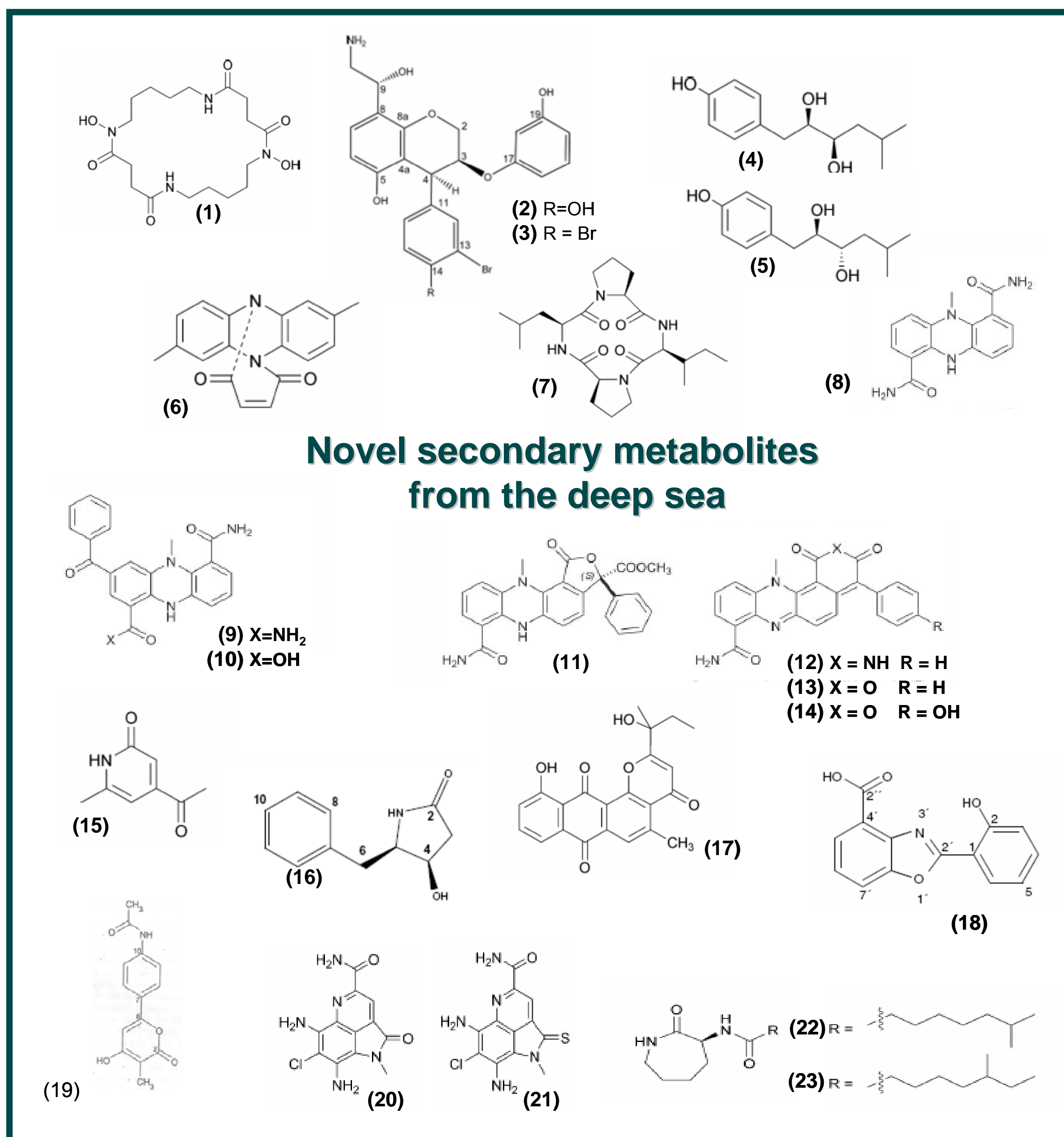

Figure 5: Chemical structures of novel secondary metabolites from the deep sea (≥ 1000m). Further information about the compounds are listed in Table 2

The dermacozines exemplarily demonstrate the enormous unexplored potential of deep-sea bacteria for recent drug discovery. Most of the novel chemical structures from deep-sea microorganisms originate from Gram-positive bacteria and in particular from members of the *Actinobacteria* (see Table 2). Like their terrestrial counterparts, representatives of marine *Actinobacteria*, even from deep-sea environments, appear to have a promising capacity for the production of secondary metabolites (Bull et al., 2005; Lam, 2006; Fenical and Jensen, 2006). Especially

members of the *Streptomycetes* appear to be well represented producers of novel bioactive structures from the deep ocean (Table 1). Streptokordin e.g. was produced by a *Streptomyces sp.* obtained from Ayu Trough sediment and displayed significant cytotoxicity against human tumor cell lines (IC_{50} < 10 µg/ml) (Jeong et al., 2006). Another strain of *Streptomyces sp*. that has been isolated from 4680 m depth at Marshall Islands produces Gamma-indomycinone, a new compound of the class of pluramycine antibiotics (Schumacher et al., 1995). Anti-angionesis activity is attributed to streptopyrrolidine which was produced by another deep-sea isolate of *Streptomyces sp.*. Further deep-sea natural products are ammosamides A and B (Hughes et al., 2009). These compounds originated form a *Streptomyces* strain from 1618 m depth and exhibit remarkable cytotoxicity against colon carcinoma cells HCT-116. As it becomes obvious, natural products of *Streptomyces sp.* are clearly dominating the list of novel deep-sea substances. This might be due to the fact, that terrestrial *Streptomycetes* produce the main part of bioactive microbial compounds, and thus marine members of *Streptomycetes* are in special focus of natural product research. There are of course also biologically active compounds from other Gram-positive and Gram-negative strains as well (Table 2), but obviously representatives of the *Actinobacteria* appear to be especially promising for novel natural product discovery from deep-sea habitats.

The importance of marine actinomycetes for the discovery of natural products is also reflected by the substantial fraction of the genome (5-10%) that is allocated to mostly cryptic secondary metabolites (Baltz, 2008). Screening for secondary metabolite biosynthetic gene clusters like the non-ribosomal peptide synthetase (nrps) and the polyketide synthases (pks) biosynthesis genes became a common tool for the selection of promising producer strains from various habitats (Metsä Ketelä et al., 1999; Martens et al., 2007; Jiang et al., 2008; Zhao et al., 2008; Schneemann et al., 2010). Non-ribosomal polypeptides, such as the antibiotic vancomycin as well as various polyketides, such as aromatic polyketides (like tetracycline), macrolides (like erythromycine), polyether (like salinomycine) and polyenes (like nystatin), are complex natural products with an immense structural diversity. They are produced by multifunctional enzymes, termed non-ribosomal peptide synthetase (NRPS) and polyketide synthases (PKS) (Cane and Walsh, 1999; Schwarzer and Marahiel, 2001). Bacterial strains from deep-sea sediments have also been effectively screened for nrps or pks gene fragments by PCR-amplification (Pathom-aree et al., 2006). Such

approaches reinforced the presence of a large amount of secondary metabolites producing bacteria in the deep-sea realm. In contrast to the relatively few secondary metabolites isolated from deep-sea environments yet, novel enzymes, lipids and exopolysaccharides with specific properties for biotechnological application have already been isolated from deep-sea bacteria, and in particular from those derived from deep-sea hydrothermal vent fields (for reviews see (Wilson and Brimble, 2009; Pettit, 2010)). Due to the extreme working conditions of biotechnological production steps (like e.g. extreme temperature and pressure), bacteria from extreme environments revealed to be a suitable source for novel compounds with biotechnological use. Thus it might also be auspicious to explore the diversity of secondary metabolites from deep-sea bacteria of different and particularly of contrasting extreme habitats.

Aim of the thesis

The Aim of this thesis was the characterization of deep-sea bacteria and the elucidation of their potential to produce natural products. Accordingly, the major part of this study was focused on cultured bacteria. As bacterial strains from deep-sea habitats are barely investigated for the production of bioactive natural products to date, the research on deep-sea bacteria was conducted in two contrasting deep-sea ecosystems: an extremely oligotrophic environment and a deep-sea hydrothermal vent field (characteristic features are summarized in Table 1).
In both habitats the focus was laid on (a) the deep-sea bacterial communities (b) the physiology and metabolic capabilities of the indigenous bacteria and (c) the discovery of novel natural products from deep-sea bacteria:

(a) Investigation of deep-sea bacterial communities:
The bacterial communities of the LHF and the Eastern Mediterranean deep sea were analyzed by culture-dependent approaches. Especially representatives of novel taxa were of interest, because they might be specially adapted to the specific living conditions and might provide novel natural products. Since the search for and the subsequent production of bioactive natural products requires up-scaling of fermentation processes and a variety of test assays running under standard

laboratory conditions, special interest was laid on the mesophilic fraction of the bacterial communities. The LHF as well as the Eastern Mediterranean deep-sea bacterial community are supposed to comprise a considerable fraction of mesophiles which were the basis of this study. In addition, the barely investigated bacterial community of diffusive fluids from the LHF was analyzed by the onstruction of a 16S rRNA gene library.

(b) Physiological and metabolic capabilities:

Deep-sea bacteria need to be physiologically adapted to the abiotic prerequisites of their habitat, e.g., elevated hydrostatic pressure. Possibly, the production of secondary metabolites may also be affected by these living conditions. One objective of this thesis was therefore the investigation of bacterial taxa cultured after incubation at *in situ* pressure, since bacteria recovered after this treatment can be expected to be specifically adapted to the deep-sea. Further objectives were up-scaled cultivation, extraction and analysis of the respective metabolite spectra as well as the presence of gene clusters responsible for the production of secondary metabolite pathways (e.g. nrps and pks) to evaluate the potential of selected strains for the production of secondary metabolites. Besides these culture-dependent approaches of the metabolic capabilities, the barely investigated bacterial community of the LHF was analyzed for functional genes responsible for autotrophic CO_2-fixation and sulfur oxidation / reduction.

(c) Discovery of novel active compounds from the deep sea:

Since the deep sea appeared to be barely investigated for active compounds to date, this thesis focused on two quite different deep-sea environments. Almost no prior knowledge was available concerning the potential of bioactive metabolites from deep-sea habitats in general and especially from the chosen habitats. Therefore, a major objective was to evaluate the potential of bacteria isolated from the deep sea for the synthesis of novel secondary metabolites.

Thesis outline

The results of this thesis are presented in the following chapters. All chapters are composed in form of manuscripts for publication, some are published (**Chapter III, IV** and **V**) others are submitted (**Chapter I**) or in preparation for submission (**Chapter II** and **IV**). Bacteria of the Eastern Mediterranean deep sea are characterized in **Chapters I – III**, while those of the Logatchev hydrothermal vent field are part of **Chapters IV – VI**.

Finally, the methods, results and future perspectives of the two investigated environments will be discussed **(Discussion)**.

(a) Investigation of deep-sea bacterial communities

Chapter I and **IV** outline the isolation of heterotrophic aerobic bacteria from the two contrasting environments. All strains were classified by 16S rRNA gene sequencing. In addition, the prokaryotic diversity of diffusive fluids from the Logatchev hydrothermal vent Irina II was analyzed by a cultivation-independent approach using environmental 16S rRNA gene clone libraries (see **Chapter VI**). Information about 16S rRNA gene clone libraries of the Eastern Mediterranean Sea sediments was already published by Polymenakou et al. (2005; 2009).

(b) Physiological and metabolic capabilities

The influence of *in situ* pressure incubation on the cultured bacterial community from the Eastern Mediterranean deep sea was an important objective in **Chapter I**. Since Gram-positive bacteria are known to produce a variety of natural products and are frequently found in cultivation-based studies of marine sediments, these organisms were in focus for further research on their metabolic potential (**Chapter II**). The bacterial strains isolated from the Logatchev hydrothermal vent field were also subjected to antimicrobial activity tests (**Chapter IV**). The physiological capabilities of one bioactive strain, strain M41^T, with only 93 % 16S rRNA similarity to the next related type strains were further investigated and the strain was assigned to the type strain of the novel genus and species *Amphritea atlantica*^{T}. A detailed description of the physiological capabilities of strain M41^T is given in **Chapter V**. In addition to studies on cultured bacterial strains the metabolic diversity of the bacterial

community of diffuse fluid from the Logatchev hydrothermal was investigated using functional key genes of CO_2-fixation pathways and the sulfur cycle (**Chapter VI).**

(c) Discovery of novel natural products from the deep sea

The isolation-based studies in order to search for novel bioactive compounds from deep-sea bacteria are described in **Chapters II** and **IV**. Since new and promising compounds could be isolated from a strain obtained from deep Eastern Mediterranean sediments, the production, isolation, structure elucidation and biological activity of these compounds are presented in this **Chapter** (**III)**.

Chapters

CHAPTER I

Isolation and characterization of bacteria from the Eastern Mediterranean deep sea

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Submitted to

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Abstract:

The Eastern Mediterranean deep sea is one of the most oligotrophic regions in the world's ocean. With the aim to classify bacteria from this special environment we isolated 107 strains affiliating to the *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* from sediments of the Eastern Mediterranean Sea. As determined by 16S rRNA gene sequence analysis, *Actinobacteria* and *Firmicutes*, in particular members of the genus *Bacillus*, were dominant and represented a remarkable diversity with 27 out of a total of 33 operational taxonomic units obtained from the untreated sediment. The considerable percentage of strains (27%) which may be considered to be new species underlines the uniqueness of the studied environment. In order to selectively enrich bacteria which are adapted to the deep-sea conditions and tolerate broad pressure ranges, enrichments were set up with a sediment sample under *in situ* pressure and temperature (280 bar, 13.5°C) using N-acetyl-D-glucosamine as substrate. Quite interestingly *Gammaproteobacteria* were significantly enriched and dominant among the strains isolated after pressure pre-incubation. Obviously, *Gammaproteobacteria* have a selective advantage under the enrichment conditions applied mimicking nutrient supply under pressure conditions and cope well with sudden changes of hydrostatic pressure. However, under the continued low nutrient situation in the Eastern Mediterranean deep-sea sediments apparently *Firmicutes* and *Actinobacteria* have a clear advantage.

Introduction

Studies on microbial communities in marine deep-sea sediments frequently focus on highly productive spots, like hydrothermally active regions. However, the major fraction of deep-sea sediments represents extremely oligotrophic environments subjected to high hydrostatic pressures. This holds especially true for the Eastern Mediterranean Sea (Lampadariou et al., 2009). The extreme depletion of nutrients in surface waters results in low primary production. Only a minor fraction of the produced organic matter is reaching the deep sea (1% according to Danovaro et al. 1999) and the sediments (Psarra et al., 2000). Organic carbon values of the Southern Cretan Margin sediments were found to be as low as 0.07- 1.55% of sediment dry weight (Polymenakou et al., 2008). The Eastern Mediterranean deep sea is furthermore characterized by comparably high temperatures of approximately 14°C opposite to most other deep-sea environments with temperatures of 2-4°C. The sediments of the Eastern Mediterranean deep sea thus combine different environmental features (extreme oligotrophy, high hydrostatic pressure and comparably high deep-water temperature) which may be in favour of the development of unique types of bacteria.

Environmental 16S rRNA gene libraries of sediment samples from the Eastern Mediterranean have revealed high bacterial diversity. Sequences assigned to the *Acidobacteria* (18%), *Gammaproteobacteria* (13%), *Planctomycetes* (11%), *Actinobacteria* (11%), *Alphaproteobacteria* (10%) and *Deltaproteobacteria* (9%) were most abundant (Polymenakou et al., 2009). There is, however, a considerable lack of cultivation-based studies of bacteria that is essential to enlighten their ecological role in natural environments (Das et al., 2006).

Low nutrient concentrations prevailing in marine deep-sea habitats have been taken into account in cultivation-based studies since the 1990s (Rappé et al., 1999; Rueger and Tan, 1992) . The use of low nutrient media turned out to significantly improve the cultivation efficiency (Koch, 2001; Rueger and Tan, 1992; Carlucci et al., 1986) and the growth of copiotrophic and fast growing bacteria is retarded in low-nutrient media giving a chance for growth of oligotrophic bacteria.

In the present study bacteria from Mediterranean deep sea sediments were characterised by culture-dependent studies using different media with low nutrient content, enrichments under pressure and with the chitin monomer N-acetyl-D-glucosamine (NAG) as supplement.

Materials & Methods

Sample collection

All samples were obtained southwards of Crete during Meteor cruise 71 leg 2 in December 2006 - January 2007. Sediment was collected from the Ierapetra Basin at 4400 m (34°30.296N, 26°11.507E) and the Herodotos Plain at 2800 m (33°42.989N, 26°20.329E) using a multiple corer. Of each sediment core the uppermost 5 cm were aseptically sub sampled. The deep-sea water column (> 500 m referring to Kontoyiannis et al. (1999)) was sampled at the same locations by a rosette sampler equipped with 24 Niskin Bottles connected to a CTD probe.

Isolation and cultivation

For standard isolation of aerobic bacteria from the sediment, dilutions of 10^{-1}- 10^{-4} in sterile Mediterranean seawater were prepared and plated on five different agar media. MW-medium: 1.5% agar added to Mediterranean seawater; MWY-medium: 1.5% agar, 0.01% yeast extract in Mediterranean seawater; TSB0.1-medium: 1.5% agar, 0.01% tryptic soy broth, 3% NaCl in $Aqua_{demin}$; CFB-medium: 1.5% agar, 0.1% tryptone, 0.05% yeast extract, 0.05% $CaCl_2x2H_2O$, 0.05% $MgCl_2x7H_2O$ in Mediterranean seawater; Chitin-medium: 1.5% agar, 0.2% chitin in Mediterranean seawater. Water samples of different depths were treated equally using dilutions of 10^0 and 10^{-1}. Agar plates (two parallels each) were incubated at onboard room temperature for up to three months and checked frequently for growth.

Pre-incubation of sediment at *in situ* pressure. In order to selectively enrich deep-sea bacteria that are adapted to elevated hydrostatic pressure and spontaneous nutrient input, we pre-incubated a sediment sample from 2800 m depth (Herodotos Plain) at 280 bar (13.5°C) in supplemented sea water and N-acetyl-D-glucosamine prior to plating on identical media and incubation at identical conditions as described above. For this purpose 10 ml of sediment were transferred into a plastic bag with 9 ml of sterile-filtered seawater (taken from the multicorer) and 1 ml solution of N-acetyl-D-glucosamine (100 µM). N-acetyl-D-glucosamine (the monomere of chitin) is supposed to be an important carbon and nitrogen source for the microbial deep-sea community. Using compression-proof steel tubes *in situ* hydrostatic pressure (280

bar) was impressed and the tubes were incubated for 6 d at *in situ* temperature (13.5°C). After this incubation, 10^{-1} and 10^{-2} dilutions were plated directly on the above-mentioned agar media and incubated under the standard conditions used throughout these experiments.

Using a binocular microscope, all colonies appearing morphologically different were transferred to fresh agar medium until pure cultures were obtained. Strains were identified and checked for purity by microscopy and sequencing of the 16S rRNA gene. For long term storage, all strains were kept at -80°C using the Cryobank system (Mast Diagnostica GmbH).

Testing the growth properties of selected strains

Selected strains of all operational taxonomic units (strains are marked by an asterisk in Table 1) were grown i.) in nutrient-rich Marine Broth medium at *in situ* temperature ii.) in oligotrophic MW-medium (containing solely autoclaved oligotrophic Mediterranean Sea water) using room temperature and iii.) in low-nutrient medium MWY (containing autoclaved oligotrophic Mediterranean Sea water with 0.01 % yeast extract) at *in situ* temperature of 13.5°C.

DNA extraction, PCR and 16S rRNA gene sequencing was performed according to Gärtner et al. (2008). 16S rRNA gene sequences determined during this study were deposited in the EMBL Nucleotide Sequence Database and were assigned accession no. FM992709-FM992846 and FN179280.

Sequence analysis. Sequences were edited by ChromasPro v.1.33 and were compared to the NCBI database using BLAST (see http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990)). Subsequently, all sequences were aligned to the ARB database (see http://www.arb-home.de, (Ludwig, 2004)) using the integrated aligner function. Type strains most closely related to the isolates according to BLAST were added to the ARB database when not already present. Alignments were refined manually and aligned sequences were added to the ARB tree using the quick-add-marked function (Parsimony). According to the results from Stackebrandt and Ebers (2006) strains showing sequence similarities < 98.7% were assigned to different operational taxonomic units (OTUs) using MOTHUR software (http://www.mothur.org/).

Phylogenetic trees. Phylogenetic trees were calculated applying maximum likelihood analysis using PHYML (Guindon and Gascuel, 2003). Maximum Likelihood analysis was performed assuming the GTR model (Keane et al., 2006) with an optimised gamma distribution parameter alpha and 100 bootstrap replicates.

Results

Phylogenetic affiliation of the strains

Strains isolated from the sediment. Using low-nutrient media, colony numbers of up to 2.1 x 10^2 CFU / cm^3 sediment were determined. Just slightly higher cell numbers (2.4 x 10^2 CFU / cm^3 sediment) were obtained with sediment samples using the CFB medium which had the highest nutrient content. Altogether 65 strains were obtained from the Eastern Mediterranean sediment (37 strains from sediment of the Ierapetra basin and 28 strains from the Herodotos plain). The strains isolated from the IB and HP sediments revealed a similar distribution among phylogenetic groups (Figure I.1).

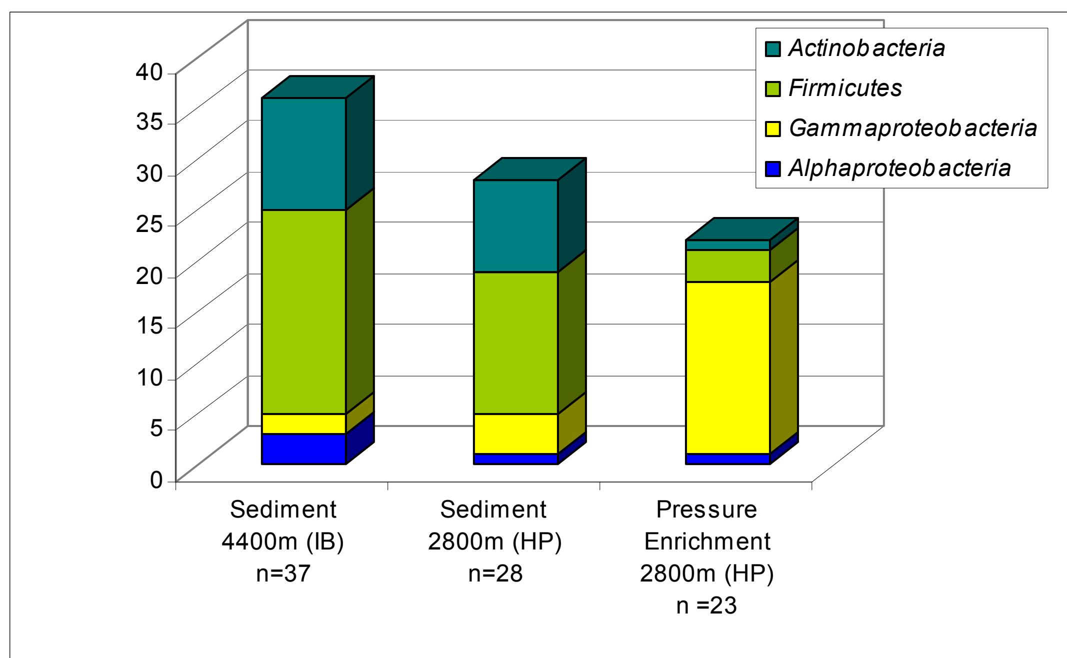

Figure I.1: Total number of strains obtained from the two sampling sites (Ierapetra Basin (IB), 4400m and Herodotos Plain (HP), 2800m) and their phylogenetic affiliation compared to the pressurized sediment enrichment.

The majority of strains affiliated to the *Firmicutes* (34 of 65 strains, 52%) and *Actinobacteria* (21 of 65 strains, 32%) with only few representatives of the *Alpha-* and *Gammaproteobacteria* (21 of 65 strains, 32%) (Figures I.2 and I.4). *Firmicutes* and *Actinobacteria* were dominant among the isolated strains of the untreated sediment and represented highly diverse groups with 17 OTUs of *Firmicutes* and 10 OTUs of *Actinobacteria* (Table I.1). Especially the genus *Bacillus* was isolated with a remarkable high number of OTUs (16 OTUs). Some of the *Firmicutes* and *Actinobacteria* were exclusively isolated using Mediterranean Sea Water medium (MW-medium) without additional nutrients (OTUs 22, 23, 24 and 30). Strains with close relationship to known species (≥98.7%) are summarized in Table I.1. Thirteen strains represented putative new species. These strains affiliated to the genera *Micromonospora* (strains S32a and S29), *Pseudonocardia* (strains S82 and S78), *Streptomyces* (strains S06 and D92), *Halobacillus* (strain S40) and *Bacillus* (strains S54, D89, S33, D94, S10, S11, D87 and S44x).

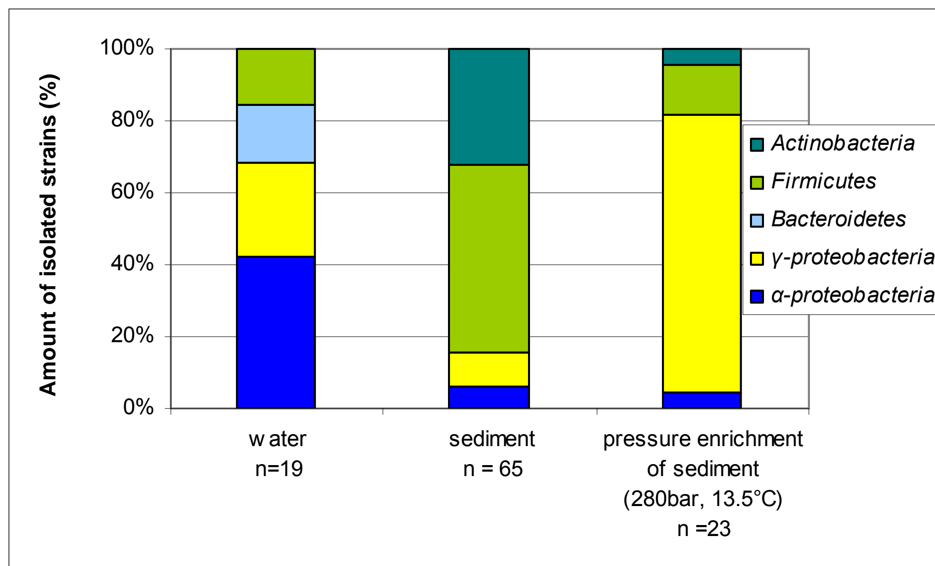

Figure I.2: Phylogenetic affiliation of all strains (%) obtained from water, sediment and the pressurized sediment enrichment.

Table I.1: Origin, isolation conditions and next related validly described type strains of all strains

Isolate	Accession-number	Next related type strain (and accession number)	Similarity	OTU	Isolation-material / location	Medium
		Gammaproteobacteria				
D34	FM992789	*Pseudoalteromonas elyakovii,* KMM162^T (AF082562)	99.5	1	PE /HP	MWY
S09 *	FM992775	*Pseudoalteromonas elyakovii,* KMM162^T (AF082562)	99.5	1	S / HP	Chitin
W03x	FM992785	*Pseudoalteromonas elyakovii,* KMM162^T (AF082562)	99.6	1	W /HP	MWY
D39 *	FM992717	*Alteromonas marina,* JCM11804^T (AF529060)	98.6	2	PE /HP	TSB
D48	FM992783	*Alteromonas marina,* JCM11804^T (AF529060)	98.8	2	PE /HP	MWY
D50x	FM992790	*Alteromonas marina,* JCM11804^T (AF529060)	98.8	2	PE /HP	TSB
N102	FM992770	*Alteromonas marina,* JCM11804^T (AF529060)	99,0	2	W / IB	MW
N105	FM992800	*Alteromonas marina,* JCM11804^T (AF529060)	99.6	2	W / IB	MWY
D42 *	FM992781	*Alteromonas marina,* JCM11804^T (AF529060)	97.7	3	PE /HP	Chitin
D33 *	FM992780	*Alteromonas litorea, JCM12188^T (AY428573)*	97.4	4	PE / HP	MWY
D45	FM992719	*Alteromonas addita,* LMG22532^T (AY682202)	97.2	5	PE / HP	MWY
D56 *	FM992724	*Alteromonas addita,* LMG22532^T (AY682202)	97.3	5	PE / HP	MWY
D47 *	FM992720	*Alteromonas addita,* LMG22532^T (AY682202)	96.5	6	PE / HP	MWY
D37	FM992715	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	99.8	7	PE / HP	MWY
D38	FM992716	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	99.7	7	PE / HP	TSB
D50	FM992722	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	99.8	7	PE / HP	TSB
D43 *	FM992782	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	100.0	7	PE / HP	Chitin
W01	FM992760	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	98.4	8	W / HP	MW
W29 *	FM992767	*Pseudomonas stutzeri,* ATCC17588^T (AF094748)	98.4	8	W / HP	MW
D06 *	FM992773	*Marinobacter flavimaris,* DSM16070^T (AY517632)	99.3	9	PE /HP	MWY
D36	FM992714	*Marinobacter flavimaris,* DSM16070^T (AY517632)	99.3	9	PE / HP	MWY
D35x *	FM992844	*Marinobacter flavimaris,* DSM16070^T (AY517632)	98.9	10	PE / HP	MWY
S30b	FM992753	*Marinobacter flavimaris,* DSM16070^T (AY517632)	98.8	10	S / IB	CFB
D95	FM992736	*Microbulbifer agarilyticus,* DSM19200^T (AB158515)	99.4	11	S / IB	MW
S58 *	FM992795	*Microbulbifer agarilyticus,* DSM19200^T (AB158515)	99.3	11	S / HP	Chitin
D02	FM992709	*Stenotrophomonas maltophilia,* DSM50170^T (AY484506)	99.4	12	PE / HP	CFB
D49	FM992721	*Stenotrophomonas maltophilia,* DSM50170^T (AY484506)	99.4	12	PE / HP	TSB
S02 *	FM992745	*Stenotrophomonas maltophilia,* DSM50170^T (AY484506)	99.0	12	S / HP	MWY
S57	FM992758	*Stenotrophomonas maltophilia,* DSM50170^T (AY484506)	99.6	12	S / HP	MWY
		Bacteroidetes				
W34	FM992769	*Leeuwenhoekiella blandensis,* CECT7118^T (DQ294290)	99.4	13	W / IB	TSB
W32 *	FM992768	*Leeuwenhoekiella blandensis,* CECT7118^T (DQ294290)	97.5	13	W / IB	MWY
W27 *	FM992766	*Pontibacter korlensis,* NRRL B-51097^T (DQ888330)	96.5	14	W / IB	MWY
		Alphaproteobacteria				
D82	FM992732	*Erythrobacter flavus,* JCM11808^T (AF500004)	100.0	15	S / IB	TSB
S36	FM992755	*Erythrobacter flavus,* JCM11808^T (AF500004)	99.9	15	S / HP	MWY
S42	FM992756	*Erythrobacter flavus,* JCM11808^T (AF500004)	99.9	15	S / IB	MWY
N106	FM992771	*Erythrobacter flavus,* JCM11808^T (AF500004)	100.0	15	W / IB	MWY
W04	FM992761	*Erythrobacter flavus,* JCM11808^T (AF500004)	99.9	15	W / HP	MWY
W20	FM992765	*Erythrobacter flavus,* JCM11808^T (AF500004)	99.9	15	W / HP	MW
N109	FM992798	*Erythrobacter vulgaris,* KMM3465^T (AY706935)	98.9	15	W / IB	CFB
W19 *	FM992764	*Erythrobacter vulgaris,* KMM3465^T (AY706935)	98.8	15	W / IB	MWY
D53 *	FM992723	*Sphingobium yanoikuyae,* ATCC51230^T (D16145)	99.4	16	PE / HP	CFB
N108 *	FM992772	*Citreicella thiooxidans,* DSM10146^T (AY639887)	95.7	17	W / HP	MWY
W14 *	FM992763	*Paracoccus alcaliphilus,* DSM8512^T (AY014177)	94.9	18	W / HP	CFB
S30a	FM992752	*Aurantimonas coralicida,* DSM14790^T (AJ86361)	99.7	19	S / IB	CFB
W05x *	FM992793	*Aurantimonas coralicida,* DSM14790^T (AJ86361)	99.7	19	W / HP	MWY

Table I.1: continued

Isolate	Accession-number	Next related type strain (and accession number)	Similarity	Phylotype	Isolation-source	Medium
		Actinobacteria				
S20	FM992749	*Micromonospora auratinigra*, DSM44815^T (AB159779)	99.2	20	S / HP	CFB
S32a	FM992754	*Micromonospora auratinigra*, DSM44815^T (AB159779)	98.3	20	S / HP	MW
S61	FM992759	*Micromonospora auratinigra*, DSM44815^T (AB159779)	99.1	20	S / HP	Chitin
S71	FM992746	*Micromonospora auratinigra*, DSM44815^T (AB159779)	99.2	20	S / IB	MWY
S51 *	FM992776	*Micromonospora endolithica*, DSM44398^T (AJ560635)	98.9	20	S / IB	CFB
S29 *	FM992751	*Micromonospora coxensis*, JCM13248^T (AB241455)	98.5	21	S / IB	CFB
S78 *	FM992777	*Pseudonocardia xinjiangensis*, DSM44661^T (EU722520)	98.3	22	S / HP	MW
S82 *	FM992788	*Pseudonocardia xinjiangensis, DSM44661*^{T} (EU722520)	97.5	23	S / HP	MW
S80 *	FM992787	*Blastococcus aggregatus*, DSM4725^T (L40614)	99.6	24	S / HP	MW
S35 *	FM992796	*Corynebacterium pseudodiphtericum*, DSM44287^T (X81918)	99.3	25	S / HP	CFB
D126	FM992744	*Streptomyces sampsonii*, ATCC25495^T (D63871)	99.9	26	S / IB	MWY
D81 *	FM992731	*Streptomyces sampsonii*, ATCC25495^T (D63871)	99.9	26	S /IB	Chitin
D84	FM992733	*Streptomyces sampsonii*, ATCC25495^T (D63871)	99.9	26	S / IB	CFB
D85	FM992734	*Streptomyces sampsonii*, ATCC25495^T (D63871)	99.9	26	S / IB	CFB
D92	FM992735	*Streptomyces sampsonii*, ATCC25495^T (D63871)	98.2	26	S / IB	Chitin
S26	FM992750	*Streptomyces sampsonii*, ATCC25495^T (D63871)	99.9	26	S / IB	TSB
S28	FM992799	*Streptomyces sampsonii*, ATCC25495^T (D63871)	100.0	26	S / IB	MWY
S06 *	FM992747	*Streptomyces flavofuscus*, ATCC19908^T (DQ026648)	97.8	27	S / HP	TSB
D44	FM992718	*Micrococcus luteus*, ATCC4698^T (AF542073)	99.3	28	PE / HP	MWY
S37	FM992797	*Micrococcus luteus*, ATCC4698^T (AF542073)	98.9	28	S / IB	MW
S43 *	FM992757	*Micrococcus luteus*, ATCC4698^T (AF542073)	99.6	28	S / IB	MW
S08 *	FM992748	*Arthrobacter tecti*, LMG22282^T (AJ639829)	98.8	29	S / HP	Chitin
		Firmicutes				
S40 *	FM992845	*Halobacillus karajiensis*, DSM14948^T (AJ486874)	98.3	30	S / HP	MW
S54 *	FM992841	*Bacillus aerophilus*, ATCC10840^T (AJ831844)	95.2	31	S / IB	CFB
D11	FM992803	*Bacillus algicola*, KMM3737^T (AJ831844)	99.8	32	S / IB	MWY
S14	FM992821	*Bacillus algicola*, KMM3737^T (AJ831844)	99.4	32	S / HP	MWY
S32	FM179280	*Bacillus algicola*, KMM3737^T (AJ831844)	99.9	32	S / HP	MW
S04	FM992843	*Bacillus algicola*, KMM3737^T (AJ831844)	99.8	32	S / HP	MWY
S38	FM992825	*Bacillus algicola*, KMM3737^T (AJ831844)	99.8	32	S / IB	MWY
S03 *	FM992831	*Bacillus algicola*, KMM3737^T (AJ831844)	99.8	32	S / HP	MWY
D26 *	FM992805	*Bacillus hawajinpoensis*, JCM11807^T (AF541966)	100.0	33	S / IB	CFB
D92x	FM992814	*Bacillus hawajinpoensis*, JCM11807^T (AF541966)	99.0	33	S / IB	MWY
D89 *	FM992834	*Bacillus decolorationis*, LMG19507^T (AJ315075)	97.0	34	S / IB	Akt
S33	FM992824	*Bacillus decolorationis*, LMG19507^T (AJ315075)	97.8	34	S / HP	Chitin
D81a	FM992811	*Bacillus barbaricus*, DSM14730^T (AJ422145)	99.6	35	S / IB	Chitin
S19	FM992822	*Bacillus barbaricus*, DSM14730^T (AJ422145)	99.6	35	S / HP	CFB
D96	FM992837	*Bacillus barbaricus*, DSM14730^T (AJ422145)	99.6	35	S / IB	MW
D79 *	FM992809	*Bacillus barbaricus*, DSM14730^T (AJ422145)	99.6	35	S / IB	MW
W15 *	FM992786	*Staphylococcus pettenkoferi*, CIP107711^T (AF322002)	99.9	36	W / HP	CFB
D35a	FM992801	*Bacillus subtilis*, DSM10^T (AJ276351)	99.9	37	PE / HP	MWY
D80	FM992810	*Bacillus subtilis*, DSM10^T (AJ276351)	99.7	37	S / IB	CFB
D88 *	FM992813	*Bacillus subtilis*, DSM10^T (AJ276351)	100.0	37	S / IB	CFB
S30	FM992823	*Bacillus subtilis*, DSM10^T (AJ276351)	99.9	37	S / IB	CFB
S52	FM992829	*Bacillus subtilis*, DSM10^T (AJ276351)	99.7	37	S / IB	CFB
W05	FM992762	*Bacillus subtilis*, DSM10^T (AJ276351)	99.9	37	W / HP	MWY
S49 *	FM992828	*Bacillus licheniformis*, DSM13^T (X68416)	99.9	38	S / IB	CFB
S47	FM992840	*Bacillus licheniformis*, DSM13^T (X68416)	99.2	38	S / IB	CFB
S53 *	FM992830	*Bacillus indicus*, DSM16189^T (AJ583158)	100.0	39	S / IB	CFB
D94 *	FM992835	*Bacillus licheniformis*, DSM13^T (X68416)	93.9	40	S / IB	MWY
S10 *	FM992819	*Bacillus novalis*, DSM15603^T (AJ542512)	96.4	41	S / HP	Chitin
D32 *	FM992832	*Bacillus boroniphilus*, DSM17376^T (AB198719)	99.9	42	S / HP	TSB
S46	FM992827	*Bacillus boroniphilus*, DSM17376^T (AB198719)	99,0	42	S / IB	CFB
S11 *	FM992820	*Bacillus foraminis*, LMG23174^T (AJ717382)	98.4	43	S / HP	Chitin
D87 *	FM992812	*Bacillus foraminis*, LMG23174^T (AJ717382)	98.3	44	S / IB	CFB
D29a *	FM992806	*Bacilllus drentensis*, LMG21831^T (AJ542506)	99.7	45	S / HP	CFB
D52 *	FM992833	*Bacillus muralis*, LMG20238^T (AJ628748)	99.5	46	PE /HP	CFB
D31	FM992807	*Bacillus muralis*, LMG20238^T (AJ628748)	99.6	46	S / HP	MWY
S05a	FM992818	*Bacillus muralis*, LMG20238^T (AJ628748)	99.6	46	S / HP	MWY
S44x	FM992826	*Bacillus muralis*, LMG20238^T (AJ628748)	98.5	46	S / IB	CFB
D04 *	FM992802	*Bacillus circulans*, DSM11^T (AY724690)	97.0	47	PE / HP	CFB
N104b *	FM992794	*Bacillus massiliensis*, CIP108446^T (AY677116)	96.8	48	W / IB	MWY
S33x *	FM992838	*Bacillus infantis*, JCM13438^T (AY904032)	99.7	49	S / HP	Chitin

S= sediment; W= water; PE = pressurized sediment enrichment; IB = Ierapetra basin; HP = Herodotos plain;**;** * = selected strains for growth experiments on MWY medium (one per OTU)

Strains isolated from the deep-sea water. To demonstrate differences between strains inhabiting the sediment and the deep water, isolates from deep water samples of both stations (cell counts were 1.7 x 10^1 CFU / ml deep-sea water, 19 strains obtained in total) were obtained and compared to those isolated from the sediment. According to their phylogenetic position, strains isolated from the deep water differed substantially from those isolated from the sediments. The 19 isolates grouped in 12 different OTUs: 3 (5 strains) *Gammaproteobacteria*, 2 (3 strains) *Bacteroidetes*, 4 (8 strains) *Alphaproteobacteria*, 3 (3 strains) *Firmicutes* (Figures I.2 – 1.4, Table I.1). Members of the *Bacteroidetes* were exclusively obtained from the deep water and all of them showed sequence similarities below 98.7 % to known species. Other isolates from the deep-sea water possibly can be assigned to new species assigned to the genera of *Pseudomonas* (strains W01 and W29), *Citreicella* (strain N108), *Paracoccus* (strain W14), *Leeuwenhoekiella* (strain W32) and *Pontibacter* (strain W27).

Strains isolated after pressure incubation. Pre-incubation was performed to selectively enrich bacteria supposed to be well adapted to the extreme conditions of the deep Mediterranean (high deep-sea temperatures, increased hydrostatic pressure, oligotrophy). Noteworthy, CFU were increased at least tenfold compared to plating of sediment samples without pre-incubation. The 23 strains obtained after pressure incubation grouped into 15 different OTUs (10 *Gammaproteobacteria*, 3 *Firmicutes*, 1 each *Alphaproteobacteria* and *Actinobacteria*). Interestingly, the *in situ* pressure pre-incubation experiment yielded a considerable increase in the number of *Gammaproteobacteria* (78% of all isolates obtained from the pressure experiment of the Herodotos Plain compared to 14% from the untreated sediment of this location (Figure I.4, Table I.1)). In contrast, the proportion of *Firmicutes* strains was drastically reduced (13% from the pressure experiment strains, compared to 50% of the Herodotos Plain sediment strains (Table I.1, Figure I.4)). Noteworthy, 8 OTUs were exclusively obtained after this treatment and could not be isolated from the untreated sediment (Figure I.3). Most of these (7) are likely to represent new species and were assigned to the genera *Alteromonas* (strains D42, D39, D33, D45, D47 and D56) and *Bacillus* (strain D04).

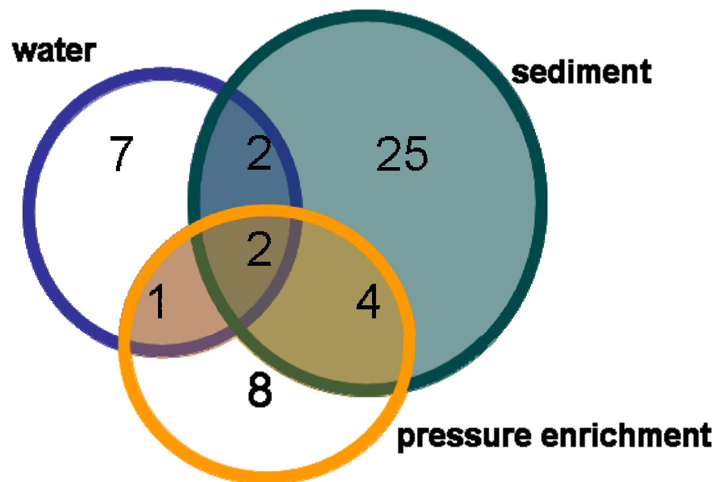

Figure I.3: Number of unique and shared OTUs from the water (n = 12), the sediment (n = 33) and the pressurized sediment enrichment (n = 15).

Growth under selected in situ conditions

The strains isolated during this study originate from media with different nutrient concentrations. All tested strains were able to grow in oligotrophic Mediterranean Sea water medium (MW) and also on Marine Broth medium with elevated nutrient concentrations. All strains could grow at room temperature as well as at the lower in situ temperature of 13.5°C. Apparently growth at the lower temperature was dependent on the medium and nutrient concentration. Cultivation experiments using MWY, which contained 0.01 % yeast extract, revealed that the great majority of the selected strains grew well under experimental low-nutrient conditions and *in situ* temperature of 13.5°C. Strains that did not grow under these conditions were *Bacillus sp*. D29a, *Corynebacterium sp*. S35 and *Staphylococcus* sp. W15. These two did grow, however, at 13.5°C when the nutrient concentration was increased. Thus, most of the isolates were able to grow under *in situ* conditions (temperature and nutrient conditions) prevailing in the Eastern Mediterranean deep sea.

This includes strains of *Alteromonas spp.* (D33, D42, D47 and D56), *Pseudomonas sp.* D43 and *Sphingobium sp.* D53, which were enriched and isolated after incubation at *in situ* pressure and *in situ* temperature with N-acetyl-D-glucosamine as nutrient source. N-acetyl-D-glucosamine, the monomere of chitin, is supposed to be an important carbon and nitrogen source for the microbial deep-sea community. Quite interestingly, most gammaproteobacterial strains recovered from the pressure enrichment were isolated from low-nutrient seawater media (MW, MWY or Chitin-medium) which are most similar to the incubation-medium during pressure enrichment.

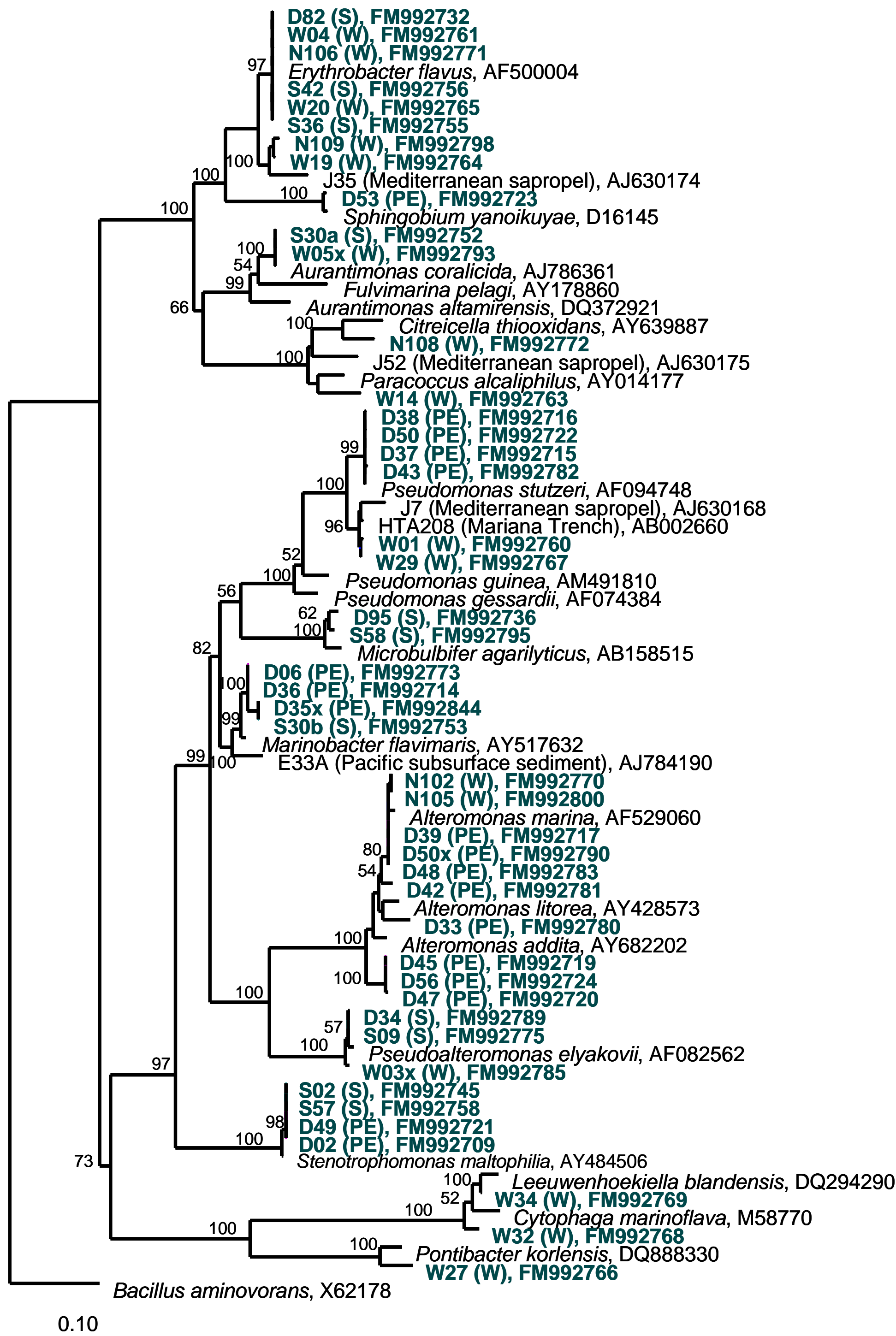

Figure I.4: Maximum likelihood tree of the 16S rRNA gene sequences of all Gram-negative strains obtained from sediment and water samples.
Numbers on nodes represent bootstrap percentages >50, calculated from 100 replicates.
(S) = isolate from sediment; (PE) = isolate from pressure experiment; (W) = isolate from water column

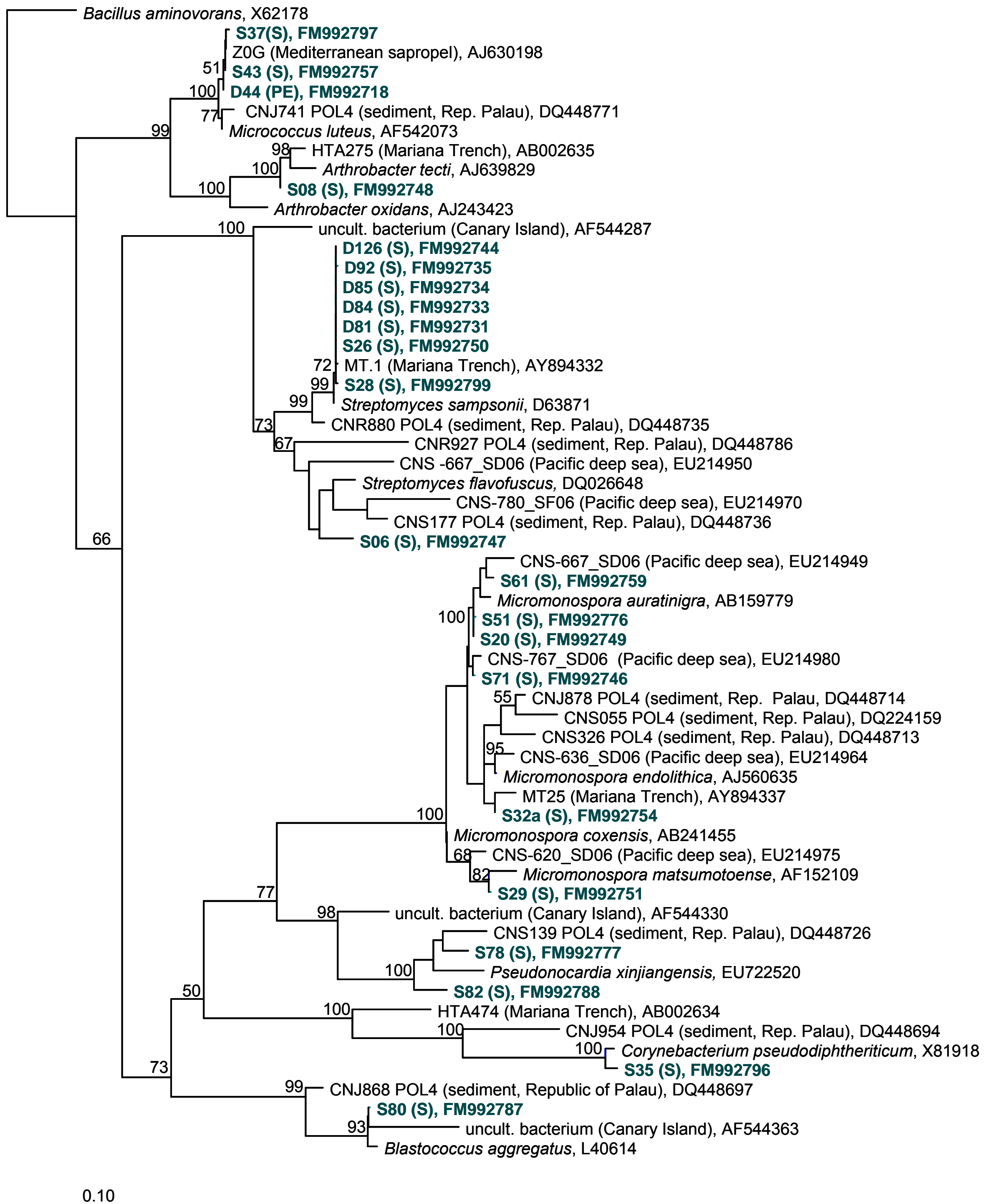

Figure I.5: Maximum likelihood tree of the 16S rRNA gene sequences of *Actinobacteria* strains obtained from sediment and water samples.
Numbers on nodes represent bootstrap percentages >50, calculated from 100 replicates. (S) = isolate from sediment; (PE) = isolate from pressure experiment; (W) = isolate from the water column

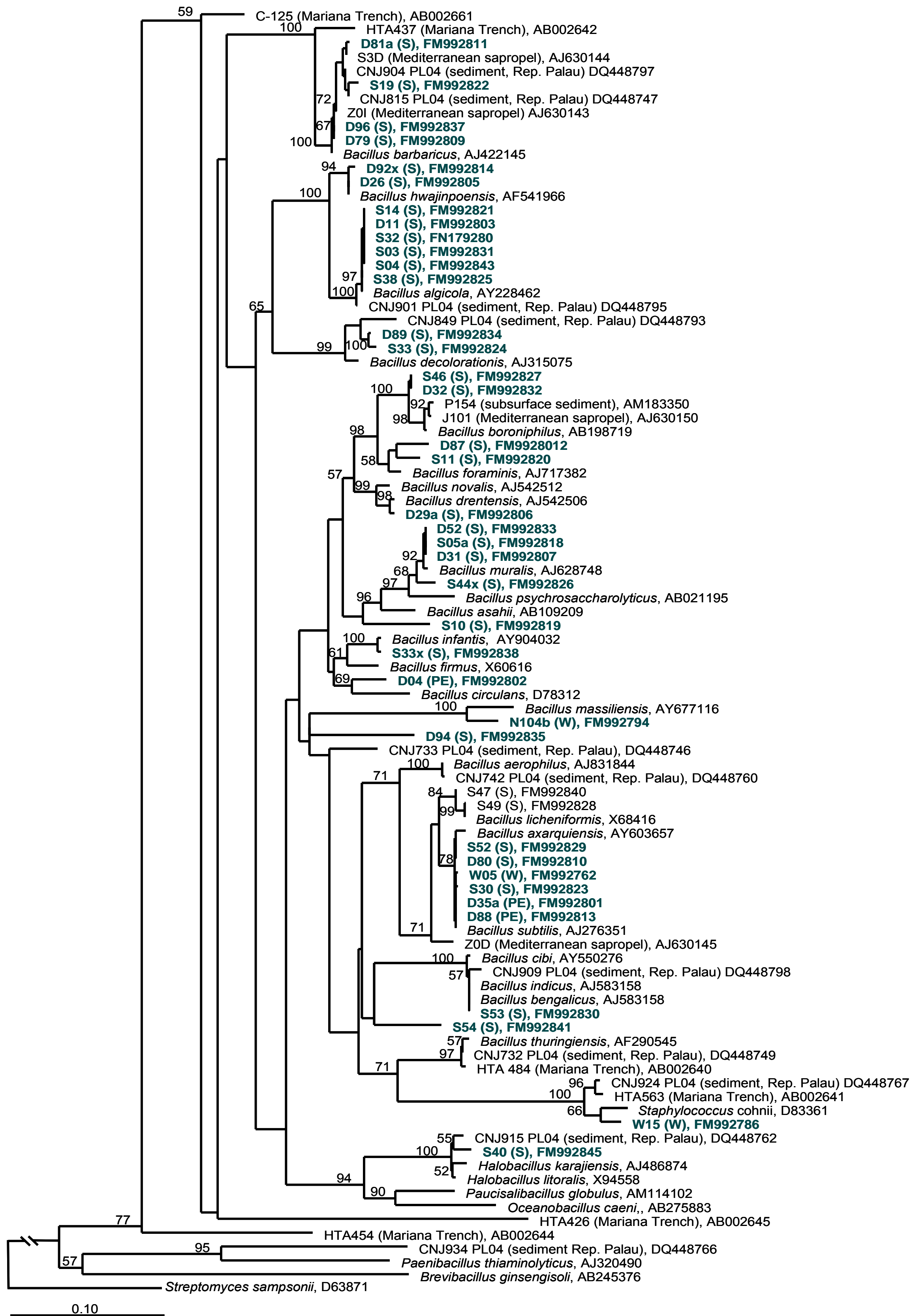

Figure I.6: Maximum likelihood tree of the 16S rRNA gene sequences of *Firmicutes* strains obtained from sediment and water samples.
Numbers on nodes represent bootstrap percentages >50, calculated from 100 replicates. (S) = isolate from sediment; (PE) = isolate from pressure experiment; (W) = isolate from the water column

Discussion

Isolation from sediment samples

Gram-positive bacteria were clearly dominant among the strains isolated from the Eastern Mediterranean deep-sea sediment. This is in accordance to the frequent isolation of mainly *Firmicutes* and *Actinobacteria* from diverse marine habitats (Prieto-Davó et al., 2008; Gontang et al., 2007; Stevens et al., 2007; Pathom-aree et al., 2006; Jensen et al., 2005; Koepke et al., 2005; Süß et al., 2004; Siefert et al., 2000; Yanagibayashi et al., 1999; Colquhoun et al., 1998; Marteinsson et al., 1996). Members of the *Firmicutes* are moreover considered to be the most frequently isolated strains from subsurface sediments (D'Hondt et al., 2004).

In contrast to the high isolation frequency of Gram-positive bacteria, they were of minor relevance in 16S rRNA gene libraries of Mediterranean deep-sea sediments (Heijs et al., 2008). *Actinobacteria* accounted for 4 – 28% of total sequences in clone libraries of Cretan margin sediments, while *Firmicutes* accounted for a maximum of 3% (Polymenakou et al., 2009). Both taxonomic groups might well be under-represented in 16S rRNA gene based molecular approaches, most likely because they often resist commonly applied DNA extraction techniques or may be missed due to primer biases (Carrigg et al., 2007; Mincer et al., 2005; McVeigh et al., 1996). The high abundance of *Firmicutes* isolated during this study and in particular the remarkable high diversity of different operational taxonomic units of the genus *Bacillus* demonstrates the importance of culture-dependent techniques as an additional tool to assess the microbial diversity.

Members of the genus *Bacillus* are ubiquitous, but very little is known about the metabolic activity of *Bacillus sp*. in marine sediments. *Bacillus* species frequently recovered from different marine environments are *B. marinus, B. badius, B. subtilis, B. cereus, B. licheniformis, B. firmus, B. lentus* and *B. pumilis* (Ivanova et al., 2010). *B. marinus* e.g. was isolated from tropical Atlantic, Antarctic and Arctic deep-sea sediments and turned out to be psychrophilic or psychrotolerant (Rüger et al., 2000). Closest phylogenetic relatives to isolates assigned to *Bacillus* obtained during this study originated from various environments and varied in their physiological properties. Though inference of physiological properties from those of closest phylogenetic relatives is possible to a limited extent, with some probability it may be used to delineate key properties of genetic relatives. With consideration of these limitations, it is interesting to see that closest phylogenetic relatives of various

sediment isolates (S10: *Bacillus novalis* DSM 15603^T, S44x: *Bacillus muralis* LMG 20238^T and S11/D87: *Bacillus foraminis* LMG 23174^T) were described as capable of reducing nitrate to nitrite (Heyrman et al. 2004, 2005, Targo et al. 2006) which may be an important metabolic process in the basically oxic oligotrophic sediment. Furthermore, *Bacillus licheniformis* as closest relative to isolate D94 is known for its specific strategy to cope with nutrient limitation (LeTi et al. 2006), which is important for adaptation to the extreme oligotrophy prevailing in the deep Eastern Mediterranean.

Interestingly, the Mariana Trench sediment was also dominated by Gram-positive bacteria and *Bacillus sp.* accounted for 25 % thereof (Takami et al., 1997) (Figure I.5). In addition, isolates from the present study assigned to *Micromonospora* and *Streptomyces* have close relatives to those recovered from the Mariana Trench (Pathom-aree et al., 2006) (Figure I.6). This close affiliation of strains indicates their possibly wide distribution in deep sea sediments. More distantly related OTUs of our isolates might represent bacteria specifically adapted to the conditions of the Mediterranean Sea.

Isolation after enrichment under *in situ* pressure

Pressure incubation of the sediments clearly shifted the composition towards *Gammaproteobacteria* which is considered to reflect their ecological importance in the nutrient degradation of the deep sea. Apparently, they cope very well with changes of hydrostatic pressure as they have been decompressed during sampling, recompressed to *in situ* pressure and again decompressed for cultivation. The majority of gammaproteobacterial OTUs (6 out of 10) were exclusively isolated after this enrichment and are regarded as specifically selected by this treatment. This refers in particular to strains affiliating to the genus *Alteromonas*, which is commonly found in marine habitats including the deep sea (Lopez-Lopez et al., 2005; Yanagibayashi et al., 1999; Li et al., 1999a; Li et al., 1999b). Most of the barophilic bacterial strains identified to date (bacteria with optimal growth at pressure >1 bar) affiliate to the *Gammaproteobacteria* (Lauro and Bartlett, 2008; Kato et al., 1999; Delong et al., 1997; Kato et al., 1995; Yayanos et al., 1979). Lopez-Lopez *et al.* (2005) compared shallow water strains of *Alteromonas macleodii* to those obtained from the deep water by detailed sequence analyses of the 16S rRNA gene, the

internal transcribed spacer and of house-keeping genes such as gyrB and rpoB. They demonstrated characteristic differences between deep-sea and shallow-water strains and ascribed them to different ecotypes (Lauro and Bartlett, 2008; Lauro et al., 2007; Lopez-Lopez et al., 2005). This hints towards the existence of specifically adapted bacterial ecotypes in the deep sea (Lauro and Bartlett, 2008).

In our experiments, the pressurized sediment was supplemented with N-acetyl-D-glucosamine (NAG), which is known to induce bacterial degradation of chitin, the (1-4)-linked ß-homopolymer of NAG and is a structural component of many organisms including fungi, protists, animals and plants. Chitin, as a valuable carbon and nitrogen source, is reported to be mainly degraded by chitinolytic bacteria, e.g. members of the genera *Pseudomonas*, *Aeromonas*, *Xanthonomas*, *Serratia*, *Cytophaga*, *Arthrobacter* as well as *Bacillus* and is supposed to be the most abundant biopolymer in the marine environment (Gooday et al., 1990). It can be assumed that many bacteria adapted to the oligotrophic conditions of the Eastern Mediterranean deep sea are able to use a broad spectrum of available carbon sources, including chitin and its degradation products such as NAG. All strains from the pressurized enrichment were grown with NAG as sole carbon source and 11 additional strains of the untreated sediment were isolated from solid chitin media. These strains belong to the *Gammaproteobacteria*, the *Firmicutes* and the *Actinobacteria*. Apparently, the ability to degrade chitin and derivatives thereof is a beneficial and widely distributed property of bacteria inhabiting the deep sea.

Growth at low nutrient concentrations

All strains tested during this study grew easily well with minor nutrient concentrations and at *in situ* temperature of 13.5°C, conditions prevailing in the deep Mediterranean Sea. The bacterial isolates apparently are well adapted to low nutrient concentrations as well as to spontaneous and occasional supply of nutrients which is a common situation in oligotrophic deep-sea sediments. An example is represented by isolates assigned to *Alteromonas* (D33, D45, D47 and D56). As demonstrated by their growth on low nutrient media, they coped well with minute nutrient concentrations but also successfully competed with others at *in situ* pressure in the presence of N-acetyl-D-glucosamine.

Members of the *Gammaproteobacteria* are frequently appointed to be *r*-strategists that can stand long periods of starvation but they outcompete others when nutrients

become available (Pinhassi and Berman, 2003; Fuchs et al., 2000). This is in accordance with D´Hondt et al. (2004) who showed that *Gammaproteobacteria* are common in sediments of the ocean margin where concentrations of organic matter and net metabolic rates are high. In contrast, *Actinobacteria* and *Firmicutes* dominated the strains obtained from the untreated sediment and some of these were exclusively isolated using low nutrient media. The suitability of low-nutrient media for the isolation of Gram-positive bacteria was also approved by other culture-dependent studies (Gontang et al., 2007; Jensen et al., 2005; D'Hondt et al., 2004). The lifestyle of these bacteria apparently is adapted to minute nutrient concentrations (*k*-strategists) and under such conditions gives them advantage over the fast growing *Gammaproteobacteria*. This is supported by a study of D`Hondt et al. (2004) who consistently isolated *Actinobacteria* and *Firmicutes* from sediments of ocean margins and open ocean sites exhibiting low concentrations of organic matter and net metabolic rates. As biopolymer degradation was shown to be of special importance in the Mediterranean deep sea (Martin-Cuadrado et al., 2009), *Actinobacteria* might be considered as important players in the degradation of complex biopolymers of the studied environment.

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CHAPTER II

Micromonospora spp. strains from the deep sea as promising sources for novel drugs

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Data in preparation for publication

Abstract

Twenty-one strains of *Micromonospora spp*. were isolated from the deep-sea sediment of the Eastern Mediterranean Sea and analyzed for their potential to produce natural products. It turned out that 48 % of the strains inhibited the growth of Gram-positive test organisms. In addition, molecular analyses enabled the detection of genes for natural product biosynthesis (nonribosomal polypeptide-sythetase, polyketid synthetase type I and type II) in 86 % of the strains. First chemical analyses of culture extracts enabled the detection of at least 2 new compounds. Thus, the deep-sea *Micromonospora* strains revealed to have a considerable potential for the discovery of new drugs.

Introduction

Members of the *Actinomycetales* (“Actinomycetes”) are widely distributed in terrestrial as well as aquatic ecosystems, where they are supposed to play an important role in the decomposition and recycling of biomaterials (Fenical and Jensen, 2006). They even have been recovered from the world’s deepest ocean trench, the Mariana Trench (Pathom-aree et al., 2006a; Pathom-aree et al., 2006b; Colquhoun et al., 1998). A controverse discussion exists about the question whether they are metabolically active in marine sediments or if they have been washed into the marine environment where they are prevailing as spores. Culture experiments using marine salinity conditions showed, that many strains grew easily well and therefore seem to be at least adapted to the marine environment (Prieto-Davó et al., 2008; Jensen et al., 1991). In fact, evidence accumulates that they are active members of the microbial community in the marine sediment (Jensen et al., 2005a; Grossart et al., 2004; Moran et al., 1995; Jensen et al., 1991). Only recently, Maldonado et al. (2005) described the first obligate marine species of the new genus *Salinispora*: *Salinispora arenicola* strain CNH-643 and *Salinispora tropica* strain CNB-440. Furthermore, two new genera were described containing solely marine isolates that tolerate high concentrations of NaCl: *Salinibacterium* (Han et al., 2003) and *Serinicoccus* (Yi et al., 2004).

A specific feature of the actinomycetes is their enormous contribution to the production of chemically diverse secondary metabolites and clinically relevant antibiotics. Novel compounds with biological activity are produced by certain members of the actinomycetes that have been isolated from the marine realm (Fenical and Jensen, 2006; Fiedler et al., 2005; Jensen et al., 2005b; Magarvey et al., 2004). This shows, that also actinomycetes from the marine environment represent a valuable source for the discovery of novel natural products as well. The majority of bioactive compounds of microbial origin comes from actinomycetes and in particular from the genera *Streptomyces* and *Micromonospora* (Berdy, 2005; Bernan et al., 2004). The genus *Micromonospora* was described by Orskov in 1923 (Kawamoto, 1989) and expanded up to now to a total of 44 species and 7 subspecies (List of prokaryotic names with standing in nomenclature; http://www.bacterio.cict.fr/). The first antibiotic isolated from *Micromonospora sp*. was micromonosporin which was described by Waksman et al. (1947). With the discovery of the aminoglycoside antibiotic gentamicin in 1963 (Figure II.1) an intensive screening for new natural

products in *Micromonospora* strains started (Weinstein et al., 1963). Up to now the Dictionary of Natural Products (CRC Press, Taylor & Francis Group, 2010) comprises about 379 compounds that have been isolated from *Micromonospora sp.*. Even though most of these strains are of terrestrial origin, it must be mentioned that culture extracts of marine members of the *Micromonosporaceae* were shown to posses the highest anti-tumor activity of marine actinomycetes isolated from different pacific deep-sea sediments (Zheng et al., 2000).

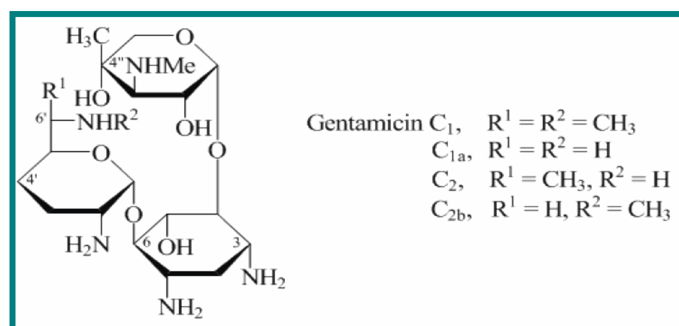

Figure II.1: Chemical structure of the aminoglycoside antibiotic gentamicin.

To our knowledge, there is no natural product described yet that originates from deep-sea strains of *Micromonospora spp.*. Therefore this study focuses on strains that have been isolated from the Eastern Mediterranean deep sea. The eastern basin of the Mediterranean Sea is one of the most oligotrophic regions of the world's oceans. The extreme depletion of nutrients, in particular phosphorus, results in low primary production in waters distant from the coast (Krom et al., 1991). Based on the poor primary production, minor amounts of organic matter reach the deep-sea floor of the Eastern Mediterranean Sea, in particular in one of the deep basins, the Ierapetra Basin, which is located approximately 30 nautic miles southeast of Crete. Studies on the macrofauna community showed, that sediments in the Eastern Mediterranean Sea can be considered as event-driven ecosystems, where the biota have to be adapted to few and pulsed nutrient input events (Kroencke and Tuerkay, 2003). Additionally, the Mediterranean deep sea is characterized by a comparably high temperature, which may exceed 13°C in deep waters (Zenetos et al., 2003). Thus, the extreme conditions of the Eastern Mediterranean deep sea can be supposed to demand specific adaptations of its inhabitants.

Aim of this study was the isolation and phylogenetic analyses of actinomycetes from the oligotrophic deep-sea sediment. Members of the genus *Micromonospora* were selected in order to evaluate the potential to produce new biologically active compounds.

Materials & Methods

Sample collection

All samples were obtained during the Meteor research cruise 71 leg 2 southwards of Crete in January 2007. Sediment was collected from two different sampling sites at 4400 m (34°30.296N, 26°11.507E) and 2800 m (33°42.989N, 26°20.329E) respectively, using a multiple corer (see Figure I.2, Chapter I). The uppermost 5 cm of each sediment core were aseptically sub-sampled and stored at 4°C until further treatment. Additionally, one sediment sample of each station was incubated at *in situ* pressure (280 bar and 440 bar) and temperature (13.5°C) for 6 days prior to further processing (see Chapter I).

Isolation procedure

For selective isolation of Gram-positive bacteria, 2 g of the sediment samples were dried in a sterile petri dish at room temperature for 3 months. Dry sediment was then heated to 120°C for 1 hour and afterwards resuspended in 19 ml $Aqua_{dest}$. 200 µl of this suspension were plated on different media: Chitin-medium: 15 g agar, 2 g chitin in 1L Mediterranean seawater added with 50 mg/ml cycloheximide and ampicillin; XJ4- medium: 15 g agar, 0.1 g histidine, 1 g raffinose, 0.5 g $NaHPO_4$, 1.7g KCl, 0.05 g $MgSO_4$ x $_4H_2O$, 0.01 g $FeSO_4$ x $7H_2O$, 0.02 $CaCO_3$, 0.5 mg each of thiamine, riboflavine, niacine, pyridoxin, Ca-panthotenate, inositol, p-aminobenzoicacid and 0.25 mg biotin and 50 ppm $K_2Cr_2O_7$ in 1 L of $Aqua_{dest}$ added with cycloheximide (50mg/ml); HA-medium: 1 g humic acid, 0.01 g $FeSO_4$, 0.5 g Na_2HPO_4, 1.7 g KCl, 0.02 g KCl, 0.02 g $CaCO_3$, 0.5 g asparagine and 50 ppm $K_2Cr_2O_7$ in 1 L of deionized water and MW+Y- medium: 1.5 % agar, 0.01 % yeast extract in 1 L Mediterranean seawater added with 50 mg/L cycloheximide and penicillin G.

Additional to this heat-treatment sediment samples were plated directly onboard on low-nutrient media (for details see Chapter I).

Agar plates were incubated at 13.5°C (*in situ* temperature) and 25°C (onboard room temperature) for up to six months and checked frequently for growth. Using a

binocular microscope all colonies appearing morphologically different were transferred to fresh agar medium until pure cultures were obtained. Isolates were checked for purity by microscopy and phylogenetically analyzed by sequencing of the 16S rRNA gene. Isolates were stored at -80°C using the Cryobank system (Mast Diagnostica GmbH, Reinfeld, Germany).

Phylogenetic analyses

DNA extraction, 16S rRNA gene amplification and sequencing were performed according to Gärtner et. al. (2008). Sequences were compared to NCBI database sequences using BLAST search (see http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences alignment of all actinobacterial strains was performed using the Arb database (Ludwig, 2004). Phylogenetic trees were calculated applying maximum likelihood analysis with PhyML software (Guindon and Gascuel, 2003). Maximum likelihood analysis was performed assuming the GTR evolution model (Keane et al., 2006) with an optimized gamma distribution parameter alpha and 100 bootstrap replicates. An additional tree of only *Micromonospora* strains and all type strains of the genus was constructed by PhyML with 100 bootstrap replicates.
For phylogenetic discussion, sequences with similarities higher than 99.5 % were grouped together using Mothur (http://www.mothur.org/wiki/Download_mothur)

Amplification of the biosynthesis genes pksI, pksII and nrps

DNA extracts of *Micromonospora* strains were analyzed for the presence of natural product producing genes such as the polyketide synthetase I and II (pks I and pks II) and as well the nonribosomal polypeptide synthase (nrps). Detection of pks II genes was performed using the primers and PCR conditions as described by Metsä-Ketelä (1999). For gene amplification 50 pMol primer, 3.5 U TaqPolymerase, 10% DMSO and 5 µl template were applied. PKS I and NRPS –PCRs were modified according to Ayuso-Sacido and Genilloud (2005) using PuReTaq® Ready-To-Go PCR Beads (GE Healthcare) and 50 pMol of the primers A3 and A7R for the NRPS gene and K1 and M6R for the PKS I gene. Additionally, primers A2f and A3r were used for amplification of NRPS according to the protocol of Martens et al. (2007). Purified PCR products were sequenced at the Institute for Clinical Molecular Biology (UK-SH, Kiel, Germany) using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems) in a 3730-DNA-Analyzer (Applied Biosystems) as specified by the

manufacturer. Sequences were edited by ChromasPro v.1.33, translated to amino acid sequences using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared to the NCBI database using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches with the highetes e-values of the blastx search were checked for high similarities and query coverage to preferably genes of *Micromonospora sp*.

Production of culture extracts

For HPLC- analysis of the metabolite profiles and for antimicrobial test assays, culture extracts of all *Micromonospora* strains were prepared. Strains were cultivated in 100 ml liquid starch - pepton medium (1 g starch; 0.5 g peptone and 1.5 g Tropic Marine® salt in 100 ml $Aqua_{dest}$) using 300 ml Erlenmeyer-flasks with one baffle. After incubation for 8 days at 28°C 100 ml ethylacetate (Prochem; Picograde®) were added and the culture broth was homogenized using an Ultra-Turrax T25 basic disperser at 17500 rpm for 30 sec. The supernatants were separated and dried. Additionally, culture extracts of strains A77 and S20 were prepared after incubation in starch-peptone medium with Mediterranean Sea water (38.6 ‰) for 8 days at 28°C.

Antimicrobial tests of culture extracts

Antimicrobial activity of the *Micromonospora* strains was tested against *Bacillus subtilis* (DSM 347)*, Staphylococcus lentus* (DSM 6672)*, Xanthomonas campestris* (DSM 2405)*,* and *Candida glabrata* (DSM 6425). 100 µl methanolic solutions of the extracts were added into a 96-well microtiter plate and dried in a vacuum centrifuge. Overnight cultures of test organisms (in liquid tryptic-soy-broth) were diluted to an OD_{630} of 0.02 - 0.06 and 200 µL were added to each well. After an incubation of 14 - 16 h at 28°C growth of the test strains was determined photometrically at $560_{Ex}/590_{Em}$ nm by reduction of nonfluorescent resazurin to fluorescent resorufin. A negative (no compound) and a positive control (100 µM of chloramphenicol for bacterial test strains and 100 µM of cycloheximid for the yeast) were measured on the same microtiter plate. All tests were run in triplicates.

HPLC analysis of culture extracts

For analyzing the metabolite profiles of our strains culture extracts were screened by HPLC-UV/MS. For reversed-phase HPLC analysis dried extracts were resolved in

MeOH. HPLC/MS measurements were carried out using a Phenomenex Onyx Monolithic C_{18} column (100 by 3.00 mm) on a VWR Hitachi Elite LaChrom system coupled to an electrospray ionization ion trap detector (Esquire 4000, Bruker Daltonics). The solvents water and acetonitrile (MeCN) were supplemented with 0.1 % HCOOH (gradient of MeCN: 0 min 5 %; 4 min 60 %; 6 min 100 %; fow rate: 2 ml/min).

Results

Isolation of *Actinobacteria*

In total, 39 isolates affiliating to the *Actinobacteria* have been isolated from the Mediterranean deep-sea sediment. While 22 strains were isolated without selective treatment by the spread agar method out of the fresh sediment (see Chapter I), additional 17 strains have been isolated from the same sediment samples by heat-treatment for selective enrichment of Gram-positive bacteria (Figure II.2). *Actinobacteria* comprised about 20 % of the cultured bacteria in both approaches with viable cell counts of < 2 x 10^2 CFU /g sediment in the heat-treatment and < 2 x 10^2 CFU /ml fresh sediment from the untreated sediment. Among them members of the genera *Micromonospora* (21 strains; 50 %) and *Streptomycetes* (7 strains; 18 %) clearly dominated. Interestingly the phylogenetic diversity of *Micromonospora* strains was higher compared to that of the *Streptomycetes*. Most of the seven strains affiliating to the genus *Streptomyces* showed 99.9 – 100 % sequence similarity to each other and to the closest related type strain *Streptomyces sampsonii* (ATCC 25495^T). According to the species concept of Stackebrandt and Ebers (2006), one strain (S06) could be assigned to a new species as it revealed to have only 97.8 % 16S rRNA gene similarity to the next related type strain *Streptomyces flavidofuscus* (DSM 41473^T). In contrast, strains affiliating to the genus *Micromonospora* could be assigned to 14 different phylotypes (≥ 99.5% similarity) (Table II.1). Interestingly, members of *Micromonospora* were successfully isolated by both treatments, while no members of the *Streptomycetaceae* and *Pseudonocardiaceae* have been isolated by heat treatment. Also members of the genus *Arthrobacter* have been recovered by both approaches. On the other hand, neither *Knoellia* nor *Marmicola* strains have been isolated without the selective enrichment procedure. Most *Actinobacteria* recovered by this study showed high sequence similarity to well known species of terrestrial origin. Therefore, all strains

was tested for growth at *in situ* temperature, low nutrient concentrations and Mediterranean salinity concentration (38.9 ‰). It was shown, that all strains grew within two months at *in situ* temperature (13.5°C) on solid Marine Broth medium and most strains (27/40) also grew with low nutrient concentrations and Mediterranean Sea water at 13.5°C.

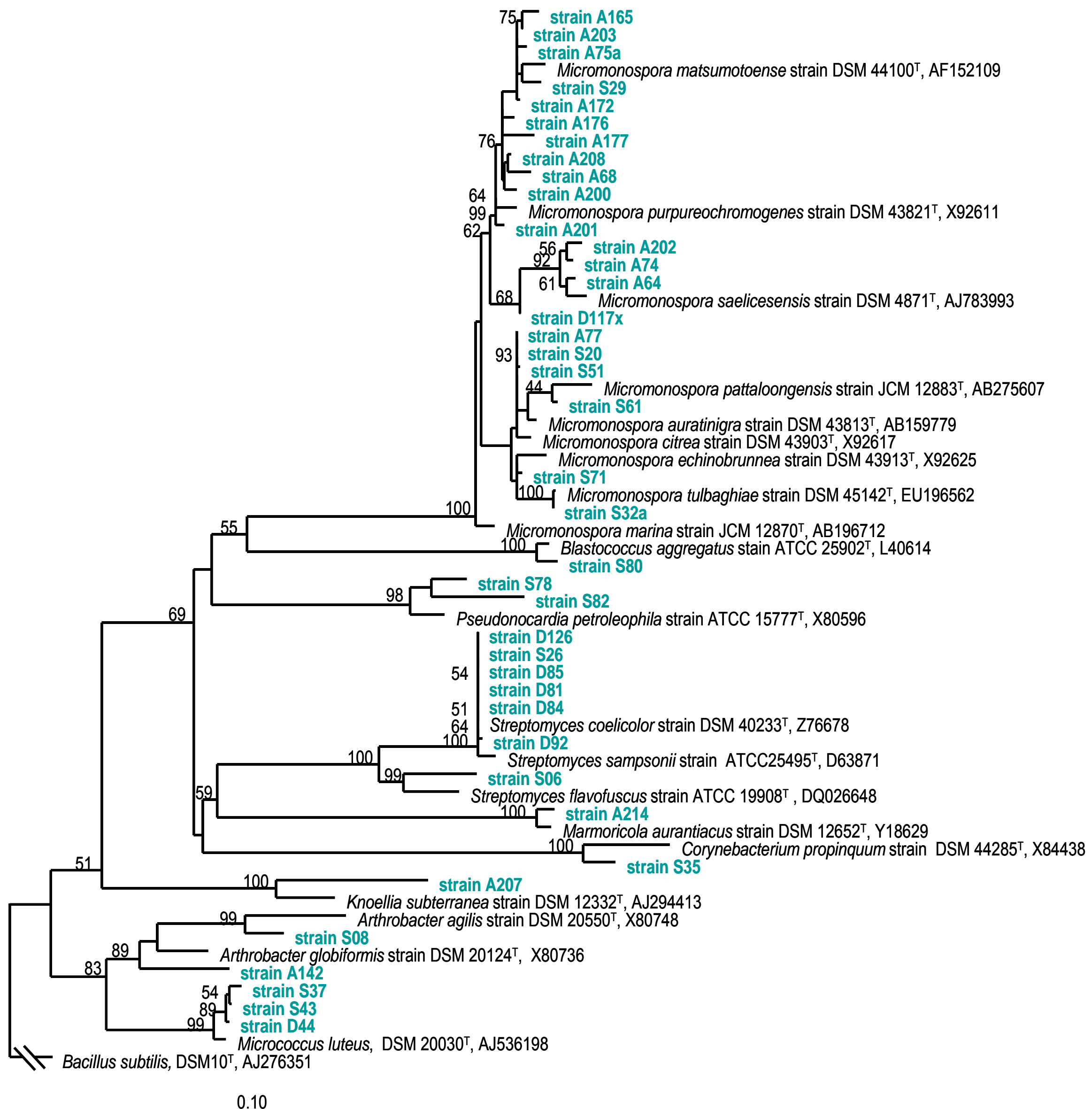

Figure II.2a: Maximum likelihood phylogenetic tree based on the 16S rRNA gene of all *Actinobacteria* isolated from the Mediterranean deep-sea sediments. Tree was constructed with 100 bootstrap replicates using phyML software. Strains marked by stars were isolated by heat-dry method. All other strains were recovered by direct plating of fresh sediment.

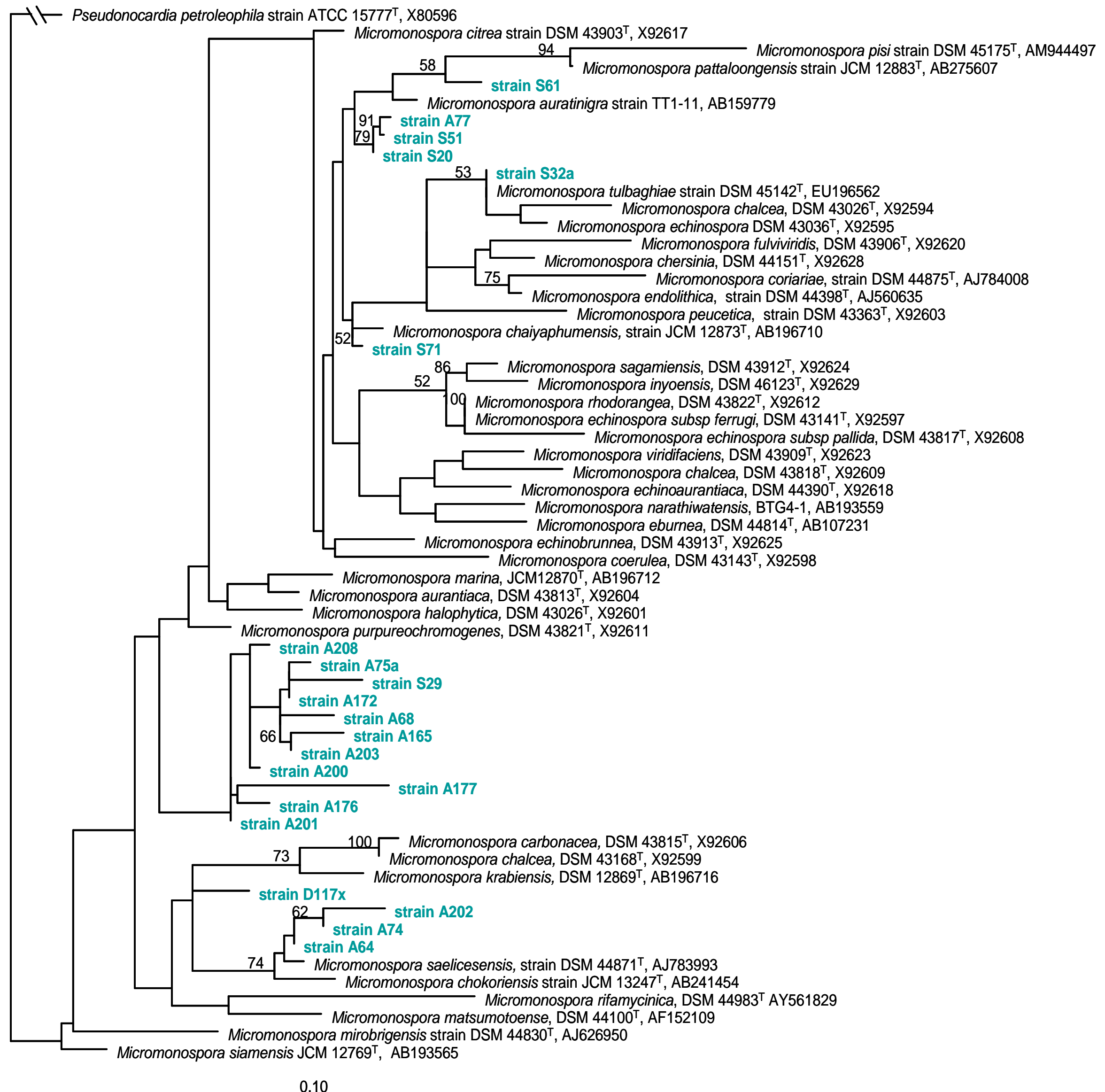

Figure II.2b: Maximum likelihood phylogenetic subtree of all type strains affiliating to the genus *Micromonospora* and the strains of *Micromonospora* spp. obtained during this study. The tree is based on 16S rRNA gene sequences and was constructed with 100 bootstrap replicates using phyML software. Numbers on nodes represent bootstrap percentages > 50. Despite the low bootstrap values, multiple calculations of the tree always resulted in identical clusters of the sequences. Strains marked by stars were isolated by heat-dry method. All other strains were recovered by direct plating of fresh sediment.

Table II.1: Cultivation conditions, phylogenetic analyses and specific characteristics of *Micromonospora* strains obtained from the Eastern Mediterranean deep sea sediment

OTU #	strain	bp	next relative strain & acc.number		Similarity (%)	Sample origin	heat treatment	Isolation medium	T (°C)	antibiotic bioactivity	Detection of biosynthesis genes via PCR and sequencing of			
											pksI	pksII	$nrps_{(short)}$	$nrps_{(large)}$
1	**M71_S29**	1436	*Micromonospora purpureochromogenes* , DSM 43821^T	X92611	98	IB	no	CFB	25	-	-	-	-	√
2	**M71_D117x**	1364	*Micromonospora saelicesensis* , DSM 44871^T	AJ783993	99	IB	no*	CFB	25	+	-	-	√	√
3	**M71_A74**	1363	*Micromonospora saelicesensis* , DSM 44871^T	AJ783993	99	IB	yes	XJ4	25	+	-	-	-	√
3	**M71_A64**	1473	*Micromonospora saelicesensis* , DSM 44871^T	AJ783993	99	HP	yes	HA	25	-	-	√√	√√	√
4	**M71_A68**	1363	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes*	XJ4	25	+	-	√√	√√	√
5	**M71_S20**	1468	*Micromonospora auratinigra* , DSM 44815^T	AB159779	99	HP	no	CFB	25	+	√	√	√	√
5	**M71_S51**	1371	*Micromonospora auratinigra* , DSM 44815^T	AB159779	99	IB	no	CFB	25	+	-	√√	√	√
5	**M71_A77**	1353	*Micromonospora auratinigra* , DSM 44815^T	AB159779	99	IB	yes	XJ4	25	-	√	√	√	√
6	**M71_A75a**	1384	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes	XJ4	25	-	-	√√	√	√
6	**M71_A172**	1472	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes*	XJ4	13	-	-	√√	√	√
6	**M71_A203**	1443	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes	XJ4	13	+	√	√	√	-
6	**M71_A165**	1371	*Micromonospora purpureochromogenes* , DSM 43821^T	X92605	98	IB	yes*	MWY	25	+	-	√√	√	√
7	**M71_A177**	1474	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes	XJ4	25	-	-	√√	√√	-
8	**M71_A200**	1476	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes	HA	13	+	√√	√√	√√	-
8	**M71_A208**	1471	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes*	HA	13	-	-	√√	√	√
9	**M71_A202**	1437	*Micromonospora saelicesensis* , DSM 44871^T	AJ783993	99	IB	yes	XJ4	13	-	√	√√	√	√
10	**M71_A176**	1471	*Micromonospora purpureochromogenes* , DSM 43821^T	X92604	99	IB	yes	XJ4	25	-	-	√√	√	√
11	**M71_S32a**	1381	*Micromonospora tulbaghiae* , DSM 45142^T	EU196562	99	HP	no	MW	25	-	-	-	-	-
12	**M71_S61**	1435	*Micromonospora pattaloongensis* , JCM 12883^T	AB257607	99	HP	no	Chi	25	+	-	√	√	√
13	**M71_S71**	1469	*Micromonospora chaiyaphumensis* , JCM 12873^T	AB196710	99	IB	no	MWY	25	+	-	-	-	-
14	**M71_A201**	1076	*Micromonospora coxensis* , JCM 13248^I	X92604	99	IB	yes	XJ4	13	-	√	√√	√√	√

IB = Ierapetra Basin; HP = Herodotos Plain; * = sediment sample was incubated at *in situ* pressure prior to cultivation (see Chapter I); NAG = N-acetyl-D-glucosamine; + = tested bioactivity against at least one of the test strains; √ = positive PCR amplification; √√ = positive PCR amplification and sequence information; - = negative test results.

Potential of *Micromonospora* strains for the production of natural products

Culture extracts of all strains affiliating to the genus *Micromonospora* have been investigated for antimicrobial activity. Interestingly, 48 % of the strains revealed bioactivity against Gram-positives (*Bacillus subtilis* or *Staphylococcus lentus*) but no strain inhibited the growth of the Gram-negative or eukaryotic test strain. Ten out of twenty-one *Micromonospora* strains showed antimicrobial activity against *Bacillus subtilis* (D117x, S20; S61, S51, S71, A165, A74, A68, A200 and A203). Most of these strains additionally exhibited antimicrobial activity against *Staphylococcus lentus* (except of strains S61 and A165) (see Table II.1, Figure II.3).

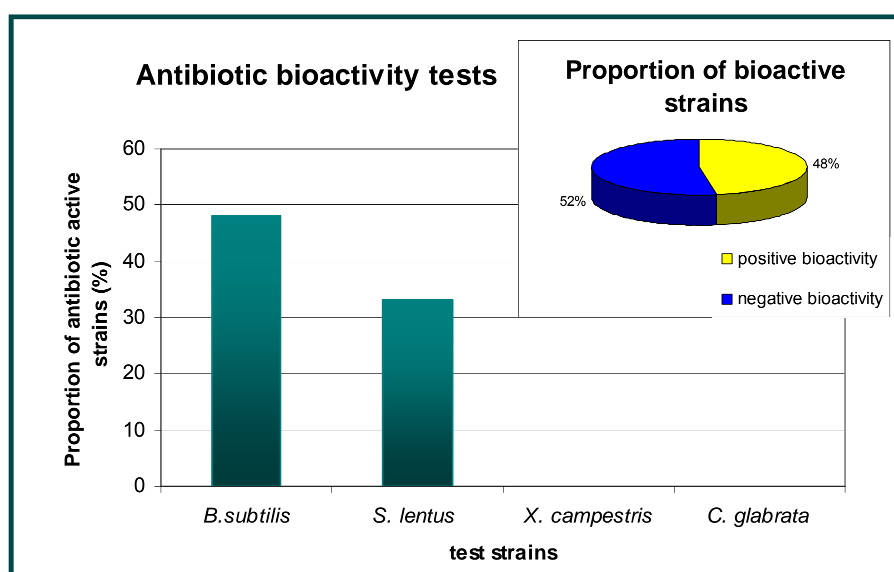

Figure II.3: Antimicrobial activity test results of *Micromonospora* strains against selected test strains: *Bacillus subtilis*, *Staphylococcus lentus*, *Xanthomonsa campestris* and *Candida glabrata*

Additional to the determination of antibiotic activity all strains have been analyzed for the presence of pks I, pks II and nrps biosynthesis genes by PCR amplification. Positive PCR amplificons were sequenced to confirm PCR specifity. As shown in Table II.1 of all genes investigated, most amplified PCR products could be identified as the correct gene fragment (Blast results see Appendix). Gene fragments of at least one of the biosynthesis genes were detected in 86% of the strains (Figure II.4). While genes for nonribosomal polyketide synthetases and also type II polyketide synthases have been revealed in 81% of the strains, type I polyketide synthase

has been found in 27% of the strains. Quite remarkable, one third (27 %) of the strains were positive for all three biosynthesis genes.

The presence of non ribosomal peptide synthetase genes was tested by PCR amplification using two different primer pairs. Both protocols revealed that 80 % of the tested strains have non ribosomal peptide synthase genes in their genomes. Results of both PCR protocols were similar. Few strains showed positive amplification in either one or the other protocol. While most of the small fragment PCR products could be confirmed as truly non ribosomal peptide synthetase genes by sequencing, most sequences of the larger fragment revealed to consist of more than one single gene fragment. In this case, cloning experiments would be necessary to separate the gene fragments and to obtain sequence information.

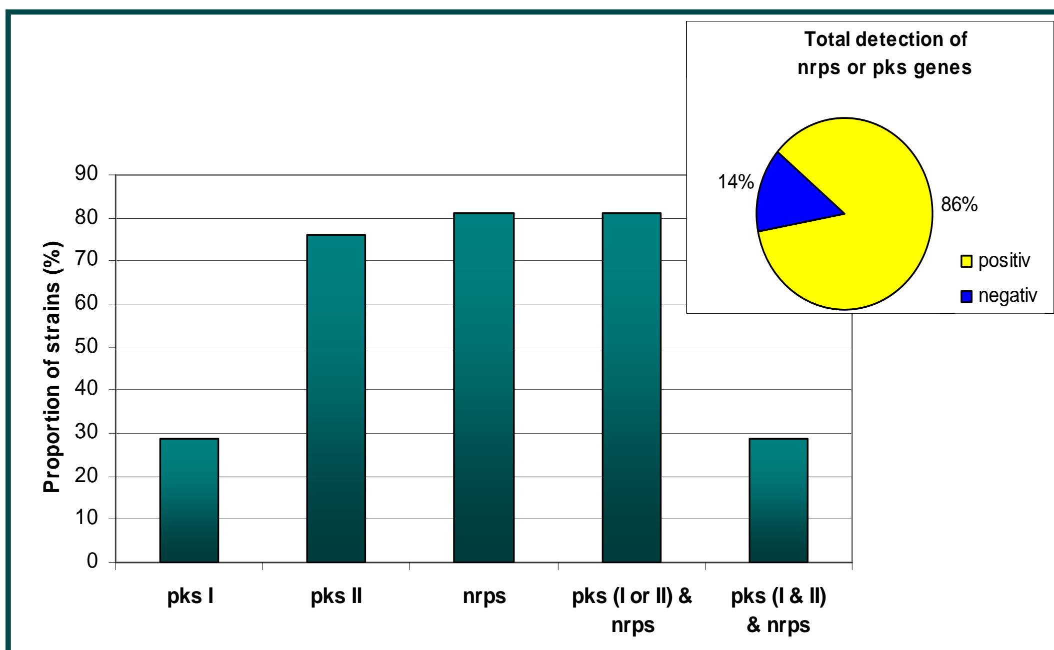

Figure II. 4: Presence of pks I, pks II and nrps genes in *Micromonospora spp.* strains of the Mediterranean deep sea

Chemical metabolite profiles of selected strains

The chemical analyses for secondary metabolites of all *Micromonospora* strains by LC-MS and UV-Vis data revealed different metabolic profiles even for closely related strains. Two strains with presumably new secondary metabolites (strain A77 and S20) were selected for further studies. Both strains could be assigned to the same species with 99.85% 16S rRNA gene sequence similarity to one another (Figure II.2a) and, noteworthy, phylogenetic calculations revealed that both strains cluster

apart from all known type strains of the genus *Micromonospora* along with strain S51 (Figure II.2b).

The culture extract of strain A77 contained an unknown compound (1) as shown by analytical HPLC. This compound had a retention time of 3.9 – 4.2 min and an UV_{max} at 260 nm with MW 508 (Figure II.5). Additionalyl a derivative of this compound (2) was found at retention times of 4.1 - 4.4 min with UV_{max} at 260 nm and MW 506. No corresponding compound was found in the database of Natural Products (CRC Press, Taylor & Francis Group, 2010).

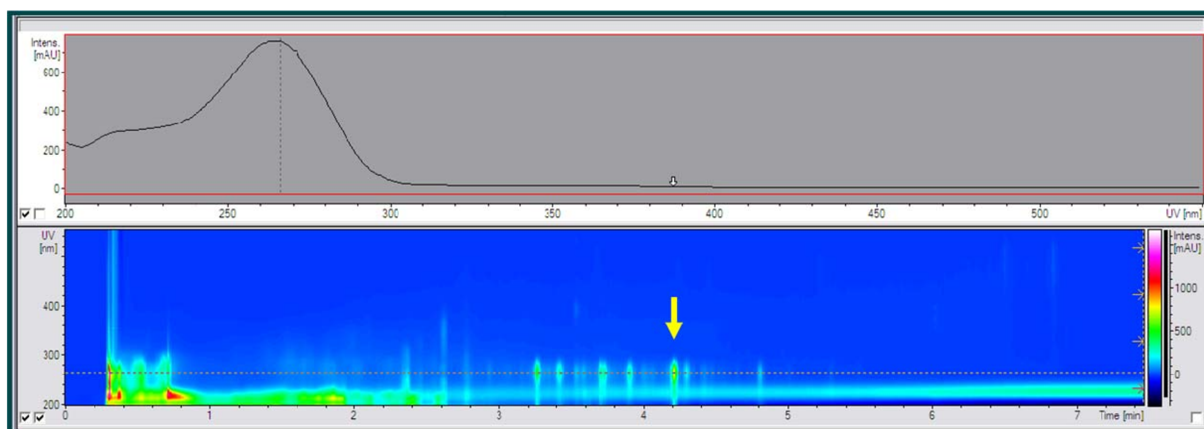

Figure II.5: UV-Vis Chromatogram of unknown compound 1 produced by strain A77.

The growth curve of a 1L culture of A77 in liquid soy-peptone medium showed, that the strain reaches the stationary phase after 4 days of incubation at 28°C. Production of the unknown substance begins at the end of the log phase and lasts throughout the stationary phase. Furthermore, salinity tests with added Tropic Marine® salt showed that the unknown substances were produced at salinities of 0, 10, 20 and 30. Also Mediterranean Sea water from the sampling site (salinity of 38.6) allowed the production of similar amounts of the compound (Figure II.6). The same compounds were also produced by two other *Micromonospora* strains, strains A74 and S51, under identical cultivation conditions.

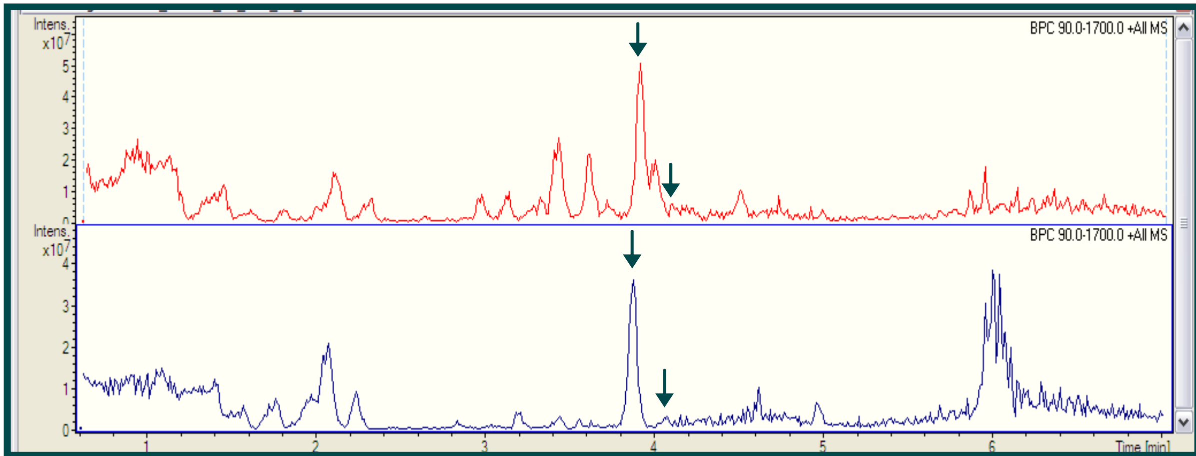

Figure II.6: Production of compound 1 and 2 by strain A77 in liquid soy-pepton medium with Mediterranean Sea water (salinity of 38.9; blue line) and with 15‰ Tropic Marine Salt (red line).

The compounds were termed levantilide A and B. A detailed description can be found in Chapter III.

The HPLC-MS profile of strain S20 revealed another unknown compound with MW = 940. This compound had an UV_{max} at 320 nm (Figure II.7).

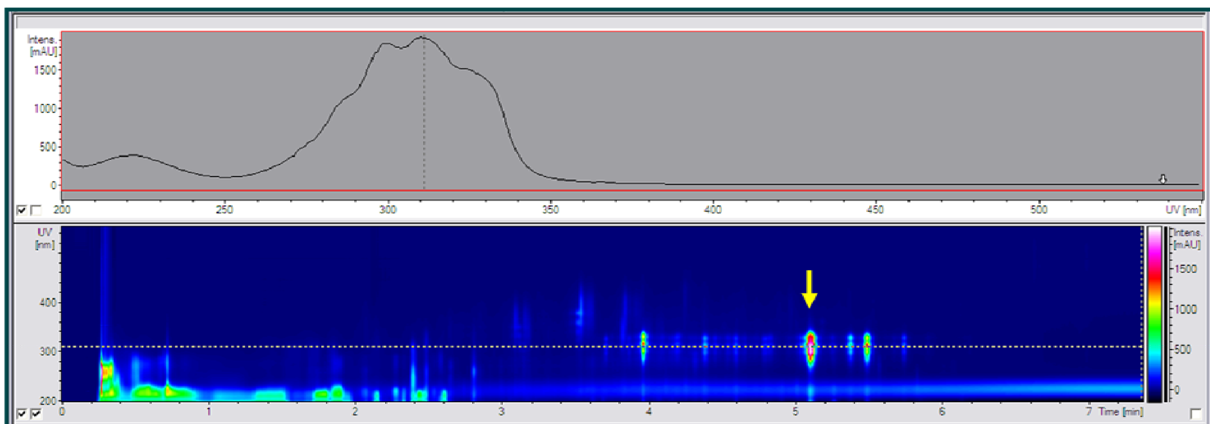

Figure II.7: UV-vis profile of unknown compound 2 produced by strain S20.

Production of the unknown substance is initiated at the end of the log-phase and lasts throughout the stationary phase. To evaluate the production of this substance in the marine milieu, salinity-dependent experiments were run. It was shown, that strain S20 produces the substance at salinities of 0, 10, 20 and 30 ‰ with Tropic Marine® salt and also when habitat seawater (salinity of 38.6) was used for cultivation (Figure II.8).

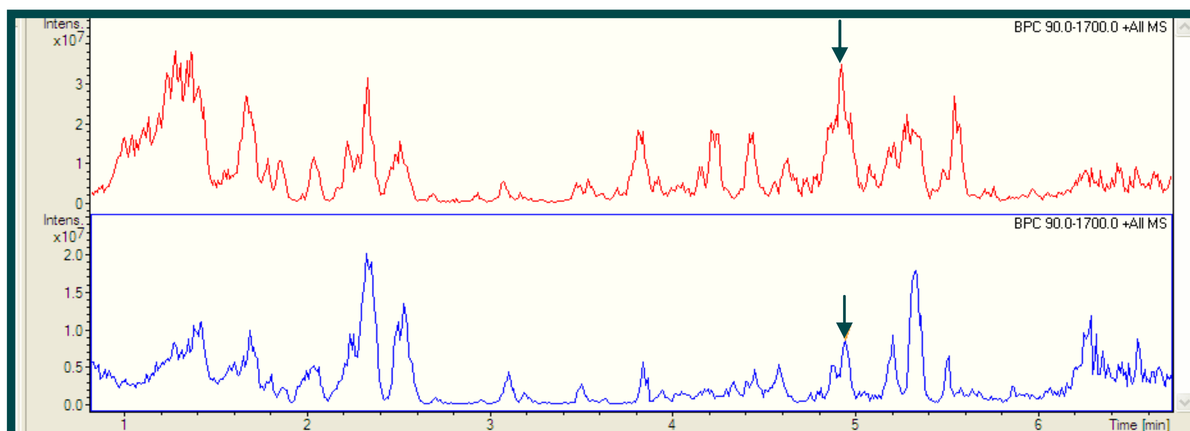

Figure II.8: HPLC-profile of the culture extract of strain S20 for comparison of the production of compound 2 with Mediterranean Sea water (salinity of 38.9; blue line) and with 15‰ Tropic Marine Salt (red line).

Besides this main compound several derivatives are produced by the strain. We identified 3 additional compounds with MW = 954, 940 and 982. Interestingly, these compounds showed variations in the bioactivity towards *Bacillus subtilis*.

Discussion

All actinobacterial strains isolated in this study originate from an extreme oligotrophic marine deep-sea habitat. Even though reports about *Actinobacteria* isolated from marine habitats are accumulating, there are few reports about *Actinobacteria* in deep-sea sediments. To our knowledge, this is the first study on actinobacterial strains in the unique environment of the oligotrophic Mediterranean Sea and, over this, the first study that analyzes deep-sea *Micromonospora* strains for their potential to produce natural products. The focus of this study was laid on marine strains that can be assigned to well known *Micromonospora* species of primary terrestrial origin. These strains revealed to posseses so far unknown secondary metabolites and thus are of great interest for the discovery of new drugs.

Isolation efficiency

Comparing both approaches (direct plating of fresh sediment and selective enrichment using dry heat) showed that members of the *Streptomycetes* were completely missing in the heat-treatment but were fairly abundant after direct plating of fresh sediment. Nonomura and Ohara showed in 1969, that dry-heat is useful for the selective enrichment of non-streptomycete actinobacteria. In this study, heat-treatment revealed to be successful for the isolation of members of further species

like *Marmicola* as well as for the isolation of further *Micromonospora* species. This demonstrates that the use of different sample treatments and cultivation conditions is essential for the cultivation of diverse actinobacterial species and genera. This is of special importance, because the diversity of this group is hardly detected by eubacterial environmental gene libraries.

Interestingly, the variety of phylotypes recovered from the genus *Micromonospora* (14 phylotypes) compared to that of other actinobacterial genera like the genus *Streptomyces* (1 -2 phylotypes) was remarkable. This is in accordance with a study of Prieto-Davo et al. (2008) who compared actinomycete recovery from shallow versus deep sediment at the coast of San Francisco. They discovered an increasing diversity of *Micromonospora* relative to *Streptomycetes* with increasing water depth. Noteworthy, most *Micromonospora* strains phylogentically cluster apart from already described type strains of the genus *Micromonospora* (Figure II.2b). Five of these strains (D117x, A165, A68, A208 and A172) were recovered from sediment incubated at *in situ* pressure (see Chapter I). Strains recovered by this approach are obviously adapted to sudden changes in hydrostatic pressure and they might be considered as specifically adapted to the conditions prevailing in the Mediterranean deep sea. Wyland et al. (1984) already reported, that *Micromonospora* strains survived long lasting treatments of high hydrostatic pressure at low temperature. We additionally tested our strains for growth at minor nutrient concentrations with habitat water and low temperature (13.5°C) which are characteristic features in the deep Eastern Mediterranean. It was shown, that most strains grew well on the applied cultivation conditions. These results showed that the strains cope well with high hydrostatic pressure, minor nutrient concentrations, reduced temperature and marine salinity. Therefore, the deep-sea strains seem to be at least adapted to the conditions of the Mediterranean deep sea and may be seen as indigenous inhabitants.

Natural products from deep-sea *Micromonospora* strains

Antimicrobial activity of the *Micromonospora* strains revealed to be targeted at Gram-positive test strains. About 48% of the tested strains inhibited growth of *Bacillus subtilis* and about half of these strains additionally inhibited *Staphylococcus lentus* in growth. As shown before (Chapter I), the strain collection of the sediments was dominated by highly diverse Gram-positives and by members of the genus *Bacillus* in paticular. All these Gram-positive strains revealed to cope easily well with minor

nutrient concentrations. Members of the genus *Bacillus* therefore seem to have similar nutrient requirements and hence may be regarded as competitors. As the deep sea is characterized by oligotrophy with spontaneous and punctual nutrient input, inhabitants must be adapted to these abiotic features and compete with others as soon as nutrients become available. This might explain why the production of antimicrobial compounds is also necessary in the deep-sea realm.
This is also supported by the detection of multiple biosynthesis genes for secondary metabolite production. All strains were analyzed for the presence of pks I, pks II and nrps and it was shown that about 86 % of the strains had at least one of the mentioned biosynthesis genes. *Actinobacteria* usually have more than one of these gene clusters. Therefore, cloning experiments of preferably larger gene fragments are necessary to separate the amplified genes and to obtain functional information of the genes by sequencing. Nevertheless, some obtained sequences were of adequate quality for comparison with NCBI database and showed best matches with appropriate biosynthesis genes of *Actinobacteria*, primarily of *Streptomyces* and *Micromonospora*. It should be considered, that not all present genes of nrps, pks I and pks II might have been amplified due to primer mismatches with presumably less nrps -PCR protocols used might have resulted by the selectivity of the different primers used. Nevertheless, this screening method allows a relatively rapid impression of the natural product producing potential of different strains. This is especially useful, if large strain collections of phylogenetically diverse strains have to be screened for metabolite producing strains.

Our data show, that the *Micromonospora* strains obtained from the Mediterranean deep sea have a broad genetic potential to produce secondary metabolites, probably more than those detected by the use of one culture extract. Cultivation conditions like temperature, shaking intensity and media composition strongly influence the production of secondary metabolites and, therefore, further cultivation experiments are supposed to enlarge the metabolite production of our strains. So far, the analyzed metabolite profiles of culture extracts by HPLC/MS enabled the detection of two unknown bioactive compounds. The identification of further compounds still is in progress. A combined screening of the chemical metabolite profile, antimicrobial activity and the genetic potential for secondary metabolite production is useful for the discovery of new natural products. This is demonstrated e. g. by strain S20 which

showed inhibitory activity against *B. subtilis* while strain A77 was not antimicrobially active towards one of the tested strains but revealed to posses a cytotoxic compound via HPLC/MS. This example further shows that phylogenetically highly related strains and identical origin can possess completely different natural products.

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CHAPTER III

Levantilides A and B, 20-membered macrolides from a *Micromonospora* strain isolated from the Mediterranean deep sea

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Abstract

Two new 20-membered macrolides, levantilides A and B, were isolated from the *Micromonospora* strain M71-A77. Strain M71-A77 was recovered from an Eastern Mediterranean deep-sea sediment sample and revealed to produce the levantilides under *in situ* salinity of 38.6 ‰. The chemical structures of the levantilides were elucidated on the basis of different one- and two-dimensional NMR experiments. Levantilide A exhibited a moderate antiproliferative activity against several tumor cell lines.

Introduction

The deep sea is an extreme environment which is still marginally investigated and harbors a great variety of so far not cultivated bacteria. Bacteria, which live in the deep sea need to adapt to the specific environmental characteristics such as high hydrostatic pressure, low temperature and only occasional nutrient supply. These constraints quite likely determine the phylogenetic diversity of the deep-sea bacterial communities and also affect the secondary metabolite production of these bacteria. Therefore, deep-sea bacteria are considered as a promising source for the discovery of new natural products. Marine members of *Actinobacteria* turned out to be highly potent producers of interesting compounds (Lam, 2006; Fiedler et al., 2005; Berdy, 2005; Jensen et al., 2005; Magarvey et al., 2004) as was already shown for their terrestrial counterparts (Bernan et al., 2004). Only recently, two strains of *Streptomyces* sp. from the Atlantic ocean deep-sea sediment were shown to produce the two new natural products caboxamycin and albidopyrone (Hohmann et al., 2009a; Hohmann et al., 2009b).
With special focus on the discovery of new natural products we selectively isolated *Actinobacteria* from the deep-sea sediment of the Eastern Mediterranean Sea (the so called Levantine Sea). This environment is characterized by a relatively high bottom temperature of 13 -14°C, salinity values of approximately 38 - 39 ‰, high hydrostatic pressure (440 bar at the sampling site) and an extreme depletion of nutrients (Thingstad et al., 2005). Among the isolated bacteria, strain M71-A77 turned out to produce two new macrolides named levantilides A (**1**) and B (**2**) which will be described in this paper.

Results and Discussion

Strain M71-A77 was isolated from the Eastern Mediterranean deep-sea sediment (4400 m) and revealed 99.3% 16S rRNA gene sequence similarity to *Micromonospora auratinigra* DSM 44815^T (AB159779). Analyses of the culture extract of this strain (incubated in liquid soy-peptone medium) by HPLC-DAD-MS led to the detection of the two unknown 20-membered macrolides, levantilide A (**1**) and B (**2**), with detected MW of 508 and 506, respectively (Figure III.1). Subsequent cultivation of the strain in larger scale (10 L) led to the isolation of the levantilides as colorless solids.

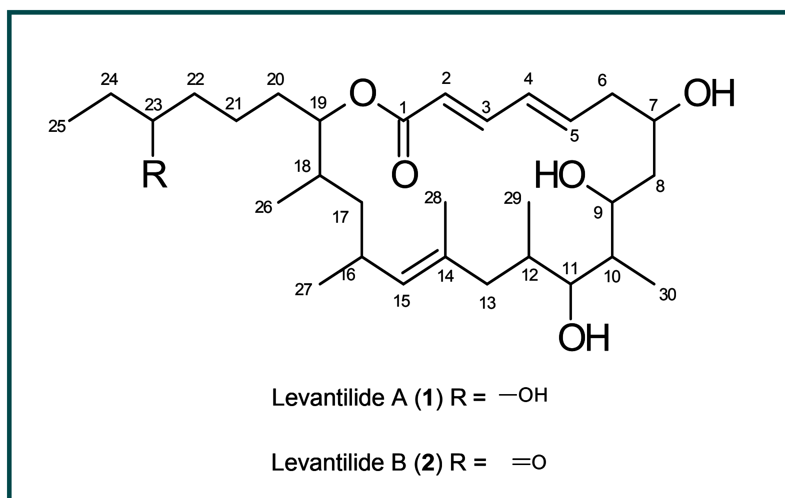

Figure III.1: Chemical structure of levantilide A and B

Levantilide A (**1**) had a measured molecular mass of *m/z* 531.3676 $[M+Na]^+$ (calculated for $C_{30}H_{52}NaO_6$ 531.3656) which yielded the molecular formula $C_{30}H_{52}O_6$ for **1** and implied five degrees of unsaturation. The structure elucidation of the compound was mainly based on one- and two-dimensional NMR spectra of **1**. The respective ^{13}C NMR spectrum displayed 30 distinct signals which were consistent with the deduced molecular formula. The carbon resonances gave evidence of one carbonyl carbon (!$_C$ 166.1 ppm), one olefinic quaternary carbon (δ_C 132.8 ppm), five olefinic methine carbons (δ_C 120.4 ppm to 143.8 ppm), five methine carbons adjacent to oxygen atoms (δ_C 67.2 ppm to 77.4 ppm), four further methine carbons (δ_C 29.4 ppm to 41.0 ppm), eight methylene groups (δ_C 22.0 ppm to 40.9 ppm) and six methyl groups (δ_C 10.1 ppm to 21.5 ppm). The ^1H-^{13}C HSQC allowed all carbon resonances to be unambiguously assigned to the resonances of their directly attached protons. The final planar structure of the molecule was deduced from ^1H-^1H COSY and ^1H-^{13}C HMBC spectra (Figure III.2). With a shift of 166.1 ppm the carbonyl carbon C-1 was very likely to belong to an ester function. ^1H-^{13}C HMBC correlations from H-3 (δ_H 7.06) and H-2 (δ_H 5.78) identified the adjacent methine groups. Accordingly, H-3 coupled with H-2 and additionally with H-4 (δ_H 6.29). H-4 also coupled with H-5 (δ_H 6.08), with H-2 to H-5 all being olefinic protons. H-5 showed ^1H-^{13}C HMBC correlations to C-3 (δ_C 143.8) as well as to C-6 (δ_C 39.6), a methylene carbon, and C-7 (δ_C 67.2), a hydroxylated methine. H-7 (δ_H 3.98) showed ^1H-^1H COSY correlations to H-6 as well as to the methylene group CH_2-8 (δ_C 33.4, δ_H 1.51; 1.16). Next to CH_2-8, there followed four methine groups, CH-9 to CH-12, each of them substituted with either a hydroxy group, CH-9 (δ_C 67.4, δ_H 3.92) and CH-

11 (δ_C 75.3; δ_H 3.00), or a methyl group, CH-10 (δ_C 41.0; δ_H 1.68) and CH-12 (δ_C 32.1 and δ_H 1.24), which could be unequivocally proven by their shifts, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC correlations. The signals of H-9 and H-11 for example showed ^{1}H-^{1}H COSY correlations to their neighboring methine protons as well as to the protons of the hydroxy groups they were attached to (9-OH δ_H 4.63 and 11-OH δ_H 4.00). Not only did the ^{1}H-^{1}H COSY correlations of the methyl groups CH_3-29 (δ_C 17.7 and δ_H 0.58) and CH_3-30 (δ_C 11.1 and δ_H 0.86) connect them to the methines CH-12 and CH-10, but their ^{1}H-^{13}C HMBC correlations also gave further evidence of the positions of the neighboring carbons and secured the sequence from CH-9 to CH-12. Furthermore, a methylene group CH_2-13 (!$_C$ 40.1, δ_H 1.87; 1.51) was shown to connect the methines CH-9 to CH-12 to the quaternary olefinic carbon C-14 (δ_C 132.8). Therefore, the last double bond evidently was located between C-14 and C-15 (δ_C 132.7). The corresponding proton H-15 (δ_H 4.76) showed long range couplings to C-17 (δ_C 40.9), C-13, C-16 (δ_C 29.4), C-27 (δ_C 21.5) and C-28 (δ_C 17.0) and coupled to H-16 and H_2-13. CH_2-17 (δ_H 1.34 and 1.05) formed the junction between the methyl-bearing methines CH-16 and CH-18 as indicated by ^{1}H-^{13}C HMBC correlations from H-17 to C-18 (δ_C 33.4), C-26 (δ_C 17.5) and C-27. The substructure was further supported by ^{1}H-^{1}H COSY correlations of the same proton signal (H-17) to the resonances of H-16 (δ_H 2.59) and H-18 (δ_H 1.75). CH-19 (δ_C 77.4; δ_H 4.71), the methine adjacent to CH-18, closed the macrolide ring by its ^{1}H-^{13}C HMBC correlation to C-1 and connected it to the side chain of the molecule by correlations to the methylene groups CH_2-20 (δ_C 27.9; δ_H 1.52 and 1.47) and CH_2-21 (δ_C 22.0; δ_H 1.30). After the carbonyl group and the three double bonds one degree of unsaturation still had to be accounted for, which was accomplished by the closure of the ring. Analysis of the NMR spectra gave evidence of a 3-hydroxy-hexyl-side chain. All double bonds, $\Delta^{2,3}$, $\Delta^{4,5}$ and $\Delta^{14,15}$, were determined to be *E*-configured. For the double bonds $\Delta^{2,3}$ and $\Delta^{4,5}$ the configuration was deduced from the ^3J coupling constants of approximately 15 Hz. $\Delta^{14,15}$ is a trisubstituted double bond, therefore the ^{1}H-^{1}H NOESY spectrum had to be consulted. As H-15 showed NOESY-correlations to H_2-13, but not to H_3-28, this double bond, too, had to be *E*-configured. Thus, the planar structure of levantilide A could be unambiguously delineated from the spectroscopic data.

Table III.1: NMR spectroscopic data (500MHz, dmso-*d*6) of levantilide A (**1**)

levantilide A (**1**)					
position	δ_C	δ_H, *J* [Hz]	COSY	HMBC	NOESY
1	166.1, C				
2	120.4, CH	5.78, d (15.5)	3	1, 3, 4, 5	3, 4
3	143.8, CH	7.06, dd (11.2, 15.5)	2, 4	1, 2, 4, 5	2, 4, 5
4	130.6, CH	6.29, dd (11.2, 15.2)	3, 5	2, 3, 6	2, 3, 5, 6b
5	139.8, CH	6.08, ddd (4.4, 10, 15.2)	4, 6	3, 6, 7	3, 4, 6a, 7
6a	39.6, CH_2	2.57, m	5, 6b, 7	4, 5, 7, 8	5
6b		2.33, dt (14.4, 9.9)	5, 6a, 7	4, 5, 7, 8	4
7	67.2, CH	3.98, m	6, 7-OH, 8		8b, 5
7-OH		4.81, d (3.8)	7	7, 8	
8a	33.4, CH_2	1.51[a], m	7, 8b, 9	6, 7, 9	8b
8b		1.16, m	7, 8a, 9	6, 7, 9	7, 8a, 11
9	67.4, CH	3.92, br. d (11.2)	8, 9-OH, 10	30	12
9-OH		4.63, br. s	9	8, 9, 10	
10	41.0, CH	1.68, m	9, 11, 30		
11	75.3, CH	3.00 br. dd (8.9, 6.0)	10, 11-OH, 12	9, 10, 12, 13, 29, 30	8b, 29
11-OH		4.00, d (6.0)	11	10, 11, 12	
12	32.1, CH	1.24, m	11, 13, 29		9
13a	40.1, CH_2	1.87, br. d (12.8)	12, 13b, 15, 29	11, 12, 14, 15, 27, 28, 29	13b
13b		1.51[a], m	12, 13a, 15, 29	11, 12, 14, 15, 27, 28, 29	13a, 15
14	132.8, C				
15	132.7, CH	4.76, d (7.8)	13, 16	12, 13, 16, 17, 27, 28	13b, 17, 29
16	29.4, CH	2.59, m	15, 17, 27	14, 17, 27	27, 28
17a	40.9, CH_2	1.34, m	16, 17b, 18	15, 16, 18, 19, 26, 27	17b
17b		1.05, ddd (13.5, 8.7, 5.0)	16, 17a, 18	15, 16, 18, 19, 26, 27	17a, 27
18	33.4, CH	1.75, m	17, 19, 26	16, 17, 19, 20, 26	
19	77.4, CH	4.71, dt (10, 2.4)	18, 20	1, 17, 20, 21,26	21, 26
20a	27.9, CH_2	1.52[a], m	19, 21		
20b		1.47[a], m	19, 21		
21	22.0, CH_2	1.30[b], m	20		19
22	36.1, CH_2	1.31[b], m	23		23, 23-OH
23	70.8, CH	3.28, m	22, 23-OH, 24	21, 22, 25	22, 25, 26
23-OH		4.25, d (5.5)	23	22, 23, 24	22, 26
24a	29.7, CH_2	1.30[b], m	23, 25		25
24b		1.27[b], m	23, 25		25
25	10.1, CH_3	0.82, t (7.4)	24	23, 24	23, 24
26	17.5, CH_3	0.88, d (6.2)	18	17, 18, 19	19, 23, 23-OH
27	21.5, CH_3	0.84, d (6.8)	16	15, 16, 17	16, 17b
28	17.0, CH_3	1.53, s		13, 14, 15	16
29	17.7, CH_3	0.58, d (6.7)	12	11, 12, 13	11, 15
30	11.1, CH_3	0.86, d (5.9)	10	9, 10, 11	9, 11

[a, b] signals are overlapping

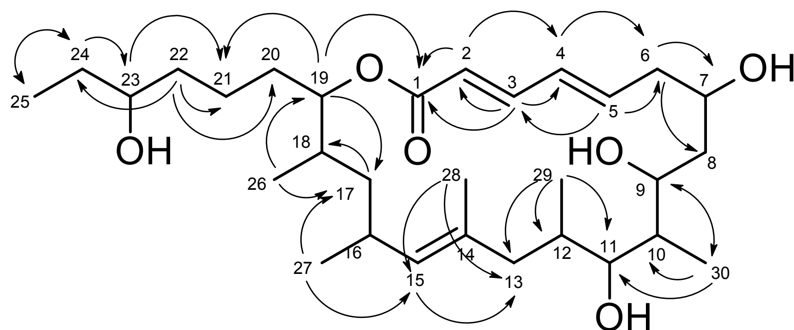

Figure III.2: Selected ^{1}H-^{13}C HMBC correlations relevant for the structure elucidation of **1**

The derivative, levantilide B (**2**) showed a mass difference of 2 in the HPLC-DAD-MS measurement, which already indicated one additional double bond, which for example can be observed, when a hydroxy-group is replaced by a carbonyl function, as it is the case here. Already in the ^{1}H NMR spectrum it was obvious that all signals belonging to protons of the macrolide ring were identical in both molecules (see Table 2). However, significant differences could be observed for the signals of the side chain. Analysis of the data showed that the methine group CH-23 was no longer present in levantilide B. Instead of it, an additional signal of a carbonyl carbon appeared, its shift of 210.5 ppm proving it to be a ketone. As a consequence of the presence of a carbonyl group instead of a methine in position 23, the signal of H_2-24 was no longer a multiplet, but appeared as a quartet as it only coupled with the methyl group CH_3-25, now. Thus, the structure of **2** was established.

Table III.2: NMR spectroscopic data of the levantilides in acetone-d6 (500 MHz)

	levantilide A (**1**)		levantilide B (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	166.9		166.9	
2	122.0	5.83	121.9	5.83
3	144.3	7.14	144.4	7.15
4	131.9	6.36	131.9	6.36
5	140.1	6.09	140.2	6.11
6a	40.6	2.69	40.6	2.66
6b		2.47		2.46
7	69.3	4.11	69.4	4.10
8a	33.8	1.73	33.9	1.72
8b		1.34		1.34
9	69.3	4.18	69.4	4.17
10	42.2	1.86	42.3	1.86
11	77.4	3.17	77.4	3.18
12	33.2	1.41	33.3	1.41
13a	41.4	2.00	41.4	1.99
13b		1.69		1.69
14	134.1		134.1	
15	134.1	4.86	134.1	4.87
16	30.7	2.70	30.7	2.69
17a	41.9	1.47	41.9	1.43
17b		1.12		1.11
18	34.7	1.87	34.7	1.87
19	78.8	4.83	78.6	4.81
20a	28.9	1.61	28.3	1.57
20b		1.53		1.50
21a	23.3	1.42	21.3	1.62
21b				1.47
22	37.6	1.42	42.1	2.45
23	72.7	3.42	210.5	
24a	31.1	1.43	36.0	2.42
24b		1.37		
25	10.4	0.90	8.0	0.96
26	18.6	0.91	18.4	0.91
27	22.0	0.86	21.8	0.87
28	17.6	1.62	17.6	1.63
29	18.5	0.70	18.4	0.71
30	10.8	0.98	11.0	0.98

The levantilides are macrolides with a 20-membered lactone ring. From a biosynthetic point of view macrolides are typical type I PKS products with very well studied biosynthetic pathways. From the structures of the levantilides a very simple assembly of a propionate starter unit, five further propionate building blocks and altogether six acetate building blocks can be deduced.

Cytotoxicity tests of **1** revealed antiproliferative activities against gastric tumor cells GXF 251L (IC_{50}= 40.9 µM), lung tumor cells LXFL 529L (IC_{50}= 39.4 µM), mammary tumor cells MAXF 401NL (IC_{50}= 28.3 µM), melanoma tumor cells MEXF 462NL (IC_{50}= 48.6 µM), pancreas tumor cells PAXF 1657L (IC_{50}= 20.7 µM) and renal tumor cells RXF 486L (IC_{50}= 52.4 µM). Antimicrobial activity against the bacterial and fungal strains in the test panel were not observed for compounds **1** and **2**.
The levantilides are 20-membered macrolides without an attached sugar and are, for example related to the cytotoxic macrolides amphidinolide A and U as well as to iriomoteolide 1a, b and c. These compounds are also 20-membered macrolides which exhibit cytotoxic activity against several human tumor cell lines (Tsuda et al., 2007; Kobayashi and Kubota, 2007; Kobayashi and Tsuda, 2004; Tsuda et al., 1999; Kobayashi and Ishibashi, 1993; Kobayashi et al., 1986) and are produced by the marine symbiotic dinoflagellate *Amphidinium sp.*. Iriomotolides 1a, 1b and 1c show remarkable cytotoxicity against B lymphozyte cells DG75 (IC_{50} = 0.0039 µM, 1.7 µM and 0.0038 µM) while amphidinolide A and U possess cytotoxic activities against murein lymphoma cells L1210 (IC_{50} = 3.7 µM and 10.7µM) and against human epidermoid carcinoma cells (IC_{50} = 10.7 µM and 35.08 µM).
Members of the *Actinomycetes* are well known to produce macrolide antibiotics (Katz and Ashley, 2005). Micromonospolides, mycinamicins, megalomicin, rosamicin and juvenimicins are, for example, macrolide antibiotics produced by members of the genus *Micromonospora*, but they all differ in the size of the macrolide ring from the levantilides (Ohta et al., 2001; Satoi et al., 1980; Hatano et al., 1976; Waitz et al., 1972; Weinstein et al., 1969).

According to Skropeta (2008), polyketide metabolites have been reported from all water depths, but interestingly only 8% of the marine natural products known so far are produced by organisms obtained at depths greater than 1000 m (Skropeta, 2008). As a matter of course this might be due to the fact that the deep sea is hardly accessible.

The macrolides levantilide A and B are produced by a *Micromonospora* strain originating from a marine deep-sea environment and show structural similarity to compounds produced by a marine dinoflagellate. It was shown by cultivation of strain M71-A77 with habitat sea water (38.6 ‰) that levantilides are also produced under the high salinity conditions occurring *in situ* in the Mediterranean Sea. Though strains of *Micromonospora spp.* were frequently isolated from deep-sea habitats (Prieto-Davo et al., 2008; Pathom-aree et al., 2006; Colquhoun et al., 1998), to the best of our knowledge, the levantilides are the first natural products described from a *Micromonospora* sp. strain isolated from the deep sea.

Experimental Section

Isolation and identification of strain M71-A77

Strain M71-A77 has been isolated from a sediment core (1.5-5 cm sediment horizon) from 4400 m depth during research cruise M71/2 with RV Meteor in the Eastern Mediterranean Sea, the so called Levantine Sea [34° 25.48 N, 26° 05.39 E]. 1 g of the sediment sample was transferred to a sterile petri dish and dried for 2 months at 20°C prior to incubation for 1 h at 120°C dry heat. Sediment was then re-suspended in demineralized water and inoculated on agar plates of XJ4-medium containing 1L $aqua_{dest,}$ 18 g agar, 0.1 g histidine, 1 g raffinose, 0.5 g natrium hydrogen phosphate, 1.7 g potassium chloride, 0.05 g magnesium sulfate, 0.01 g iron sulfate, 0.02 g calcium carbonate, 0.5 mg thiamine hydrogen chloride, 0.5 mg riboflavine, 0.5 mg niacine, 0.5 mg piridoxin, 0.5 mg calcium pantothenate, 0.5 mg inositol, 0.5 mg paraamino-benzoic acid and 0.25 mg biotin. After 2 months of incubation at 28°C strain M71-A77 was isolated by transferring to fresh XJ4-medium. The strain was classified by 16S rRNA gene sequence analysis according to Gärtner *et al.* (2008). The 16S rRNA gene sequence was deposited in the EMBL Nucleotide Sequence Database and was assigned the accession no. FR714833.

Chemical analysis

General experimental procedures. The optical rotation was measured on a Perkin Elmer model 241 polarimeter. UV-spectra were obtained on a NanoVue (GE Healthcare). NMR spectra were recorded on a Bruker DRX500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 2.04 and δ_C 28.9 ppm for

acetone-*d*6; δ_H 2.50 and δ_C 39.51 ppm for DMSO-*d*6). High-resolution mass spectra were acquired on a benchtop time-of-flight spectrometer (MicrOTOF, Bruker Daltonics) with positive electrospray ionization. Analytical reversed phase HPLC-UV/MS experiments were performed using a C_{18} column (Phenomenex Onyx Monolithic C18, 100 × 3.00 mm) applying an H_2O (A) / MeCN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL/min) on a VWR Hitachi Elite LaChrom system coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics). Preparative HPLC was carried out using a Phenomenex Gemini C18 110A AXIA, 100 x 50.00 mm column.

Isolation of levantilides A and B. 10L of liquid starch-peptone medium (1L $aqua_{dest}$, 10 g starch, 5 g soy-peptone, 15 g Tropic Marine® sea salt and 1 g calcium carbonate) were used for cultivation of strain M71-A77. After 8 days of incubation (28°C, 125 rpm), the culture supernatant was separated from the cells by centrifugation at 10.000 rpm for 10 min (Beckmann_J2-MC). Cell pellets were suspended in methanol and homogenized three times with an Ultra Turrax T25 basic (IKA-Werke GmbH & Co., Staufen, Germany) at 17.500 U/min for 1 min. After additional centrifugation the methanol extract was decanted and dried. The culture broth supernatant was extracted with ethylacetate (1:1). The dried extracts were dissolved in methanol and analyzed by HPLC-UV/MS. Levantilides A and B were detected at 4.2 and 4.5 min with a maximum UV-absorption at 260 nm. For structure analysis **1** and **2** were separated by reversed phase HPLC. For that purpose HCOOH (0.1%) was added to the solvents H_2O (A) and MeCN (B) and a gradient from 10% B over 60% B (reached after 17 min) to 100% B was applied (flow 15 ml/min). Levantilides A and B were detected at 16.6 and 17.8 min. Thus, 7 mg of **1** and 3 mg of **2** were obtained.

Levantilide A (1): colorless, amorphous solid; $[\alpha]^{20}_D$ -72.4 (*c* 0.145, MeOH); UV (MeOH) λ_{max} (log ε) 262 (4.61); for 1D and 2D NMR data see Table III.1 and Appendix; HRESIMS *m/z* 531.3676 $[M + Na]^+$ ($C_{30}H_{52}NaO_6$, 531.3656).

Levantilide B (2): colorless, amorphous solid; $[\alpha]^{20}_D$ -97.5 (*c* 0.04, MeOH); UV (MeOH) λ_{max} 261 (log ε) (4.48); ^{1}H NMR (Aceton-*d6*, 500 MHz) ! 7.15 (1H,dd, *J*=15.1, 11.0, H-3), 6.36 (1H, dd, *J*=15.1, 11.0, H-4), 6.11 (1H, ddd, *J*=15.1, 9.8, 4.4, H-5), 5.83 (1H, d, *J*=15.1, H-2), 4.87 (1H, d, *J*=8.4, H-15), 4.81 (1H, dt, *J*=10.2, 2.5, H-19), 4.17

(1H, dt, *J*=11.9, 3.1, H-9), 4.10 (1H, m, H-7), 3.18 (1H, m, H-11) 2.69 (1H, m, H-16), 2.66 (1H, m, H-6a), 2.46 (1H, m, H-6b), 2.45 (2H, m, H_2-22), 2.42 (2H, q, *J*=7.5, H_2-24), 1.99 (1H, m, H-13a), 1.87 (1H, m, H-18), 1.86 (1H, m, H-10), 1.72 (1H, m, H-8a), 1.69 (1H, m, H-13b), 1.63 (3H, s, H_3-28), 1.62 (1H, m, H-21a), 1.57 (1H, m, H-20a), 1.50 (1H, m, H-20b), 1.47 (1H, m, H-21b), 1.43 (1H, m, H-17a), 1.41 (1H, m, H-12), 1.34 (1H, m, H-8b), 1.11 (1H, ddd, *J*=14.6, 9.0, 5.1, H-17b), 0.98 (3H, d, *J*=7.0, H_3-30), 0.96 (3H, t, *J*=7.5, H_3-25), 0.91 (3H, d, *J*=7.5, H_3-26), 0.87 (3H, d, *J*=7.0, H_3-27), 0.71 (3H, d, *J*=7.0, H_3-29); ^{13}C NMR (Aceton-*d6*, 125 MHz) ! 210.5 (C, C-23), 166.9 (C, C-1), 144.4 (CH, C-3), 140.2 (CH, C-5), 134.1 (CH, C15), 134.1 (C, C14), 131.9 (CH, C-4), 121.9 (CH, C-2), 78.6 (CH, C-19), 77.4 (CH, C-11), 69.4 (CH, C-7), 69.4 (CH, C-9), 42.3 (CH, C-10), 42.1 (CH_2, C-22), 41.9 (CH_2, C-17), 41.4 (CH_2, C-13), 40.6 (CH_2, C-6), 36.0 (CH_2, C-24), 34.7 (CH_2, C-18), 33.9 (CH_2, C-8), 33.3 (CH, C-12), 30.7 (CH, C.16), 28.3 (CH_2, C-20), 21.8 (CH_3, C-27), 21.3 (CH_2, C21), 18.4 (CH_3, C-26), 18.4 (CH_3, C-29), 17.6 (CH_3, C-28), 11.0 (CH_3, C-30), 8.0 (CH_3, C-25); HRESIMS *m/z* 529.3509 $[M + Na]^+$ ($C_{30}H_{50}NaO_6$, 529.3500).

Production of levantilide A (1) and B (2) at *in situ* salinity

Strain M71-A77 was tested for the production of secondary metabolites at habitat salinity (38.6 ‰). For that purpose, Tropical Marine® salt and $Aqua_{dest}$ were replaced by Mediterranean Sea water obtained from the sampling site. After 8 days of cultivation at 28°C, the culture broth was extracted with ethylacetate and analyzed by analytical HPLC-UV/MS as described above.

Antimicrobial tests

Antimicrobial activity of compound **1** and **2** (100 µM) was tested against *Bacillus subtilis* (DSM 347), *Pseudomonas syringae* (DSM 50252), *Staphylococcus lentus* (DSM 6672), *Xanthomonas campestris* (DSM 2405), *Escherichia coli* (DSM 498), *Erwinia amylovora* (DSM 50901), *Pseudomonas fluorescens* (NCIMB 10586), *Ralstonia solanacearum* (DSM 9544), *Candida glabrata* (DSM 6425) and *Septoria tritici* as described by Lang et al (2007). The results were compared to a positive (100 µM chloramphenicol for bacteria and 100 µM cycloheximide for *C. glabrata* and *S. tritici*) and a negative (no compound) control on the same plate.

Cytotoxic tests

The *in vitro* antiproliferative activities of compound **1** against the gastric cancer cell line GXF 251L cells, lung cancer cell line LXFL 529L, melanoma cancer cell line MEXF 462NL, mammary cancer cell line MAXF 401NL, renal cancer cell line RXF 486L and pancreatic cancer cell line PAXF 1657L were determined by Oncotest GmbH (Freiburg, Germany) using a modified propidium iodide monolayer assay (Dengler et al., 1995). Compound **2** was not tested by Oncotest GmbH, for there was not enough left of the compound.

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Supplementary Information: ^{1}H-NMR spectra, ^{13}C-NMR spectra, ^{1}H-^{1}H COSY spectra and ^{1}H-^{13}C HMBC spectra of compounds **1** and **2** as well as the NMR spectroscopic data of compound **2** are supplied in the appendix.

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CHAPTER IV

Short note:

Bacteria from the Logatchev hydrothermal vent field exhibit antibiotic activities

Andrea Gärtner, Jutta Wiese and J.F. Imhoff

Manuscript in preparation for publication as short note

Abstract:

With the aim to recover bioactive heterotrophic bacteria from the Logatchev hydrothermal vent field, 100 bacterial strains affiliating to *Gammaproteobacteria* (91%), *Firmicutes* (6%), *Alphaproteobacteria* (2%) and *Actinobacteria* (2%) were isolated. One third of the strains had less than < 98.7% 16S rRNA gene sequence similarity to the next related type strain and thus might be considered as representatives of novel species. 50 % of the strains inhibited growth of at least one of the Gram-positive test strains (*Bacillus subtilis, Staphylococcus lentus*) and nine strains additionally inhibited the growth of *Escherichia coli*. One of the bioactive strains was assigned to a novel bacterial genus, *Amphritea atlantica*^{T} (Gärtner et al., 2008). The present study demonstrates that deep-sea hydrothermal vent fields harbor a considerable number of novel and bioactive bacterial strains as a promising source for the discovery of bioactive compounds.

Today the deep sea is coming into focus of recent drug research for the discovery of novel bioactive compounds (see review (2008)). Especially in regard of the high recovery rate of already known substances, the exploration of novel habitats with high species diversity seems very promising. Deep-sea hydrothermal vent fields are unique ecosystems that represent hot spots of biodiversity in contrast to the scarce deep ocean biosphere. These systems are characterized by a dense macrofauna biomass supply of e.g. mussels, shrimps, crabs, tubeworms and fishes. Furthermore, steep gradients of temperature, pH, oxygen, heavy metals and reduced chemical compounds characterize these systems and offer a broad range of microhabitats. Thus, a dense and highly diverse microbial community can be found, with the primary production of chemolithotrophic bacteria at the basis of the comprehensive food web (Van Dover, 2000).

It is proposed, that the extreme living conditions present at hydrothermal vent ecosystems do not solely affect the physiological capabilities but also the secondary metabolite production of the inhabitants. Quite a lot of novel biotechnological relevant lipids, enzymes, proteins and polysaccharides already have been isolated from deep-sea hydrothermal vent microbes (for review see Wilson and Brimble (2009)). In contrast, the discovery of microbial bioactive secondary metabolites from hydrothermal vent systems is just in the beginning. Only two years ago the first novel natural products from hydrothermal vent environments have been reported. The amphiphilic siderophores lohichelins A-F were isolated from a strain of *Halomonas sp.* LOB-5, from the Loihi seamount (Homann et al., 2009). In addition, two brominated hydroxyethylamine chromans, ammonificins A and B, were isolated from a strain of *Thermovibrio ammonificans* (Andrianasolo et al., 2009). Unfortunately, the bioactive features of these compounds are not yet elucidated. Thus, microbes isolated from hydrothermal vent fields are an unexplored and highly promising source for the discovery of novel natural products.

The aim of the present study was therefore the isolation and classification of antimicrobially active bacteria from the Logatchev hydrothermal vent field, which is located at 14°45´N 45°58´W on the Mid-Atlantic Ridge in 2960-3060 m water depth (Batuyev et al., 1994; Kuhn et al., 2004). With the intent to isolate bacteria from different hydrothermally influenced habitats, several samples of sediment, stones and *Bathymodiolus* sp. mussels were plated on solid TSB3-S25 medium (3 g/L tryptic soy broth, 25 g/L NaCl, $Aqua_{dest}$) and GPYNS-S30 medium (1 g/L glucose, 0.5 g/L

peptone, 0.1 g/L yeast extract, 30 g/L NaCl, 1L $Aqua_{dest}$). In total, 100 strains were isolated after incubation at 28°C for 2 - 10 weeks. These strains were tested to inhibit the growth of selected Gram-positive and Gram-negative test strains *Bacillus subtilis* (DSM347), *Staphylococcus lentus* (DSM6672), *Escherichia coli* (DSM498) and *Candida glabrata* (DSM6425), respectively, by an overlay method according to Wiese *et al.* (2009). Half of the strains (50) revealed antimicrobial activity against at least one of the test strains. These biologically active strains were classified by 16S rRNA gene sequencing as described by Gärtner *et al.* (2008). Most bioactive strains affiliated to the *Gammaproteobacteria* (90%). Further three strains affiliated to the *Firmicutes* and one strain each affiliated to the *Alphaproteobacteria* and the *Actinobacteria,* respectively (Figure IV.1).

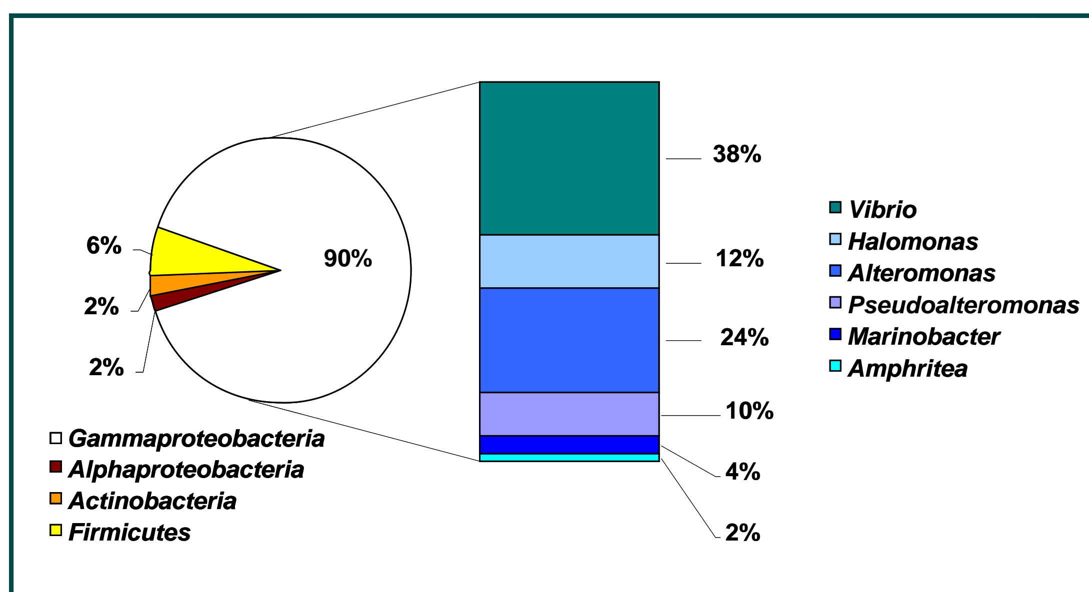

Figure IV.1: Phylogenetic affiliation of strains isolated from the Logatchev hydrothermal vent field

The dominating *Gammaproteobacteria* were represented by six different genera: *Vibrio* (18 strains), *Halomonas* (6 strains), *Alteromonas* (12 strains), *Pseudoalteromonas* (5 strains), *Marinobacter* (3 strains) and the novel genus *Amphritea* (Gärtner et al., 2008) as well. One third of these strains (17) had less than 98.7 % 16S rRNA gene similarity to the next related type strain and thus can be

considered as representatives of novel species (Table I.1) (Stackebrandt and Ebers 2006).

The growth inhibition activity of these phylogenetically identified strains was predominantly targeted towards the Gram-positive test strains. All strains revealed to inhibit the growth of at least one of the Gram-positive test strains, with 70% inhibiting growth of *Bacillus subtilis and* 42% inhibiting the growth of *Staphylococcus lentus.* Additional 8 strains (16%) were also active against *E. coli* (Table IV.1 and Figure IV.2).

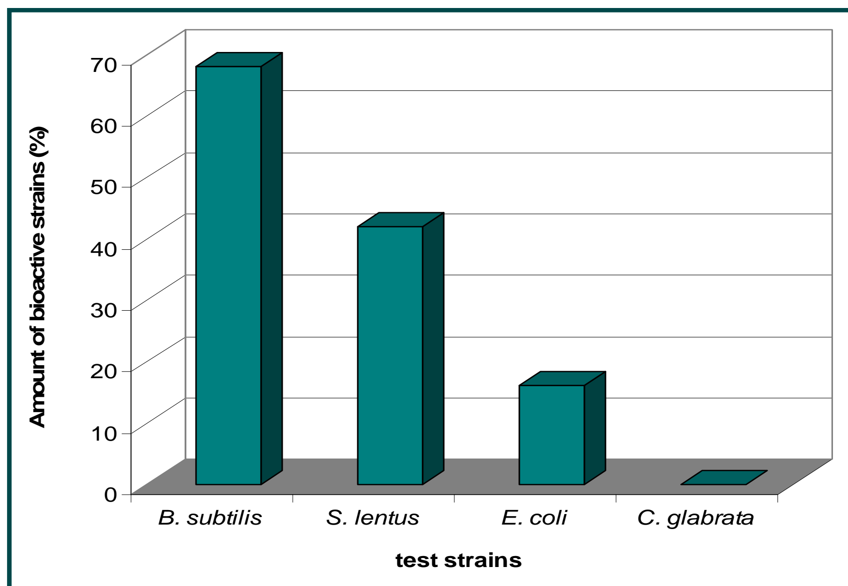

Figure IV.2: Antimicrobial activities of 50 strains isolated from the Logatchev hydrothermal vent field.

The strain collection of the Logatchev hydrothermal vent field comprised bacterial taxa which are commonly found at diverse deep-sea habitats (Süß et al., 2004; Rathsack et al., 2009; Zeng et al., 2010). Especially *Gammaproteobacteria* represented by members of *Vibrio sp.*, *Alteromonas sp.*, *Pseudoalteromonas sp. Marinobacter sp.* and *Halomonas sp.* dominated the strain collection of the present study. These taxa are frequently assigned to the lifestyle of r-strategists and thus the enormous biomass supply at hydrothermal vent sites displays optimal growth conditions. Moreover, the gammaproteobacterial strains were antimicrobially active against Gram-positive as well as Gram-negative test strains. Studies about the ecological function of antibacterial compounds in hydrothermal environments are rare. It is hypothesized, that vent metazoans use the antibacterial substances of associated bacteria as chemical defense against grazing (Kicklighter et al., 2004).

This assumption was based on the observation, that shallow-water fishes denied food supplemented with tissue of vent polychaetes and bivalves, while food supplemented with the H_2S-rich blood of *Riftia pachyptila* was not rejected by the fishes. Also, crude extracts of the deterred tissues revealed unpalatable for the predator. Members of *Vibrio sp.*, *Alteromonas sp.*, *Pseudomonas sp.* and *Halomonas sp.,* which have been isolated in the present study as antimicrobial active strains, were already shown to produce antimicrobial compounds but as far as known to date, no bioactive compounds have been isolated from microbial representatives of deep-sea hydrothermal vent ecosystems until now (see Dictionary of Natural Products, CRC Press, Taylor & Francis Group, 2010).

It turned out further that 34 % of the strains showed less than 98.7 % 16S rRNA gene sequence similarity to the next related type strains and therefore can be assigned to novel species (Stackebrandt and Ebers, 2006). This especially refers to gammaproteobacterial strains. The novel bioactive strains recovered from the LHF might be specifically adapted to the extreme living conditions and thus there is considered to be a high potential for the discovery of unique metabolite structures.
Among the presumably novel strains, strain M41^T turned out to be a representative of a new genus (Gärtner et al., 2008). Strain M41^T was isolated from a mussel of *Bathymodiolus sp.* and showed antimicrobial activity against *Bacillus subtilis*. One of the next related clone sequences, with 98% 16S rRNA sequence similarity, was assigned to an uncultivated clone sequence R21 of a heterotrophic symbiont from the whale bone eating polychaete *Osedax japonicus* (AB293970). Meanwhile, two additional species have been ascribed to the genus *Amphritea*: *A. balanea* (type strain JAMM 1525^T) and *A. japonica* (type strain JAMM 1866^T) (Miyazaki et al., 2008). These strains have been recovered from sediment adjacent to sperm whale carcasses in the Japan Sea at 230 m depth. Strain JAMM 1866^T revealed to have 100% 16S rRNA gene sequence similarity to the symbiotic bacterial clone R21 of *Osedax japonicus* (AB293970). Whale falls create sulfide-rich habitats analogous to hydrothermal vent systems (Smith et al., 2003). The environment of whale carcasses is similar to that of hydrothermal vent fields. Due to the high concentrations of H_2S as well as the enormous supply of biomass in contrast to the scarce deep sea, whale carcasses are discussed as stepping stones in the dispersal of the hydrothermal community (Smith 1989). The close affiliation of the related strains JAMM 1866^T and

JAMM 1525 to the *Osedax japonicus* symbionts and as well the temperature (4 - 40°C) and pH (4.6 - 9.5) range for growth of strain M41^T lead to the assumption, that M41^T might be a typical member of the microbial hydrothermal vent community. Further information about strain M41^T will be given in Chapter V.

Our data demonstrate that microbes from hydrothermal vent ecosystems are a highly promising source for the discovery of bioactive substances. The extreme living conditions of these unexplored ecosystems as well as the phylogenetic, metabolic and physiological diversity of the microbial community considerably increase the potential for the recovery of novel bioactive metabolites.

Table IV.1: Phylogenetic affiliation and antibacterial bioactivity of bacterial strains isolated from the Logatchev-hydrothermal vent field. Test results of antibiotic test assays are quoted with the size of the inhibition zone (mm)

Information about the strain			phylogenetic affiliation			Antibacterial bioactivity test results		
Strain ID	bp	Source of isolation	Name of closest related type strain	Acc.Nr	Similarity (%)	*B. subtilis*	*S. lentus*	*E. coli*
M60_ M5a	1472	sulfidic sediment	*Halomonas axialensis*, DSM 15723^T	AF212206	99.8	2	-	-
M60_ M5b	1483	sulfidic sediment	*Vibrio natriegens*, ATCC14048^T	X74714	**98.3**	5	-	-
M60_ M11	1515	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	100.0	-	2	-
M60_ M12	1459	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	98.9	-	2	-
M60_ M13b	1486	hot sediment	*Halomonas salari*, DSM 18044^T	AM229316	99.0	2	-	-
M60_ M13c	1312	hot sediment	*Paracoccus alcaliphilus,* DSM 8512^T	AY014177	**94.8**	3	-	-
M60_ M14a	1482	hot sediment	*Halomonas salari*, DSM 18044^T	AM229316	98.8	2	-	-
M60_ M16	1494	hot sediment	*Bacillus subtilis subsp. subtilis,* DSM 10^T	AJ276351	100.0	3	8	1
M60_ M17a	1497	hot sediment	*Halomonas meridiana*, DSM 5425^T	AJ306891	99.6	3	-	-
M60_ M17b	1425	hot sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**98.4**	3	-	-
M60_ M18a	1496	hot sediment	*Marinobacter aqueolei,* DSM 11845^T	AJ000726	99.5	3	-	-
M60_ M18b	1437	hot sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**98.3**	1	-	-
M60_ M18c	1484	hot sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**98.2**	1	-	-
M60_ M19b	1395	hot sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**97.2**	5	-	-
M60_ M26	1494	stone with bacterial mat	*Pseudoalteromonas alien,* KMM 3562^T	AY387858	**95.6**	3	-	-
M60_ M27	1466	stone with bacterial mat	*Pseudoalteromonas alien,* KMM 3562^T	AY387858	**96.0**	2	-	-
M60_ M31a	1520	part of a chimney	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99.1	-	1	-
M60_ M34a	1520	part of a chimney	*Vibrio fluvialis,* LMG 7894^T	X76335	**98.5**	2	1	-
M60_ M34b	1488	part of a chimney	*Bacillus subtilis subsp. subtilis,* DSM 10^T	AJ276351	99.8	-	1	-
M60_ M35a	1473	part of a chimney	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	100.0	-	2	-
M60_ M35b	1501	part of a chimney	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	100.0	-	2	-
M60_ M36b	1475	part of a chimney	*Bacillus subtilis subsp. subtilis,* DSM 10^T	AJ276351	100.0	-	1	-
M60_ M39	1508	*Bathymodiolus mussel*	*Pseudoalteromonas arctica*, DSM 18437^T	DQ787199	**97.4**	5	-	-
M60_ M40	1500	*Bathymodiolus mussel*	*Pseudoalteromonas paragorgicola*, KMM 3548^T	AY040229	**96.8**	5	-	-
M60_ M41	1325	*Bathymodiolus mussel*	*Oceanospirillum multiglobuliferum*, ATCC 33336^T	AB006764	**93**	5	-	-
M60_ M42a	1460	*Bathymodiolus mussel*	*Vibrio natriegens*, ATCC14048^T	X74714	99.2	-	1	-
M60_ M42b	1421	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	100.0	-	2	-
M60_ M46	1488	hot sediment	*Alteromonas marina*, JCM 11804^T	AF529060	98.9	5	-	-
M60_ M52	1471	sediment	*Alteromonas marina*, JCM 11804^T	AF529060	98.8	2	-	-
M60_ M53	1473	sediment	*Alteromonas marina*, JCM 11804^T	AF529060	98.8	2	-	-
M60_ M54	464	sediment	*Halomonas axialensis*, DSM 15723^T	AF22206	99	2	-	-
M60_ M59	1496	sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**98.3**	2	-	-
M60_ M60	1455	sediment	*Alteromonas marina*, JCM 11804^T	AF529060	98.7	2	-	-
M60_ M62	1448	stone with bacterial mat	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99	1	2	-
M60_ M63	1480	stone with bacterial mat	*Marinobacter vinifirmus*, DSM 17747^T	DQ235263	**98.0**	1	-	-
M60_ M64a	1440	stone with bacterial mat	*Marinobacter aqueolei,* DSM 11845^T	AJ000726	**98.0**	2	-	-
M60_ M64b	1290	stone with bacterial mat	*Alteromonas marina*, JCM 11804^T	AF529060	99.6	3	-	-
M60_ M65	1472	stone with bacterial mat	*Halomonas axialensis*, DSM 15723^T	AF212206	99.9	2	-	-
M60_ M70	1374	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	98.9	-	1	-
M60_ M72a	1460	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99.1	-	2	3
M60_ M72c	447	*Bathymodiolus mussel*	*Pseudoalteromonas haloplanktis,* DSM 6060^T	X67024	100.0	2	4	-
M60_ M73	1508	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99.07	-	2	3
M60_ M74b	1428	*Bathymodiolus mussel*	*Vibrio natriegens* strain ATCC 14048	X74714	99.6	-	2	4
M60_ M74c	1421	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99.0	-	2	4
M60_ M75b2	1481	*Bathymodiolus mussel*	*Microbacterium oxidans,* DSM 20638^T	AJ717357	100.0	1	2	3
M60_ M76b	1457	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	**97.9**	4	-	-
M60_ M75a	1093	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	99	-	2	3
M60_ M77	1433	*Bathymodiolus mussel*	*Vibrio parahaemolyticus*, ATCC 17802^T	AF388387	100.0	-	2	1
M60_ M82	1383	sulfidic sediment	*Alteromonas marina*, JCM 11804^T	AF529060	98.8	2	-	-
M60_ M84	1491	sulfidic sediment	*Alteromonas marina*, JCM 11804^T	AF529060	**98.4**	2	-	-

the term “hot sediment” refers to an onboard temperature of about 60°C of the recovered sediment sample.

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CHAPTER V

Amphritea atlantica gen. nov., sp. nov., a gammaproteobacterium from the Logatchev hydrothermal vent field

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Abstract

A new Gram-negative, motile, aerobic rod-shaped bacterium was isolated from a *Bathymodiolus* sp. specimen collected from the Logatchev hydrothermal vent field at the "Mid-Atlantic" Ridge. The bacterium is catalase and oxidase positive and metabolizes various carbohydrates and amino acids. It grows well on Marine Broth with an optimal growth temperature of 31°C to 34°C (range 4 to 40°C) and salinity requirements of 3% (range 0.3 - 9%). The pH range for growth is 4.6 and 9.5 with an optimum at pH 8.0. Predominant fatty acids are $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$. The DNA base composition of strain $M41^T$ is 52.2 mol% G+C. The 16S rRNA gene sequence is 94% similar to that of the type strain of *Oceanospirillum beijerinkii* as the closest cultivated relative. Other related type strains are *Oceanospirillum multiglobuliferum (93%), Neptunomonas naphthovorans* (92%) and *Marinobacterium jannaschii* (92%). According to phylogenetic analysis and physiological characteristics, the description of a novel genus and species is proposed, which is designated *Amphritea atlantica gen. nov., sp. nov*. and is represented by type strain $M41^T$ (= DSM 18887^T= LMG 24143^T).

INTRODUCTION

The deep sea is an extreme habitat in which organisms need to be adapted e.g. to high pressure, to low nutrient concentrations and temperatures, to darkness and irregular food availability (Jannasch *et al.*, 1984). At deep-sea hydrothermal vent fields the organisms additionally have to deal with steep physicochemical gradients such as temperature over an extremely large range, heavy metal concentrations, oxygen supply and pH (Kelley *et al.*, 2002). Along these gradients, many microhabitats are formed and a high diversity of microorganisms can develop. Even though an increasing number of bacterial clone sequences in the databases originate from deep-sea habitats and also from deep-sea hydrothermal fields (Hoek *et al.*, 2003; López-García, 2003; Radjasa *et al.*, 2001; Reysenbach, 2000), at present only a small number of deep sea bacteria have been cultivated.

Phylogenetic information derived from 16S rRNA gene sequences does not give ecological or physiological information that could support the functional characterization of these extreme habitats. Hence, cultivation approaches are necessary and need to be applied. Moreover, microorganisms from extreme marine environments are considered as a rich source for the detection of new secondary metabolites, new metabolic pathways and new enzymes, and therefore have become a special focus of research in recent years (Faulkner, 2000; Haefner, 2003; Jensen *et al.*, 1994; Kelecom, 2002).

As part of a study of the microbial biodiversity of hydrothermal vents at the "Mid-Atlantic" Ridge, we have isolated numerous bacteria living in habitats influenced by hydrothermal activity. Among these, a new Gram-negative, mesophilic rod shaped *Gammaproteobacterium*, strain M41^T, showed less than 94% 16S rRNA gene sequence similarity to any other validly described bacterium. In this study, the isolate was characterized by phenotypic and phylogenetic analyses and is proposed as the new species and genus *Amphritea atlantica* with the type strain M41^T (= DSM 18887^T = LMG 24143^T = JCM 14776^T).

MATERIALS AND METHODS

Isolation and storage of strain M41^T

Strain M41^T was isolated by dilution-plating method from a *Bathymodiolus sp.* mussel sample collected at the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge (14°45,19N and 44°58,75W) in about 3000 m depth. The mussels were taken from a

TV-grab on research cruise M60/3, which recovered several mussels and other typical hydrothermal fauna like crabs, snails and ophiurids. One of the mussels was directly stored in sterile seawater at 4°C for further isolation procedures. Later the storage water was diluted and plated on TSB medium (3.0 g/l Difco tryptic soy broth, 15 g/L Difco agar, 25 g/L NaCl) and incubated at 22°C for 14 days. Strain M41^T was isolated and maintained at -20°C using the Cryobank System (Mast Diagnostica GmbH).

Morphological characterization

The cell morphology was determined by scanning electron microscopy. Strain M41^T was cultivated for 24 h in liquid Marine Broth (Difco) at 28°C on a rotary shaker with 100 rpm, followed by fixation with a final concentration of 1% formol and filtered through 0.2 µm polycarbonate filters (Sarstedt). The filters were applied in a subsequent ethanol series for dehydration (50%, 70%, 90% and three times 100% for 10 min each) (Boyde *et al.*, 1969), critical-point drying with CO_2 and sputter-coated with Au/Pb and examined with a Zeiss DSM 940 scanning electron microscope.

Physiological characterization

Strain M41^T was cultivated aerobically on Marine Broth (Difco). The temperature range for growth was tested by incubation in liquid Marine Broth medium at 4°C, 15°C, 20°C, 27°C, 29°C, 31°C, 33°C, 35°C, 37°C, 39°C, 41°C, 43°C and 45°C. The salinity requirements were tested from 0% to 13% in liquid medium consisting of different amounts of marine salt (Tropic Marine), 1 g/L peptone (Difco) and 5 g/L yeast extract (Difco) at 28°C. The pH range for growth was tested in the range from pH 3.5-10 in steps of 0.5. To test, whether NaCl can replace the salt requirement of natural seawater, strain M41^T was incubated at 28°C in a medium consisting of 1 g/L peptone (Difco), 5 g/L yeast extract (Difco) and 15 g/L agar (Bacto) in 1 L of pure water and supplemented with 0 -100 g NaCl. The Na^+ requirement was tested in a Marine Broth, in which the NaCl was replaced by either $CaCl_2$ or $MgCl_2$.

Gram-staining using KOH according to Gregersen (1978), poly-ß-hydroxybutyrate (PHB)-staining with Sudan black according to Simbert *et al.*, (1994) and catalase production (detected with 5% H_2O_2) were all performed with overnight cultures incubated at 28°C in Marine Broth and determined by phase-contrast microscopy

(Axiophot, Zeiss). Luminescence was tested on liquid and solid Marine Broth supplemented with 3% glycerol.

The aerobic oxidation of various compounds was tested using the BIOLOG GN2 system (Oxoid). Liquid cultures of strain M41^T were diluted in 1% NaCl solution to obtain an optical density of 0.3 - 0.35 (OD_{600nm}). This solution was used as inoculum for the tests which were done in triplicate and checked daily for one week. A positive test result was assessed in case of three positive reactions. Additional tests were performed with the API 20NE test system for Gram-negative bacteria (Biomerieux) and API ZYM (Biomerieux). The inoculum was prepared as described before and incubated for at least 3-7 days at 32°C. Both tests were run in triplicates.

Chemotaxonomic properties

The G + C content and the fatty acid composition of strain M41^T were analyzed at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) using the HPLC-method (Mesbah *et al*., 1989) and the MIDI-System (Sasser, 1990).

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA from the culture was extracted as follows: cell material was transferred from agar plate into 500 µl DNA-free water (Sigma-Aldrich) and homogenized (2 x 6300 rpm/min for 20 sec) in the Precellys24 homogenisator (PEQLAB Biotechnologie GmbH, Erlangen, Germany). After centrifugation (10 min; 8.000 g) the supernatant was directly used for PCR. Amplification of the 16S rRNA gene was performed using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) with the eubacterial primers 27f and 1492r (Lane, 1991). The PCR conditions were: initial denaturation (2 min at 94°C) followed by 30 cycles of primer extension (90 s at 72°C), primer annealing (40s at 50°C) and denaturation (40s at 94°C) and a final step of primer annealing (1 min at 42°C) as well as a final extension step (5 min at 72°C).
Purification of PCR products was carried out with Exonuclease I (Exo I, GE Healthcare) and Shrimp Alkaline Phosphatase (SAP, Roche). For each reaction 1.5 U of Exo I and 0.3 U of SAP were added to the PCR product and incubated for 15 min at 37°C, followed by heat inactivation of the enzymes for 15 min at 72°C. Sequencing was performed using the BigDye Terminator v1.1 Sequencing Kit

(Applied Biosystems) in a 3730-DNA-Analyzer (Applied Biosystems) as specified by the manufacturer. To obtain sequence information of both DNA strands, sequencing was performed with the primers 27f, 342f (Lane, 1991), 790f (5`-GATACCCTGGTAGTCC-3`), 543r (Muyzer *et al*., 1993), 907r (Lane *et al.*, 1985) and 1492r. The 16S rRNA genesequence was submitted to the EMBL/GenBank Database.

The 16S rRNA gene sequence of strain M41^T was compared to other gene sequences in the NCBI GenBank database using BLAST search (Altschul *et al.*, 1990), the Ribosomal Database Project II (Cole *et al.*, 2007) using the sequence match tool and the EMBL-EBI online tool FASTA (Pearson, 1990). The sequence was aligned using the ARB software package (Ludwig, 2004). A maximum parsimony tree and a neighbor joining tree were calculated within ARB (data not shown). Additionally a maximum likelihood tree was calculated with the online version of PhyML (Guindon *et al.*, 2005) using the general time reversible (GTR) model with 500 bootstrap replicates.

RESULTS AND DISCUSSION

Phenotypic and physiological characteristics

Using solid Marine Broth medium, strain M41^T formed flat and circular beige colonies with entire whitish edges. The cells were Gram-negative, rod-shaped, 0.3 – 0.5 x 0.5 – 2 µm in dimensions and possess a single monopolar flagellum in overnight cultures (Fig. V.1). In contrast, members of the phylogenetically closest genus *Oceanospirillum* form helical cells with bipolar tufts.
After one week of incubation coccoid bodies, so called microcysts, occurred in the cultures. The occurrence of microcysts in older cultures of *Spirillum lunatum* has been described earlier and it was found that the majority of coccoid bodies present in old cultures are viable and can germinate when placed into fresh medium (Rittenberg *et al.*, 1956).

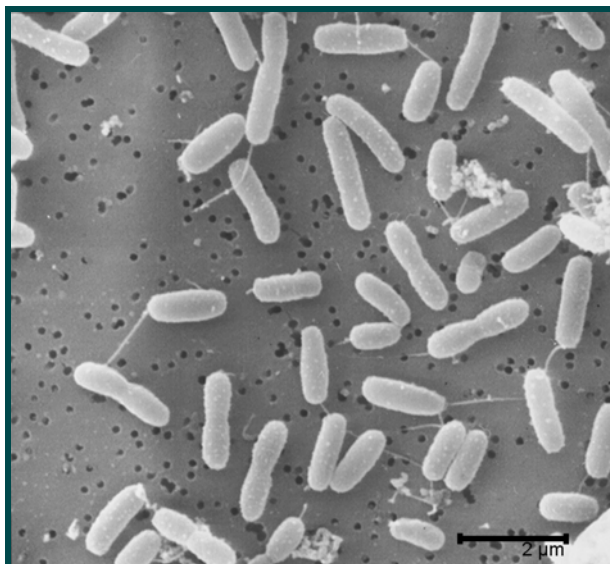

Figure V.1: Scanning electron micrograph of cells of strain M41T cultivated in Marine Broth for 24 h at 28°C. Scale bar: 2 µm

Strain M41T was able to grow at 4°C (allowing incubation of more than 7 days) but could not grow at more than 40°C. Optimal growth occurred between 31 - 34°C and 3% salinity. The salinity range for growth was 0.3 and 9% if sea salt was used. NaCl could replace the complex mixture of sea salt, but no growth occurred in the absence of NaCl or sea salts. Growth in medium with NaCl as sole salt source occurred from 0.3 – 6% NaCl. An obligate requirement for the sodium ion NaCl was indicated by the inability to replace the sodium ion by magnesium or calcium chloride. The optimum pH for growth was 8, the growth range from pH 4.6 – 9.5.
Further detailed phenotypic data are given in Table V.2 and in the species description.

Chemotaxonomic characteristics

The fatty acid profile of strain M41T (Table V.1) was characterized by mainly straight-chain saturated and unsaturated fatty acids with approximately 8% of short-chain 3-hydroxy-fatty acids. $C_{16:1}\omega 7c$ was the predominantly fatty acid and made up 40% of the total fatty acid profile. Other fatty acids were: $C_{16:0}$ (29%); $C_{18:1}\omega 7c$ (22%); $C_{12:0}$ (2%); $C_{18:0}$ (0.8%) and $C_{10:0}$ (0.3%). The hydroxy fatty acids comprised approximately 8% of total fatty acids, $C_{10:0}$ 3OH (5%) and $C_{12:1}$3OH (3%).

Table V.1: Fatty acid composition of strain M41^T and closely related genera.
Percentages of all non-polar and all hydroxy fatty acids are considered separately.
1 = strain M41^T; 2 = *Oceanospirillum beijerinckii*; 3 = *Oceanospirillum linum* 4 = *Marinobacterium jannaschii*; 5 = *Oceanobacter kriegii*; 6 = *Oceanospirillum multiglobuliferum*; 7 = *Reinekea marinisedimentorum.*

Fatty acids	1[a]	2[b]	3[b]	4[b]	5[b]	6[b]	7[c]
				Non-polar			
$C_{10:0}$	0.3						
Unknown	2						
$C_{12:0}$	2	4	3	2	7	3	<1
$C_{12:1}$			2		4	2	
$C_{14:0}$		4	1	1	1	2	2
$C_{14:1}$					1		
$C_{15:0}$		1			2		4
$C_{16:0}$	29	32	16	19	16	28	32
$C_{16:1}$	43	50	48	46	36	44	27
$C_{17:0}$					2		6
$C_{17:1}$					3		3
$C_{18.0}$	0.8			1	1		<1
$C_{18:1}$	22	9	30	31	27	20	19
$C_{19:1}$							
				Hydroxy			
$C_{10:0}$ 3-OH	64	63	100	100	19	100	
$C_{10:1}$ 3-OH							
$C_{12:0}$ 3-OH					54		
$C_{12:1}$ 3-OH	36						
$C_{14:0}$ 3-OH		30					2
$C_{14:1}$ 3-OH							
$C_{16:0}$ 3-OH		6			27		

[a] data from this study
[b] data obtained from Hylemon *et al.* (1973)
[c] data obtained from Romanenko *et al.* (2004)

The fatty acid profile was similar to that of members of the closely related genus *Oceanospirillum* (Hylemon *et al.*, 1973) and to those of other related genera like *Neptunomonas* (Hedlund *et al.*, 1999), *Marinobacterium* (Satomi *et al.*, 2002) and to the more distantly related *Reinekea* (Romanenko *et al.*, 2004). Predominant fatty

acids were $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$. Characteristic is the presence of $C_{12:1}$3OH in strain M41^T (Table V.1).The DNA base ratio of strain M41^T is 52.2 mol% G + C.

Phylogenetic analysis

Sequence analysis of the 16S rRNA gene and phylogenetic calculations showed, that *Oceanospirillum beijerinckii* IFO13614^T (93.8%) and *Oceanospirillum multiglobuliferum* IFO13614^T (93.4%) are the closest related cultivated organisms to strain M41^T. Figure V.2 illustrates the maximum likelihood tree that is supported by the topology of the neighbor joining tree and the maximum parsimony tree (data not shown).

Moreover, the 16S rRNA gene sequence of *Neptunomonas naphtovorans* ATCC700637^T has 92.6% similarity to that of strain M41^T. Four uncultivated clone sequences from a sea dyke in Isahaya Bay (Japan) have 100% sequence similarity to strain M41^T. One representative of these clone sequences (AY910901) is included in Fig. 2. Other uncultivated clone sequences, that have equal or higher sequence similarity to strain M41^T than any of the cultivated bacteria are represented by i) a sediment clone (AY375069), that originates from a warm pool of the Western Pacific (95%), ii) an uncultivated carbazole degrading bacterium (AB086227) (93.4 %) of unknown origin, and iii) a tributyl-resistant, biofilm-forming bacterium (AF505721) from Boston harbor surface water (93.1%). The 16S rRNA gene sequence of strain M41^T is available at the GenBank/EMBL/DDBJ databases under the accession number AM156910.

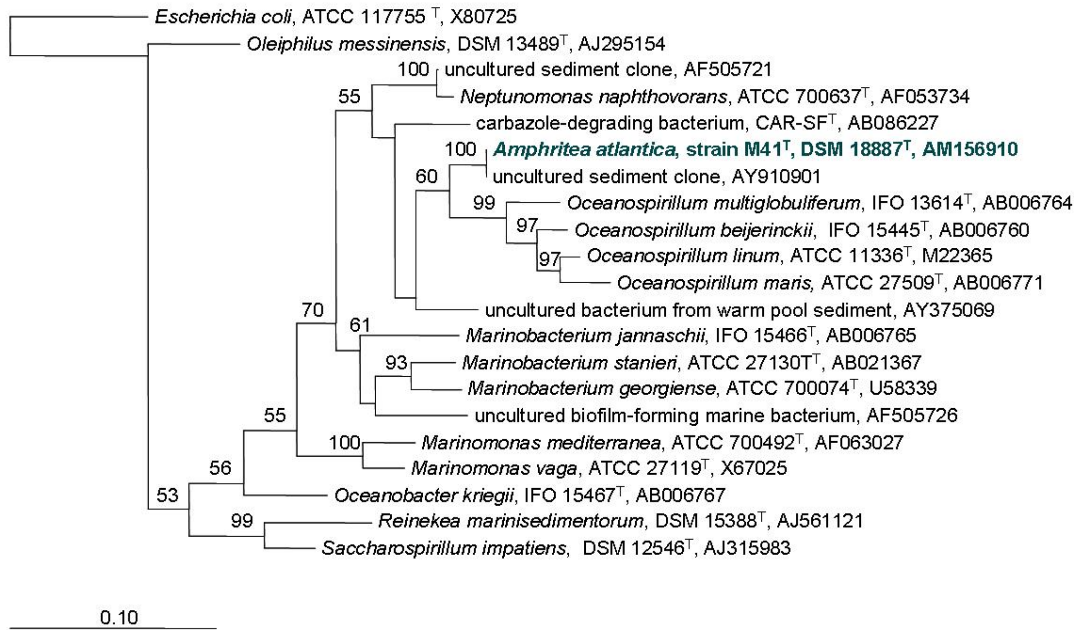

Figure V.2: Phylogenetic maximum likelihood tree of strain M41^T and related type strains. Some selected uncultivated clone sequences with close relationship to M41^T were also included into phylogenetic calculation. Tree was calculated with the online version of PhyML with 500 bootstrap replicates using the GTR evolution model. Bootstrap values expressed as percentage of 500 replications are given next to the branching points, when higher than 50%.

Consistent with the phylogenetic placement, strain M41^T shared some physiological properties with *Oceanospirillum* spp., such as the formation of coccoid bodies in old cultures, accumulation of poly-ß-hydroxybutyrate, catalase and oxidase reaction. But there were also some major phenotypic differences (Table V.2). Members of *Oceanospirillum* spp. have helical cells with bipolar tufts and the G+C content is as low as 42-48 mol%. They do neither oxidize nor ferment sugars (González et al., 2001; Krieg, 1976). In contrast, strain M41^T had a G+C content of 52.2 mol% and formed straight rods with a monopolar flagellum. Furthermore, strain M41^T was able to oxidize some sugars. The low sequence similarity of strain M41^T to representatives of all related genera of *Oceanospirillaceae* (< 95%) in addition to significant physiological differences necessitates the description of a new genus for which the name *Amphritea* is proposed. The physiological properties characterize *Amphritea* as a typical marine bacterium with an obligate requirement for sodium, a growth optimum close to seawater salinity and a temperature range from cold deep ocean temperatures up to the elevated mesophilic range in proximity of hydrothermal vent systems of the deep sea. In addition, the substrate utilization (carbohydrates, amino

acids) and enzymatic activities characterize this bacterium as a degrader of organic matter available in the vicinity of hydrothermal mussel fields.

Table V.2: Properties of strain M41^T in comparison to related genera.
1 = M41^T (data from this study); 2 = *Oceanospirillum (Hylemon et al., 1973);* 3= *Neptunomonas* (Hedlund *et al.*, 1999; Hylemon *et al.*, 1973); 4 = *Marinobacterium* (Hylemon *et al.*, 1973; Satomi *et al.*, 2002);

	1	2	3	4
morphology	straight rods	helical	straight rods	straight rods
cell size	0.5x 0.5-2µm	0.4-1.2 x 2-4	0.7-0.9	0.5-0.7 x 1.6- 2.3
flagella	single polar	bipolar tufts	single polar	1-2 polar
Coccoid body formation	+	+	+	-
Growth at 4°C	+	-	+	+
G+C-content (mol%)	52,2	47-49	46	54,9
PHB	+	+	+	-

nd, not determined; +, presence; -, absence.

Description of *Amphritea* gen. nov.

Am.phri´te. (N.L. fem. n. Amphritea from Gr. Fem. N. Amphrite, *a nymph of the ocean in Greek mythology*; referring to the habitat of the bacteria).

Cells are Gram-negative rods, motile by monopolar flagella. Coccoid bodies may be formed in old cultures. Catalase and oxidase positive and accumulate poly-ß-hydroxybutyrate. Growth range is from <4°C - 40°C, from 0.3 - 9% salinity and from pH 4.6 – 9.5. Various sugars and carboxylic acids are oxidized. Predominant fatty acids are $C_{18:1}$ ω7c; $C_{16:1}$ ω7c and $C_{16:0}$. 16S rRNA gene sequence analysis positions the new genus in close proximity to *Oceanospirillum* and *Neptunomonas* within the *Oceanospirillaceae*. The type species is *Amphritea atlantica.*

Description of *Amphritea atlantica sp.* nov.

at.lan´ti.ca. (L. fem. Adj. atlantica, of or pertaining to the Atlantic Ocean).

Cells are rod-shaped with a monopolar flagellum. The mean cell size is 0.5 x 0.5-2.0 µm. Colonies on marine broth agar are circular beige with entire whitish edges. Optimal growth occurs at 31-34°C with 3% salinity and pH 8. The sodium ion Na+ is

obligate for growth. Catalase and oxidase reactions are positive and poly-ß-hydroxybutyrate is accumulated. Luminescence is negative. The main fatty acids are $C_{16:1}$ ω7c, $C_{16:0}$ and $C_{18:1}$. Hydroxy fatty acids are represented by $C_{10:0}$3OH and $C_{12:1}$3OH.

Carbon sources oxidized (Biolog GN2) are: tween80, tween40, D-fructose, alpha-D-glucose, sucrose, methyl pyruvate, mono-methyl succinate, p-hydroxy-phenylactic acid, alaninamid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-phenylalanine, L-proline, L-pyroglutamic acid and gamma-amino-butyric acid. Enzyme activities are positive for alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase. Negative reactions are obtained for esterase lipase, lipase, valin arylamidase, cystine arylamidase, trypsine, alpha-chymotrypsine, alpha-galactosidase, beta-galactosidase, beta-glucoronidase, alpha-glucosidase, beta-glucosidase, N-acetyl-beta-glucosamidase, alpha-mannosidase and alpha-fucosidase.

Furthermore, the API 20NE test system shows strong activities of arginin dehydrolase, lysine decarboxylase, ornithine decarboxylase and urease. Acetoin production and citrate utilization are also positive. Negative reactions occur for indol production, beta-galactosidase, H_2S production, tryptophane deaminase, gelatinase.

The G+C content is 52.2 mol%.

The type strain is M41^T (= DSM 18887^T = LMG 24143^T) and was isolated from warm sediment samples of 3000 m depth at the Mid-Atlantic Ridge.

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CHAPTER VI

Functional genes as markers for sulfur cycling and CO_2 fixation in microbial communities of hydrothermal vents of the Logatchev field

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Abstract

Life at deep-sea hydrothermal vents depends on chemolithoautotrophic microorganisms as primary producers mediating the transfer of energy from hydrothermal fluids to higher trophic levels. A comprehensive molecular survey was performed with microbial communities in a mussel patch at the Irina II site of the Logatchev hydrothermal field by combining the analysis of 16S rRNA gene sequences with studies of functional key genes involved in biochemical pathways of sulfur oxidation/reduction (soxB, aprA) and autotrophic carbon fixation (aclB, cbbM, cbbL). Most significantly, major groups of chemoautotrophic sulfur oxidizers in the diffuse fluids differed in their biosynthetic pathways of both carbon fixation and sulfur oxidation. One important component of the community, the *Epsilonproteobacteria*, has the potential to grow chemoautotrophically by means of the reductive tricarboxylic acid cycle and to gain energy through the oxidation of reduced sulfur compounds using the Sox pathway. The majority of soxB and all retrieved aclB gene sequences were assigned to this group. Another important group in this habitat, the *Gammaproteobacteria*, may use the APS pathway and the Calvin-Benson-Bassham cycle, deduced from the presence of aprA and cbbM genes. Hence, two important groups of primary producers at the investigated site might use different pathways for sulfur oxidation and carbon fixation.

Introduction

Deep-sea hydrothermal vent environments represent highly productive ecosystems, fueled solely by a number of reduced inorganic substances (e.g. reduced sulfur compounds, hydrogen or methane) contained in the hydrothermal fluids. Through the oxidation of such compounds chemolithoautotrophic microorganisms gain energy, which can be used for the fixation of inorganic carbon. Thereby these microorganisms mediate the transfer of energy from the geothermal source to higher trophic levels and thus form the basis of the unique food chains existing in these environments (for recent reviews see Kelley et al., 2002; Huber & Holden, 2008). Hydrogen sulfide represents one of the major energy sources used by chemolithoautotrophs and is regarded as key feature for the development of hydrothermal vent communities (Kelley et al., 2002; Shock & Holland, 2004; Nakagawa & Takai, 2008; Sievert et al., 2008a). The Logatchev hydrothermal field is an ultramafic-hosted system on the Mid-Atlantic Ridge, with the Irina II complex as main structure consisting of a large mound with several black smoker chimneys at the top and mussel fields surrounding the base of the chimneys (Petersen et al., 2009). Previous studies at the Irina II complex examined the microbial community structure of high-temperature fluids and chimney structures based on 16S rRNA gene sequences (Perner et al., 2007a; Voordeckers et al., 2008). Venting of diffuse fluids is an important process at this site. The fluids which reach the seafloor with moderate temperatures provide energy for free-living and symbiotic chemolithoautotrophic bacteria, nourishing the dense mussel and shrimp populations. Furthermore, these fluids provide a window into the subseafloor biosphere which can not be easily assessed directly (Huber & Holden, 2008).

Microbial communities in vent fluids at different hydrothermal habitats have been studied previously (Reysenbach et al., 2000; Huber et al., 2003, 2007; Brazelton et al., 2006; Perner et al., 2007a, 2007b). However, most of these studies used 16S rRNA gene sequences and therefore could not conclude on physiological properties and specific biochemical pathways acting in the studied communities. In order to obtain insight into the metabolic potential of vent communities, functional genes need to be studied. Though, specific genes involved in nitrogen fixation, methanogenesis, sulfate reduction, and carbon fixation have been analyzed (Dhillon et al., 2003, 2005; Teske et al., 2003; Campbell & Cary, 2004; Nakagawa et al., 2004a, 2004b;

Nercessian et al., 2005; Moussard et al., 2006; Perner et al., 2007b; Voordeckers et al., 2008), functional gene studies of energy-yielding processes in hydrothermal vent communities remain scarce (Nercessian et al., 2005; Elsaied et al., 2007). Only recently, a GeoChip-based study provided deeper insight into the metabolic diversity of microbial communities in vent chimneys (Wang et al., 2009).

Bacterial sulfur oxidation pathways have been studied in a variety of sulfur-oxidizing bacteria over the last few years and the biochemistry behind these pathways is quite complex (for a recent review see Ghosh & Dam, 2009). Neutrophilic sulfur-oxidizing bacteria use two types of sulfur oxidation pathways: one involving a multienzyme complex catalyzing the complete oxidation of reduced sulfur compounds to sulfate (Sox pathway) (Kelly et al., 1997; Friedrich et al., 2001), and another implementing elemental sulfur and sulfite as intermediates (Pott & Dahl, 1998; Kappler & Dahl, 2001; Ghosh & Dam, 2009). Components of a fully functional Sox complex involve SoxB, SoxXA, SoxYZ, and SoxCD. In earlier studies, the SoxB encoding gene has been used as a marker gene and its presence has been demonstrated in reference strains from the *Alpha*-, *Beta*-, *Gammaproteobacteria* and *Chlorobi* (Petri et al., 2001; Meyer et al., 2007). The sox genes have also been found within the genomes of certain *Epsilonproteobacteria*, strongly indicating that they use the Sox pathway for sulfur oxidation (Nakagawa et al., 2007; Sievert et al., 2008b). Only recently initial biochemical evidence for the operation of the Sox system has been reported for the *Epsilonproteobacterium Sulfurovum sp*. NBC37-1 (Yamamoto et al., 2010). The alternative pathway of sulfur oxidation starts with the transformation of sulfide to polysulfide. Sulfur globules are formed and again remobilized by the dissimilatory sulfite reductase (DSR) yielding sulfite (Pott & Dahl, 1998). Subsequently, sulfite is either oxidized to sulfate by sulfite: acceptor oxidoreductase (SOR), or adenosine 5´-phosphosulfate (APS) is formed as an intermediate (APS pathway). The APS pathway involves the enzymes APS reductase and ATP sulfurylase (Kappler & Dahl, 2001). Dissimilatory APS reductase (AprBA) also is a key enzyme of the dissimilatory sulfate-reduction pathway in sulfate-reducing prokaryotes (Meyer & Kuever, 2007b). However, the enzyme is found in a wide variety of sulfur-oxidizing prokaryotes, where it is most likely involved in the reverse process, the transformation of sulfite to APS (Meyer & Kuever, 2007a). Primers designed for the amplification of a fragment of the aprA gene allowed the positive aprA amplification in sulfate-reducing as well as sulfur-oxidizing prokaryotes (Blazejak et al., 2006; Meyer & Kuever, 2007a, 2007b).

Since chemolithoautotrophs are the primary producers at deep-sea vent habitats, a few studies have put focus on the autotrophic potential of these biotopes (Nakagawa & Takai, 2008 and references therein). Genes encoding key enzymes of two important carbon fixation pathways, namely the Calvin-Benson-Bassham (CBB) cycle (key enzyme: ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I and II, genes: cbbL and cbbM, respectively) and the reverse tricarboxylic acid (TCA) cycle (key enzyme: ATP citrate lyase, genes aclA and aclB) have been studied to demonstrate the autotrophic potential of deep-sea vent communities (Campbell et al., 2003; Campbell & Cary, 2004; Moussard et al., 2006; Elsaied et al., 2007; Perner et al., 2007b; Voordeckers et al., 2008).

In this study, comprehensive analyses of the microbial community within hydrothermal fluids has been performed studying 16S rRNA gene sequences as well as sequences of genes for different pathways of carbon fixation (aclB, cbbM, cbbL) and sulfur metabolism (soxB, aprA) within one and the same sample.

Materials and methods

Sampling site, sample collection and fluid characteristics

The Irina II chimney complex in the Logatchev hydrothermal vent field is located at 14°45´N, 44°58´W on the Mid-Atlantic Ridge (MAR) in water depths of about 3,000 m (Petersen et al., 2009). About 600 mL of diffuse hydrothermal fluids with a temperature range of 24 to 43°C were retrieved from a mussel bed during a dive with the remotely operated vehicle (ROV) Jason II (Woods Hole Oceanographic Institution, MA, USA) during the cruise MSM 04 with R/V Maria S. Merian (Borowski et al., 2007). On board the fluids were immediately processed. Sulfide and oxygen measurements resulted in 12 µM and 60 µM, respectively (see Borowski et al., 2007 for further details). As reference sample 2 L seawater were retrieved with a rosette sampler attached to a CTD probe (location: 13°30´N, 45°00´W; depth: 2660 m).

DNA extraction and amplification

For bacterial community analyses 400 mL of hydrothermal fluids were concentrated on polycarbonate filters (type: GTTP, pore size 0.1 µm, Millipore, Eschborn, Germany) and kept at -20°C. DNA was extracted from filters using the UltraClean Soil DNA Isolation Kit (MoBio, Solana, CA, USA) according to the manufacturer’s instructions. Bacterial 16S rRNA genes were PCR-amplified with PuReTaq Ready-

To-Go-PCR Beads (GE Healthcare, Munich, Germany) using the primer pair 27F and 1387R (Lane, 1991; Marchesi et al., 1998). Primers (10 pmol/µL), 10 – 100 ng of DNA template and sterile water were added to PuReTaq Ready-To-Go-PCR Beads (GE Healthcare, Munich, Germany) to a total volume of 25 µL. An initial denaturation step (92°C for 2 min) was followed by 30 cycles of 92°C for 40 s, 50°C for 45 s, 72°C for 60 s. For amplification of gene fragments of the SoxB component of the periplasmic thiosulfate-oxidizing Sox enzyme complex (soxB) and the alpha subunit of the adenosine 5'-phosphosulfate (APS) reductase (aprA), primer sets soxB432F/soxB1446B and aps1F/aps4R and PCR conditions were used as described previously (Petri et al., 2001; Blazejak et al., 2006). A fragment of the beta subunit of the ATP citrate lyase genes (aclB) was amplified using the primer set 892F/1204R, applying the conditions as described before (Campbell et al., 2003). For the amplification of fragments of the genes coding for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) cbbL and cbbM (RubisCO form I and form II, respectively) the primer sets cbbLF/cbbLR and cbbMF/cbbMR were applied using the conditions according to Campbell & Cary (2004). Each functional gene fragment was amplified in five parallel PCR reactions, which were subsequently pooled for the construction of the gene libraries.

Cloning and sequencing

The amplified and pooled PCR products were gel-purified using the QIAGEN QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into pCR4-TOPO plasmid vectors with the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. An environmental clone library for each gene was constructed. From each library clones were randomly chosen and analyzed for the insert-containing plasmid by direct PCR with the vector primers M13F and M13R followed by gel electrophoreses of the amplified products. PCR products of the correct size were sequenced using the M13 primer set (see Table 1 for the number of sequenced clones for each library). Sequencing was performed using the BigDye Terminator v1.1 sequencing kit in a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer, resulting in sequence lengths of ~1350 bp (16S rRNA genes), ~1050 bp (soxB), ~390 bp (aprA), ~330 bp (cbbM), and ~340 bp (aclB).

Phylogenetic analysis

All sequences were edited with ChromasPro c.c1.33 and compared to the NCBI database using BLAST (Altschul et al., 1990). The 16S rRNA gene sequences were aligned with the ARB software (www.arb-home.de) using the ARB FastAligner utility (Ludwig et al., 2004). If not already present, the closest relatives according to BLAST search were also added to the ARB database. The sequence alignment was manually adjusted, taking known secondary structures into account. Amino acid sequences of functional genes were aligned using Clustal X (Thompson et al., 1997) and manually adjusted using BioEdit (Hall, 1999). Neighbor-Joining trees were calculated using PAUP, version 4.0b10. Maximum-Likelihood based trees were constructed using PhyML (Guindon & Gascuel, 2003). Bootstrapping included 1.000 replicates for Neighbor-Joining based trees and 100 replicates for Maximum-Likelihood based trees. Calculated 16S rRNA gene trees were re-imported into ARB and shorter sequences were added using the "quick add marked" tool. Operational taxonomic units (OTUs) were defined based on 97 % nucleotide sequence identity for the 16S rRNA gene sequences. OTUs based on 99 % nucleotide sequence identity were also calculated (Table 1). In order to define OTUs (referred to as sequence types) for the functional genes (soxB, aprA, cbbM, aclB) inferred amino acid sequences and 16S rRNA gene sequences from isolates possessing the respective genes were used to produce matrices of pairwise distance values (as described by Weber & King, 2010). As a result sequence types based on 97 % (aprA, aclB), 95.5 % (cbbM) and 94 % (soxB) amino acid sequence identity were defined for the respective functional genes. Rarefaction curve of the 16S rRNA gene clone library and rank abundance analyses of 16S rRNA, soxB, aprA, ccbM and aclB gene libraries are available at FEMS Microbiology Ecology as supporting information, see: http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6941.2010.00919.x/suppinfo.

Nucleotide sequence accession numbers

Sequence data have been submitted to EMBL/GenBank/DDBJ databases under accession numbers FN562806 – FN562916 (16S rRNA genes), FN562666 – FN562697 (aclB), FN562917 – FN562931 (cbbM), FN562762 – FN562805 (soxB), FN562698 – FN562761 (aprA).

Results

Phylogenetic analyses of the bacterial community

Diffuse fluid from a mussel field at the base of the Irina II structure of the Logatchev hydrothermal field showed a maximum temperature of 43.3°C at the point of discharge. Sulfide and oxygen concentrations were 12 µM and 60 µM, respectively (Borowski et al., 2007). The bacterial 16S rRNA gene was amplified and a clone library was constructed to analyze the microbial community structure in these fluids. The majority of the 53 detected operational taxonomic units (OTUs) were related to Epsilonproteobacteria (34 %), most of which clustered within the marine group 1, also called group F (Table VI.1, Figures VI.1 and VI.2). So far only a few strains of this highly diverse group have been cultured. The closest cultured relative to our sequences was *Sulfurovum lithotrophicum* (Inagaki et al., 2004). Three OTUs fell into group D (*Nautilia/Caminibacter* group) and one into group B (*Sulfurimonas* group) (classification according to Corre et al., 2001).

Table VI.1. Number of 16S rRNA gene and functional gene sequences associated with distinct phylogenetic groups (see Material and Methods for details)

Gene library	Clones sequenced/ OTUs (iden.)	Total	Epsilon-	Gamma-	Delta-	Alpha-	Others
				Proteobacteria			
16S rRNA gene	Clones	11	50	19	1	1	40
	OTUs (97%)	53	18	6	1	1	27
	OTUs (99%)	67	27	8	1	1	30
soxB	Clones	44	34	-	-	4	6
	OTUs (94%)	10	7	-	-	2	1
aprA	Clones	64	-	45	11	4	4
	OTUs (97%)	26	-	12	7	3	4
cbbM	Clones	15	-	9	-	4	2
	OTUs (95.5%)	7	-	4	-	1	2
aclB	Clones	32	32	-	-	-	-
	OTUs (97%)	11	11	-	-	-	-

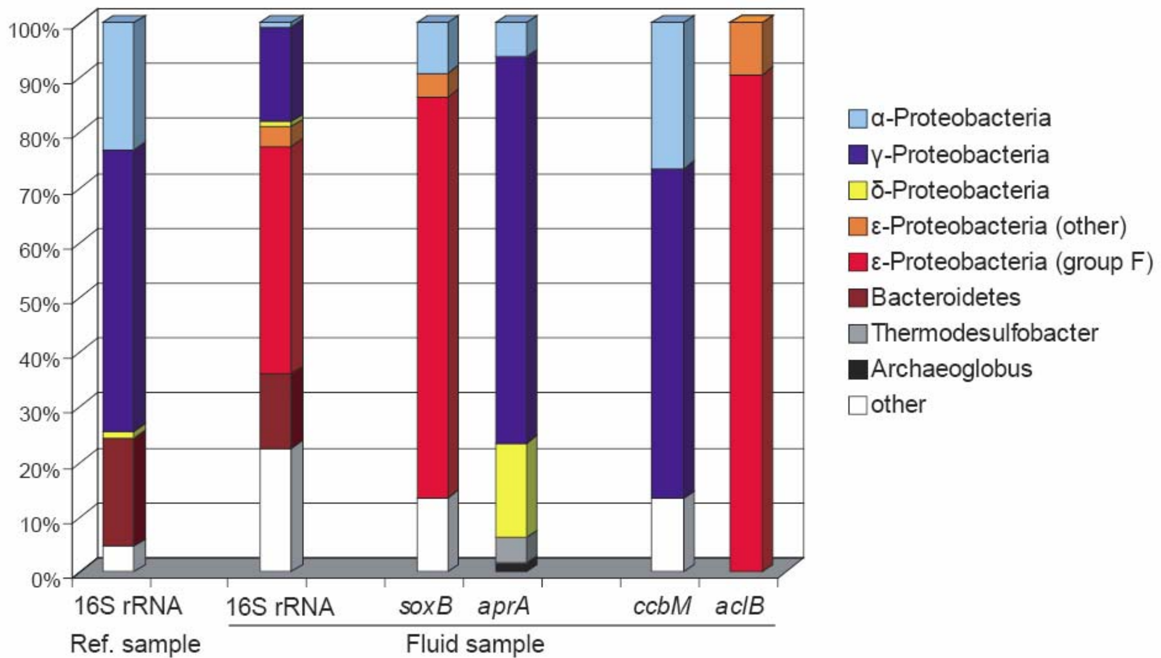

Figure VI.1: Percentage composition of 16S rRNA gene and functional gene (soxB, aprA, cbbM, aclB) libraries. Note that a bacterial 16S rRNA gene library was constructed from DNA isolated from the fluid sample, as well as from DNA isolated from a deep-sea water sample without hydrothermal influence (reference sample).

Sequences of Gammaproteobacteria (17 % of all retrieved sequences) were distributed in six different OTUs (Figure VI.2). The most frequently found phylotype showed > 99 % identity to the 16S rRNA gene sequence of the intranuclear bacterium Candidatus *Endonucleobacter bathymodioli* of *Bathymodiolus spp*., also occurring at the Irina II site (Zielinski et al., 2009). This bacterium has been proposed to represent an intranuclear parasite of these mussels (Zielinski et al., 2009). Other sequences cluster with heterotrophic *Gammaproteobacteria* of the genus *Psychromonas* or show highest similarities to methanotrophic or methylotrophic isolates, respectively.

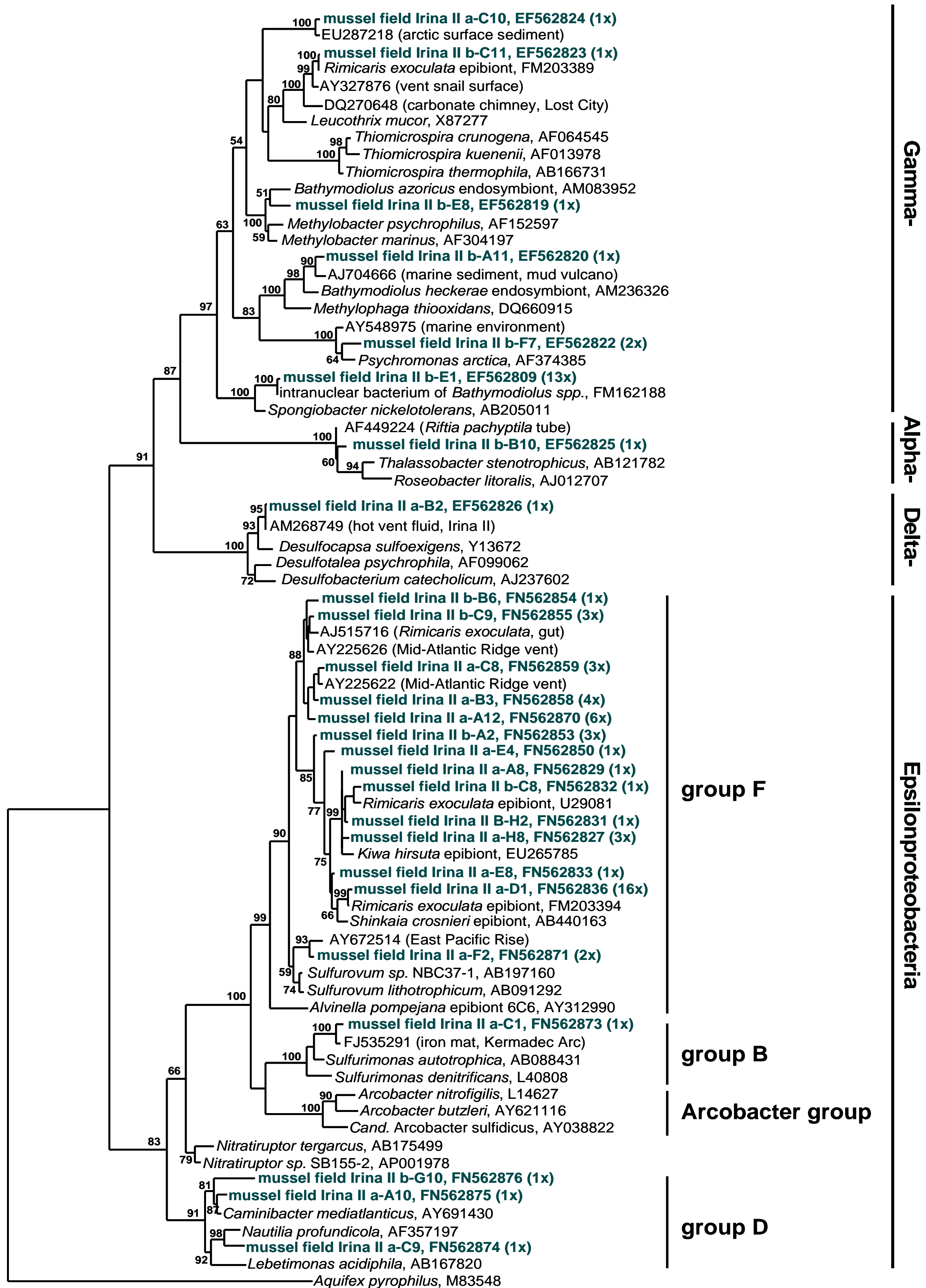

Figure VI.2: Phylogenetic tree based on 16S rRNA gene sequences of *Proteobacteria*. The tree was calculated using the Maximum-Likelihood method. Bootstrap values are shown as percentages of 100 bootstrap replicates. Sequences obtained in this study are indicated in bold.

Further groups present in the clone library included *Bacteroidetes* (12 OTUs), *Fusobacteria* (1 OTU), *Deltaproteobacteria* (1 OTU), *Alphaproteobacteria* (1 OTU), *Verrucomicrobia* (2 OTUs), *Spirochaeta* (1 OTU) *Mollicutes* (1 OTU) and *Caldithrix* (1 OTU), as well as candidate divisions RE-1 (3 OTUs), SR1 (2 OTUs) and OD1 (3 OTUs).

A reference clone library of bacterial 16S rRNA gene fragments from the water column nearby showed a significantly different composition. It completely lacked *Epsilonproteobacteria* and was dominated by *Gammaproteobacteria* (48 %, sequences related to the genera *Alteromonas*, *Halomonas*, *Chromohalobacte*r, *Marinobacter*, *Acinetobacter*, *Alcanivorax*) and *Alphaproteobacteria* (30 %, sequences related to the genera *Erythrobacter, Sulfitobacter*, *Citromicrobium*, *Leisingera*, *Hirschia*) followed by *Bacteroidetes* (16 %) and 5 % other groups (data not shown).

Genes involved in sulfur cycling

The genetic potential of microbial sulfur metabolism was analyzed by amplifying key genes of known sulfur oxidation pathways. A gene encoding an essential component of the Sox enzyme system, namely soxB (SoxB, sulfate thiohydrolase) was chosen along with the gene encoding the alpha subunit of APS reductase (aprA).

The majority of identified soxB sequences (77 %) retrieved from the fluid sample were affiliated to those of *Epsilonproteobacteria* (Figures VI.1 and VI.3a) and formed 7 distinct sequence types. Among the cultured strains, soxB of *Sulfurovum sp*. NBC-37-1 was most similar to the majority of these sequences.

One sequence type (soxE8) was related to soxB *of Sulfurimonas denitrificans* and another one (soxG10) to the soxB gene of *Arcobacter spp.*. Notably, the soxB genes of *Arcobacter spp*. and soxG10 formed a distinct cluster separate from all other soxB sequences of *Epsilonproteobacteria* (Figures VI.3). Apart from epsilonproteobacterial sequences, two other, novel clusters of soxB genes were identified. One cluster (represented by soxG8) was distantly affiliated with genes of *Gammaproteobacteria*, the other (represented by soxB3) with *Alphaproteobacteria*. In addition, a single sequence (soxF6) was included into the soxB cluster of the Rhodobacter / *Roseobacter* group of the *Alphaproteobacteria*.

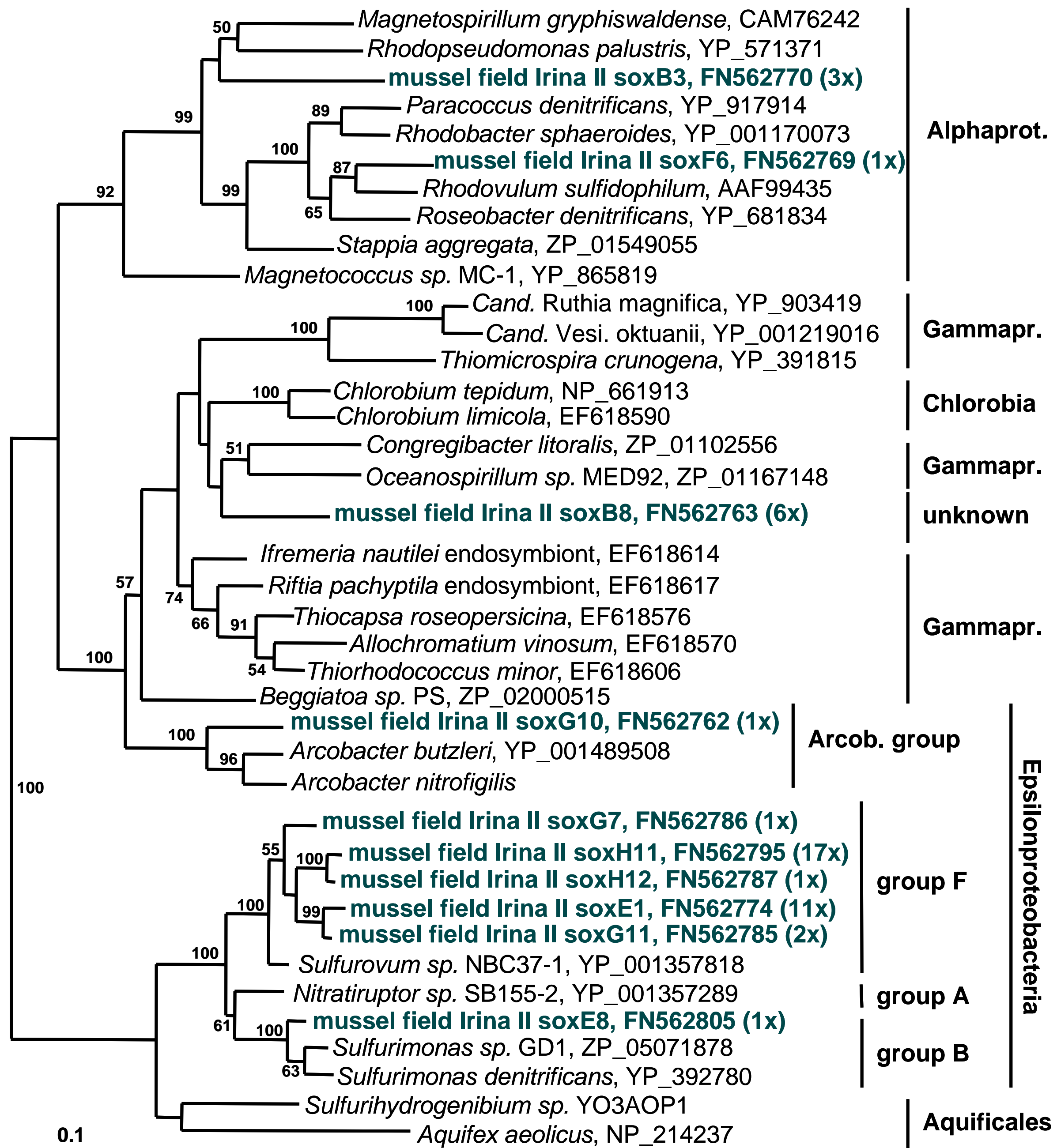

Figure VI.3. Phylogenetic tree based on the amino acid sequences of the *soxB* (A) and *aprA* (B) genes. The trees were calculated using the Maximum-Likelihood method. Bootstrap values are shown as percentages of 100 bootstrap replicates. Sequences obtained in this study are indicated in bold.

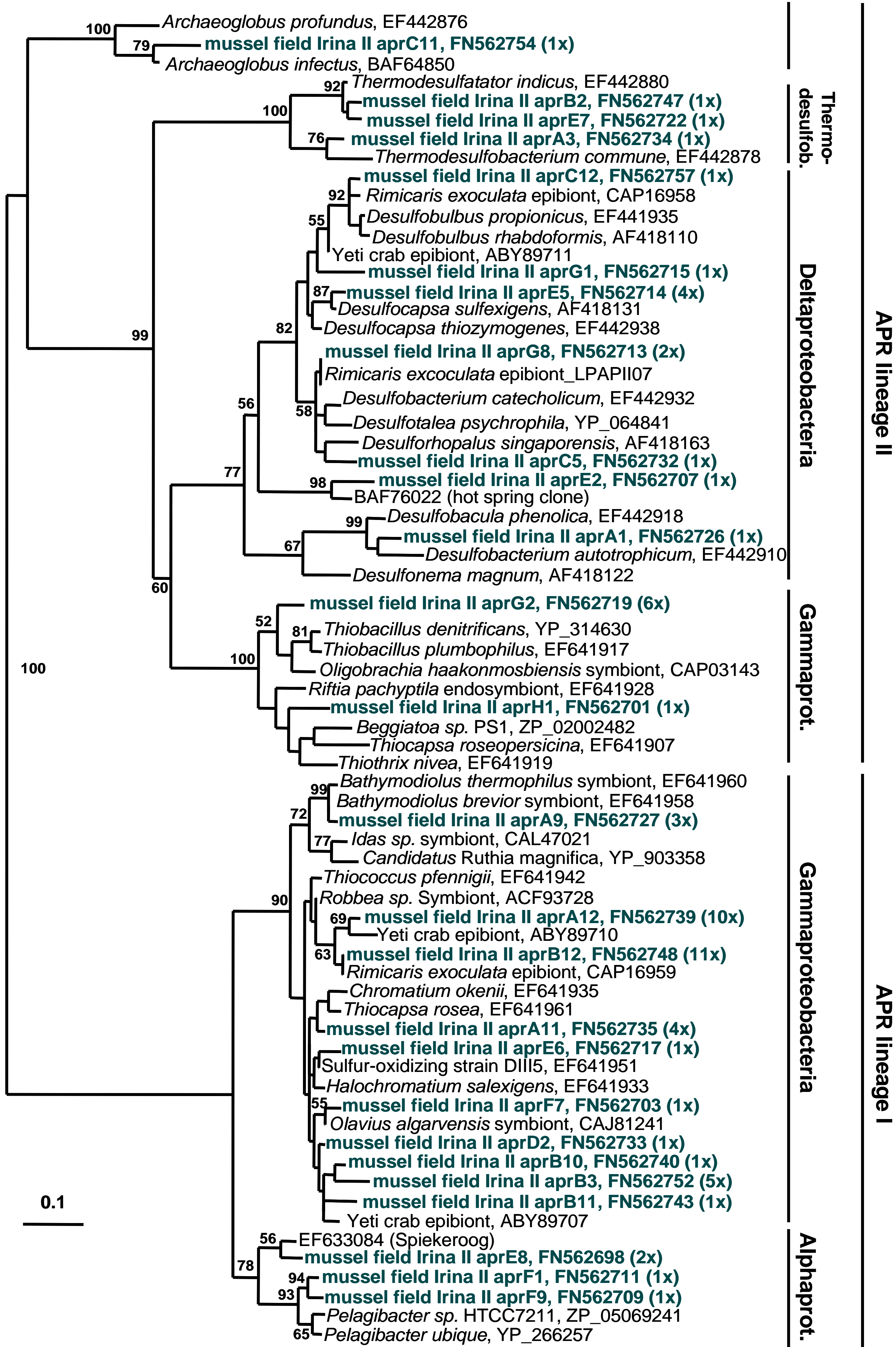

Figure VI.3: continued.

Based on the topology of the tree, the aprBA genes of sulfur-oxidizing and sulfate-reducing prokaryotes form two phylogenetic lineages (Meyer & Kuever, 2007a). The aprA sequences obtained in this study were highly diverse and sequences of both lineages were found in the aprA clone library (Figure VI.3b). Since sulfate-reducing as well as sulfur-oxidizing prokaryotes use APS reductase, the genes of this reversible enzyme can be found in both groups. Yet, the majority of the obtained sequences (77 %, 15 sequence types) could be clearly attributed to sulfur-oxidizing bacteria, while 11 sequence types (23 % of the obtained sequences) clustered with the aprA gene of sulfate-reducing prokaryotes. Among the sequences assigned to sulfate-reducing prokaryotes, the majority were related to aprA sequences from *Deltaproteobacteria* (Figure VI.3b). Others (aprE7, aprB2, aprA3) showed high similarities to aprA genes of the deeply-rooting *Thermodesulfobacteria* and to an archaeal aprA gene (aprC11). Among aprA sequences attributed to sulfur-oxidizing bacteria, more than 80 % of the sequence types could be assigned to gammaproteobacterial groups. The others clustered with aprA of *Pelagibacter spp.* (SAR11 group, *Alphaproteobacteria*). Despite the existence of a large database of aprA sequences from gammaproteobacterial isolates, almost all sequences from the Irina II fluids formed new lineages within the *Gammaproteobacteria* distinct from those of cultured strains (Figure VI.3b). Quite interestingly, these sequences were most similar to a number of aprA sequences of epi- and endosymbionts from vent mussels (*Bathymodiolus spp.*), vent crabs (*Kiwa hirsuta*), vent shrimps (*Rimicaris exoculata*) and oligochaetes (e.g. *Olavius algarvensis*) (Blazejak et al.; 2006; Meyer & Kuever, 2007a; Goffredi et al., 2008; Zbinden et al., 2008).

Genes involved in autotrophic CO_2 fixation

In order to obtain insights into the potential of the Irina II fluid bacterial community to fix CO_2 via the CBB cycle or the reductive TCA cycle, genes encoding key enzymes of these two autotrophic carbon fixation pathways were amplified, including cbbL and cbbM, encoding the large subunit of RubisCO form I and form II, respectively, and the gene for the beta subunit of ATP citrate lyase aclB.

A faint cbbM amplification product could be retrieved, while all attempts to amplify a cbbL gene fragment failed despite repeated trials using different DNA template concentrations. The obtained cbbM sequences were related to those of *Alpha-* and *Gammaproteobacteria* (Figures VI. 1 and VI.4), with sequences most similar to those

from *Thiomicrospira pelophila* and *Thiomicrospira thermophila* (cbbMA11 and cbbMB12, respectively) and the symbiont of *Calyptogena magnifica*, Candidatus *Ruthia magnifica* (cbbMB5). The most frequently found sequence type (cbbMA8) was related to cbbM sequences retrieved from tubeworm symbionts. Another sequence type (cbbMB10) was most similar to the cbbM gene of the *Alphaproteobacterium Rhodobacter sphaeroides*. Two sequences (cbbMA10, cbbMB3) were not affiliated to any known cbbM gene of cultured bacterial strains. They form a distinct lineage together with cbbM sequences obtained from other environmental samples (Figure VI.4).

A gene fragment encoding the small subunit of ATP citrate lyase (aclB) could be successfully amplified and the construction of the clone library yielded many positive clones. All sequences of the aclB gene were related to those of phylogenetically different groups of *Epsilonproteobacteria* (Figures VI.1 and VI.5). The majority of the aclB sequences (9 sequence types) were associated with several novel clusters of which the closest related aclB gene retrieved from a pure culture is that of *Sulfurovum lithotrophicum* (group F). Other aclB sequences were related to those of *Caminibacter mediatlanticus* of group D (aclBA6) or *Thioreductor micantisoli* of group G (aclBE3). The sequence aclBB4 forms a novel lineage together with environmental aclB sequences from high-temperature Irina II fluids and from chimney samples of vents in the Guaymas Basin (Campbell & Cary, 2004).

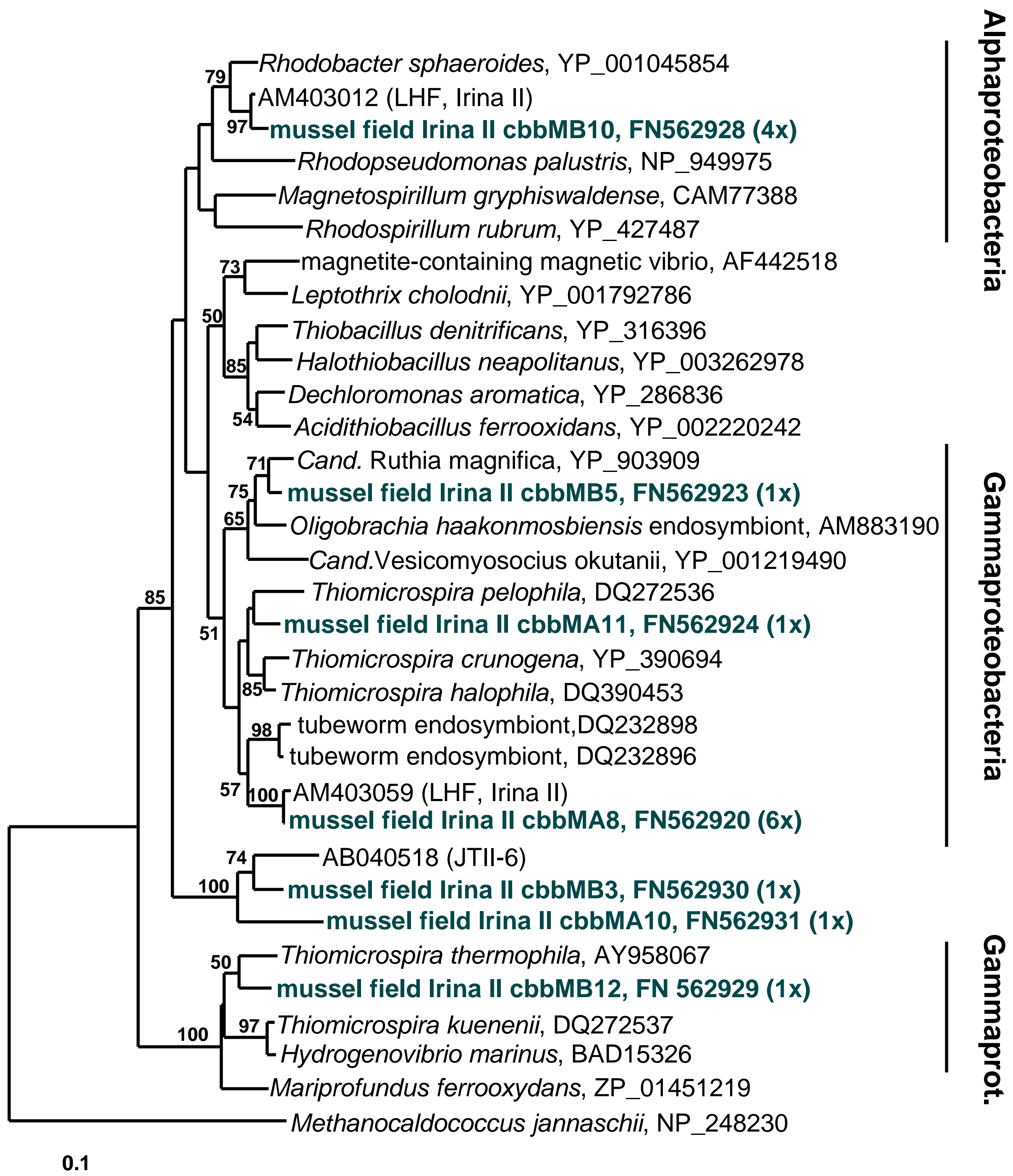

Figure VI.4: Phylogenetic tree based on the amino acid sequences of the *ccbM* gene. The tree was calculated using the Neighbor-Joining method. Bootstrap values are shown as percentages of 1000 bootstrap replicates. Sequences obtained in this study are indicated in bold.

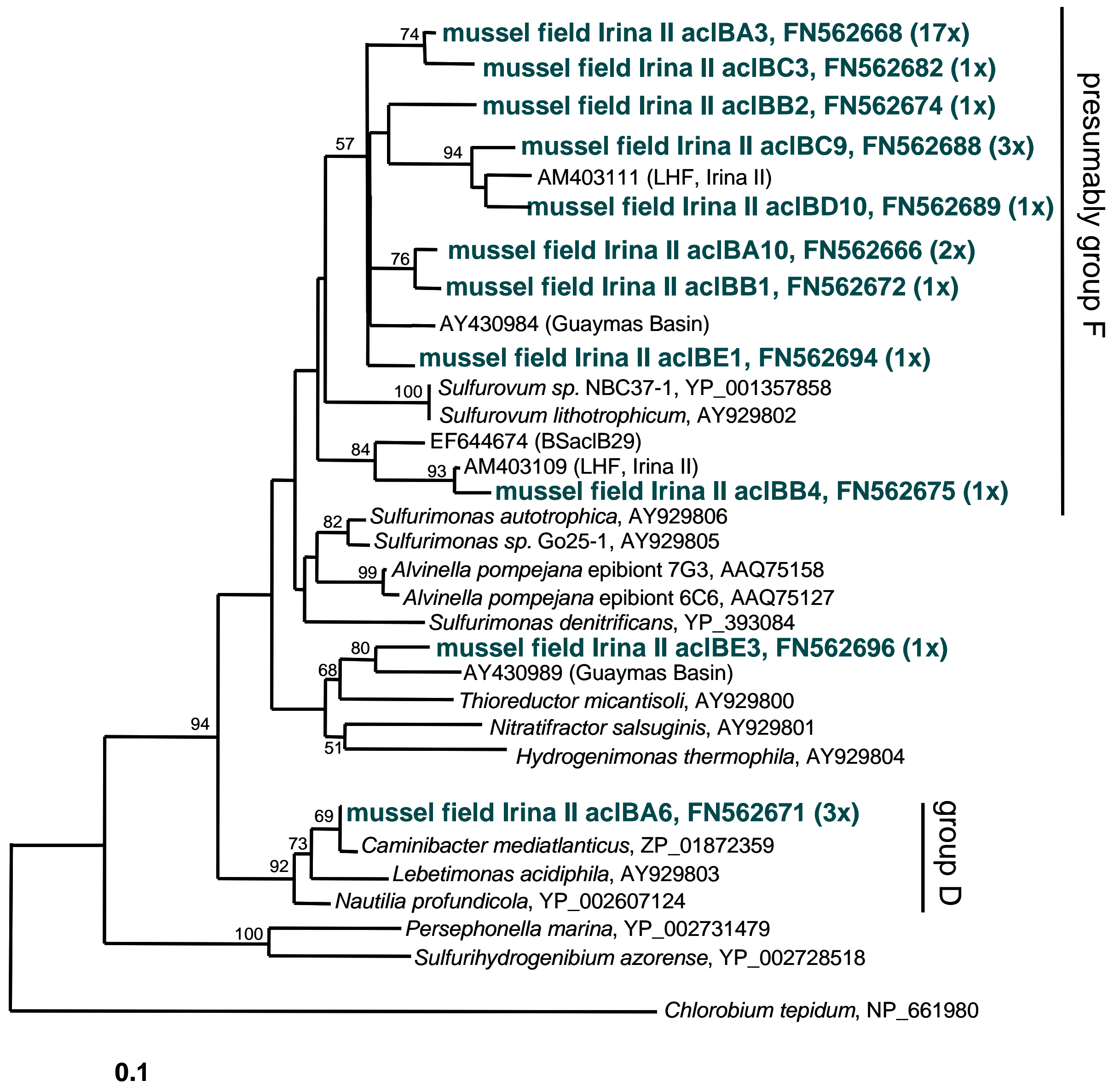

Figure VI.5: Phylogenetic tree based on the amino acid sequences of the *aclB* gene. The tree was calculated using the Neighbor-Joining method. Bootstrap values are shown as percentages of 1000 bootstrap replicates. Sequences obtained in this study are indicated in bold.

Discussion

The microbial community structure within hydrothermal fluids from the Irina II structure of the Logatchev vent field was analyzed using genes involved in different pathways of sulfur metabolism and CO_2 fixation as functional markers and the 16S rRNA gene as a phylogenetic marker. For the first time, different key genes of

important energy-yielding processes were analyzed together with key genes of different carbon fixation pathways in order to reveal the genetic capabilities of the chemolithoautotrophic primary producers at this deep-sea vent site. The 16S rRNA gene sequences obtained clearly differ from those of a reference sample apart from the hydrothermal fluids and demonstrate overall similarities in the community composition to those of similar previously studied hydrothermal vent habitats. In fact evidence accumulates that *Epsilon*- as well as *Gammaproteobacteria* constitute the major part of the bacterial community at the Irina II site and at other deep-sea hydrothermal vent habitats (e.g. Reysenbach et al., 2000; Corre et al., 2001; Campbell et al., 2006; Perner et al., 2007a; Nakagawa & Takai, 2008; Wang et al., 2009).

Epsilonproteobacteria as important primary producers

Phylogenetic analyses of the 16S rRNA, aclB and soxB genes demonstrated that *Epsilonproteobacteria* most likely are an important part of the microbial community at the Irina II site able to oxidize reduced sulfur compounds via the Sox pathway and fixing CO_2 via the reductive TCA cycle. During the last few years, the great importance or even dominance of *Epsilonproteobacteria* has been pointed out in a number of studies using the 16S rRNA gene as a marker (for a review see Campbell et al., 2006). These studies included free-living bacterial populations in vent fluids, on surfaces of vent structures, or in the shallow subsurface where mixing between vent fluid and ambient sea water occurs (Alain et al., 2004; Kormas et al., 2006; Moussard et al., 2006; Huber et al., 2007). Only a small number of epsilonproteobacterial isolates has so far been obtained. These are able to generate energy through the oxidation of reduced sulfur compounds or hydrogen (for an overview see Takai et al., 2005; Nakagawa & Takai, 2008; Sievert et al., 2008a). Most of the isolates were chemolithoautotrophs using the reductive TCA cycle for carbon fixation (Hügler et al., 2005; Takai et al., 2005). The first whole genome sequences of autotrophic free-living epsilonproteobacterial isolates recently became available and provide insight into their metabolic capabilities (Nakagawa et al., 2007; Sievert et al., 2008b; Campbell et al., 2009).

According to the phylogeny of the 16S rRNA gene, *Epsilonproteobacteria* in the Irina II fluids were members of the groups B, D, F, with the majority of the sequences related to group F. The trees of the functional genes (soxB, aclB) add up very well.

The main fraction of amplified soxB genes could be assigned to *Epsilonproteobacteria* and most of these clustered with *Sulfurovum sp*. NBC37-1 (group F). A single sequence type of both 16S rRNA and soxB genes was affiliated with *Sulfurimonas denitrificans* (group B). These results demonstrate the good congruence between soxB phylogeny and 16S rRNA gene phylogeny. At the same time they point towards the potential of *Epsilonproteobacteria* to oxidize sulfur via the Sox pathway, which so far has been suggested for individual strains only from genomic and initial enzymatic studies (Nakagawa et al., 2007; Sievert et al., 2008a, 2008b; Yamamoto et al., 2010). In this context it should be mentioned, that *Epsilonproteobacteria* of group D can not oxidize reduced sulfur compounds, but rely on hydrogen as energy source. Consequently, their genomes lack the sox genes (Campbell et al., 2009).

Autotrophic *Epsilonproteobacteria* have been shown to use the reductive TCA cycle for CO_2 fixation with a bona-fide ATP citrate lyase as key enzyme (Hügler et al., 2005; Takai et al., 2005; Voordeckers et al., 2008). In this study, various epsilonproteobacterial aclB sequences were found clustering mainly with sequences from the groups F and D. This indicates the potential of *Epsilonproteobacteria* at the Irina II site to fix CO_2 via the reductive TCA cycle.

Role of *Gammaproteobacteria*

Gammaproteobacteria were another abundant group in our clone libraries and represented major fractions of the sequences of 16S rRNA, cbbM and aprA genes (Figure VI.1). Functional gene analyses revealed their potential to (i) oxidize sulfur mainly via the APS pathway and (ii) to fix CO_2 via the CBB cycle. A large variety of aprA genes could be assigned to *Gammaproteobacteria* (Figure VI.3b). Interestingly, these genes clustered with aprA genes amplified from diverse epi- and endosymbionts, including mussel symbionts (Meyer & Kuever, 2007a), crab and shrimp epibionts (Goffredi et al., 2008; Zbinden et al., 2008), and symbionts of gutless marine worms (Blazejak et al., 2006). Our results suggest, that at the Irina II site, oxidation of sulfur via the APS pathway with sulfite as free intermediate is performed by a variety of *Gammaproteobacteria*.

Sulfate reducing bacteria

It is worth mentioning that deltaproteobacterial sequences related to the genus *Desulfocapsa* were found in the 16S rRNA as well as in the aprA gene library (Figures Vi.2 and VI.3b). *Desulfocapsa* species can grow chemolithoautotrophically as sulfate reducers or by the disproportionation of thiosulfate, sulfite or elemental sulfur (Finster et al., 1998). APS reductase in these bacteria is involved in the dissimilatory sulfate reduction pathway (Meyer & Kuever, 2007b). Due to the different phylogenetic grouping of aprA from sulfur-oxidizing and sulfate-reducing bacteria a clear distinction of the two groups can be made with respect to environmental samples. AprA genes of sulfur-oxidizing bacteria form APR lineage I, while sequences from sulfate-reducers form lineage II (Figure VI.3b). However, the aprA genes of several sulfur-oxidizing bacteria, like those of *Beggiatoa spp*., *Thiobacillus spp*. or the endosymbiont of *Riftia pachyptila*, cluster within lineage II indicating lateral gene transfer of the apr genes (Meyer & Kuever 2007a).
Analyses of the aprA gene fragments revealed that in addition to *Deltaproteobacteria* other sulfate-reducing prokaryotes occur at the Irina II site, including thermophilic *Thermodesulfobacteria* and hyperthermophilic *Archaeoglobales* (Figure VI.3b). As fluid temperatures of up to 43°C at the point of discharge are unlikely to support growth of these thermophilic microbes, it is anticipated that they thrive in the subsurface where higher temperatures are reached and are carried to the seafloor within the fluids.

Ecological implications

By analyzing key genes involved in sulfur cycling and carbon fixation we have gained insight into the metabolic reactions driving the biogeochemical cycles in these fluids and the adjacent subsurface habitats and into the organisms involved in these reactions. *Epsilonproteobacteria* of group F appear to be the most abundant bacterial group in the investigated hydrothermal fluid. Their potential to gain energy from the oxidation of reduced sulfur compounds is supported by a variety of epsilonproteobacterial soxB gene fragments. In addition, as indicated by the diverse aclB gene sequences, most strains have the capability to grow autotrophically by means of the reductive TCA cycle, supporting previous studies at other hydrothermal vent sites (Campbell & Cary, 2004; Moussard et al., 2006; Voordeckers et al., 2008). Notably, investigations of high-temperature fluids of the Irina II site showed quite

similar results with *Epsilonproteobacteria* related to group F representing the most abundant bacterial clones (Perner et al., 2007a). Taken together, these results indicate that group F *Epsilonproteobacteria* might be important primary producers within the fluids and in the adjacent subsurface of the Irina II site. Furthermore, studies from other hydrothermal vent sites yielded similar results (e. g. Alain et al., 2004; Kormas et al., 2006; Huber et al., 2007). Why do these organisms succeed in various hydrothermal habitats? Compared to other epsilonproteobacterial isolates, members of the groups F and B can tolerate relatively high oxygen concentrations (Nakagawa et al., 2005), which might be beneficial in ecosystems were intensive mixing of hydrothermal fluid and seawater takes place, like e.g. the shallow subsurface. In addition their versatile metabolism, i.e. growing anaerobically and/or microaerobically with reduced sulfur species and/or hydrogen as energy sources (Takai et al., 2005; Campbell et al., 2006), together with the use of the energy-efficient reductive TCA cycle for autotrophic CO_2 fixation (Hügler et al., 2005; Takai et al., 2005) certainly is a competitive advantage for this bacterial group. Consequently, they are perfectly adapted to the steep gradients of sulfide and oxygen found at the Irina II site and in other hydrothermal vent environments.

Although sulfur-oxidizing members of the *Gammaproteobacteria* have been isolated frequently from vent sites, our knowledge about their sulfur-oxidation pathways is rather limited. Some strains, e.g. *Thiomicrospira crunogena* XCL-2, seem to use the complete Sox enzyme system (Scott et al., 2006), while others like *Beggiatoa* and the endosymbiont of the tubeworm *Riftia pachyptila* use alternative pathways (Hagen & Nelson, 1997; Markert et al., 2007). The great variety of gammaproteobacterial aprA genes found in this study indicates, that *Gammaproteobacteria* at the Irina II site mainly use the APS pathway for sulfur oxidation. At least some strains have also the capability to use the CBB cycle for carbon fixation since we found several gammaproteobacterial cbbM gene sequences, encoding RubisCO form II (Table VI.1, Figure VI.4). It has been postulated that RubisCO form II is used in niches with high CO_2 and low O_2 levels, while RubisCO form I works best in a low CO_2 and high O_2 environment (Badger & Bek, 2008). In line with this hypothesis we were only able to amplify the cbbM gene. Thus, the organisms present in the Irina II fluids seem to be well-adapted to the prevailing environmental conditions.

Conclusions

Based on the results obtained in this study, we propose that *Epsilonproteobacteria* are of major importance as microbial players in sulfur oxidation and CO_2 fixation in the shallow subsurface and hydrothermal fluids of the Irina II vent site. By using the energetically cheap reductive TCA cycle (less then half of the energy demand for CO_2 fixation compared to the CBB cycle) as major route of CO_2 fixation and the Sox pathway for sulfur oxidation, they have a distinct advantage over *Gammaproteobacteria*, which use the CBB cycle for CO_2 fixation and the APS pathway for the oxidation of reduced sulfur compounds. Notably, our results indicate, that the two important groups of primary producers present at the investigated site, the *Epsilon-* and the *Gammaproteobacteria* may use different pathways for sulfur oxidation and carbon fixation.

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Discussion

The aim of the present study was the culture-dependent characterization of bacterial communities from two rather contrasting deep-sea ecosystems (the Logatchev hydrothermal vent field (LHF) and the oligotrophic Eastern Mediterranean sediment) with special focus on antimicrobially active strains. In addition, culture-independent genetic methods were used to analyze the bacterial community and the diversity of functional genes responsible for CO_2-fixation and sulfur oxidation / reduction in diffusive fluids of the LHF leaking from a mussel patch.

The methods used for isolation and characterization of bacterial communities were adapted to the respective environments and thus, the bacterial strains isolated from both environments can hardly be compared: due to the variety of different habitats at hydrothermal vent fields several samples (stones, sediment and mussels) and two different media were used for isolation of bacteria. In contrast, the isolation of bacteria of the Eastern Mediterranean deep sea was focused on sediment samples applying five different low-nutrient media and variations in cultivation conditions to enlarge the number of bacterial taxa and phylotypes.

Nevertheless the chosen methods for isolation and characterization of bioactive bacteria revealed to be successful for both environments. 50 bioactive bacterial strains were isolated from the LHF and one third revealed to be representatives of novel species (Chapter IV). Moreover, one of these strains was assigned to the novel genus and species *Amphritea atlantica*^{T} (Gärtner et al. 2008). On the other hand also the actinomycetes recovered from the Mediterranean Sea revealed to be promising producers of natural products. The cytotoxic compound levantilide A and its derivative, levantilide B, turned out to be the first novel natural products produced by deep-sea strains of *Micromonospora sp.*.

In the following the advantages and disadvantages of culture-dependent and -independent approaches for the characterization of bacterial deep-sea communities will be discussed. The discussion will consider further about the efficiency to investigate deep-sea bacteria for drug discovery, since deep-sea bacteria are not as easy available as their terrestrial counterparts. In addition, the biological functions of bacterial secondary metabolites will be subject of the discussion.

Discovering the microbial life of deep-sea habitats by cultivation and cultivation-independent approaches

The isolation of bacteria is essential to analyze their physiological capabilities and metabolites and thus to make assumptions about their ecological function as well as to investigate bacteria in search for secondary metabolites. Therefore, the main part of the present study relied on cultivation-based investigations.

Changes in the cultivation strategies, like the composition of the media, have an essential influence on the bacterial taxa that can be recovered. Yet remarkable is the recovery of diverse Gram-positive phylotypes from the oligotrophic sediment and the repeated isolation of *Vibrio* strains from the hydrothermal vent field. In strong contrast, these genera are of minor importance or even missing in 16S rRNA gene libraries of these ecosystems (Polymenakou et al., 2005; Polymenakou et al., 2009; Hügler et al., 2010). This demonstrates the discrepancies between culture-based and molecular studies of biodiversity. Even if these frequently isolated taxa may represent a minor fraction of the bacterial community, their ecological function might not be necessarily of minor importance. For example, members of *Vibrionaceae* have been isolated from hydrothermal polychaete annelids and revealed to have a remarkable resistance to high concentrations of different heavy metals (Jeanthon and Prieur, 1990). It has been suggested that these bacteria can assist in detoxifying the heavy-metal-rich environment of the tubeworms.

On the other hand, the establishment of culture-independent strategies enabled a completely new impression of the bacterial diversity (Amann et al., 1995). It is supposed, that e.g. Mediterranean deep-sea sediments harbor an incredibly high and unique prokaryote diversity (different from that of other benthic environments (Danovaro et al., 2010). 16S rRNA gene libraries established by Polymenakou et al. (2005) and (2009) of the Mediterranean deep-sea sediments revealed a highly diverse bacterial community which is reflected by many sequences (12%) that did not fall into any taxonomic division previously identified (Polymenakou 2009). Furthermore, clone sequences of *Alpha-, Gamma*- and *Deltaproteobacteria* as well as *Acidobacteria* and *Planctomycetes* were frequently recovered from the Mediterranean sediments. Quite interestingly, the comparably high bottom temperature of around 13.5°C clearly delimits the Mediterranean deep sea from other deep-sea environments but is comparable to the water temperature surrounding

hydrothermal vent systems (Danovaro et al., 2010). Nevertheless, comparison of environmental 16S rRNA gene libraries of the two studied ecosystems revealed a completely different composition of the detected bacterial taxa (Polymenakou et al., 2005; Hügler et al., 2010). While the well-oxygenated deep-sea sediment of the Mediterranean Sea was composed of mainly heterotrophic bacteria, the Logatchev hydrothermal environment was based on autotrophic metabolism and dominated by free-living, epibiotic and symbiotic *Epsilonproteobacteria and Gamma-proteobacteria* (Hügler et al., 2010).

Besides phylogenetic diversity based on 16S rRNA gene sequences also functional gene diversity was analyzed within this study. The bacterial community, of the Logatchev hydrothermal vent field was investigated for the presence and diversity of genes responsible for carbon fixation and sulfur metabolism. According to Pedros-Alio (2006) sequences obtained by culture-independent approaches are supposed to be “core” sequences, defining those sequences that are responsible for carbon and energy flux in the ecosystem. This example reflects how important and likewise difficult it is to combine these approaches. Hence, studies on biodiversity should always link culture-dependent and –independent surveys to obtain information about the core species and the so called “rare biosphere” as well (Pedros-Alio, 2006).

New natural products from the deep

While the discovery of novel natural products stocks, the recovery of already known substances steadily increases. Since the deep sea is still marginally investigated, it is reckoned as promising environment for new natural products. Unfortunately, as already mentioned, deep-sea research is very cost intensive and time consuming. Moreover, deep-sea habitats are often event-driven ecosystems and in particular hydrothermal vent fields are extremely instable, hence it is quite difficult to re-sample identical bacteria from deep-sea habitats. It should be considered further, that cultivation of organisms (macrofauna in particular) recovered from the deep sea often is difficult. Therefore, this study focused on deep-sea bacteria that are adapted to the prevailing conditions of the investigated ecosystems but can be easily cultivated at laboratory conditions.

In total, 173 strains were isolated and phylogenetically classified. Selected strains were further analyzed for their potential to produce bioactive compounds. The levantilides as well as further bioactive compounds produced by members of the genus *Micromonospora* were discovered (Chapters II and III). Cultivation experiments demonstrated that the producer strains, A77 and S20, are well adapted to the limited nutrient conditions, high salinity and *in situ* temperature of the Mediterranean deep sea. Phylogenetic analyses of the producer strains revealed further that they cluster separately from the described type strains of the genus. Over this, an antimicrobially active strain could be assigned to a novel genus. Phylogenetic classification and physiological characterization of this strain, *Amphritea atlantica* M41^T, revealed that the strain might be considered as typical prokaryotic member of the hydrothermal community. This clearly demonstrates that the deep sea is indeed a promising habitat for the discovery of new bacterial taxa and natural products.

Due to the broad set of bacteria that have been successfully cultivated from the different deep-sea samples, efficient screening methods were necessary to select promising strains. Of course, screening methods are always targeted to selected features and it is likely, that quite interesting strains are not detected by the screening methods used. Nevertheless, these methods are essential to overcome the number of bacterial isolates. All bacterial strains obtained from the Logatchev hydrothermal vent field were selected for classification by 16S rRNA gene sequencing due to their growth-inhibiting potential against at least one of the test organisms *Bacillus subtilis, Staphylococcus lentus or Escherichia coli*. This approach enabled the classification of 49 bioactive strains including strain M41^T, the type strain of *Amphritea atlantica*^{T}. Obviously, this approach led to the successful selection of promising strains and therefore can be highly recommended. Nevertheless it should be considered, that the pre-selection of all strains was targeted to the antimicrobial activity towards three selected test organisms. This means, that all bacteria without detected biological activity against the selected test strains were rejected, even though they might have produced other interesting bioactive substances. Moreover, the applied cultivation conditions favored the repeated selection of strains with similar growth properties and metabolic capabilities, like the frequently isolated *Vibrio* strains from mussel samples. Diverse samples of sediment, stones and mussels were taken for isolation using two different media.

A different selection strategy was chosen for the Mediterranean sediments (Chapters I and II): with the aim to enlarge the set of phylogenetically distinct bacterial strains, all colonies appearing morphologically different were selected for sub-cultivation. Consequently a considerable amount of bacteria was sub-cultivated to obtain pure cultures and all pure cultures were classified by sequencing of the 16S rRNA gene. The selection for promising natural product producing strains was done according to their phylogeny. Since members of *Actinobacteria* are well known as producers of bioactive natural products, strains affiliating to this taxonomic group were selected and subjected to several parallel screening assays: (a) Genetic analyses of the presence of nrps and pks biosynthesis genes, (b) antimicrobial test assays of culture extracts and (c) HPLC/MS analysis of the chemical metabolite profile. As a matter of fact, these screening methods used are highly selective to the tested targets and a certain loss of promising strains must be taken into account. Results of the genetic analyses for example strongly depend on the primers used which in turn depend on the sequences of already known gene clusters. Nevertheless, the combination of these three screening methods enabled the detection of novel bioactive compounds and thus the chosen strategy can be considered to be highly successful. Furthermore, all strains can easily be subjected to further screening procedures, like other bioactivity tests at any time, and thus even more promising strains and bioactivities might be discovered. Therefore, a combination of a diverse set of screening methods (including cultivation conditions, primer pairs, and test organisms) is a highly recommended strategy to discover natural products.

Secondary metabolites and their biological function in deep-sea habitats

The bacterial strains recovered from the Logatchev hydrothermal vent field inhibited the growth of the Gram-positive test strain *B. subtilis*. Most of these strains affiliated to the *Gammaproteobacteria* (Chapter IV). As already mentioned, *Gamma-proteobacteria* are typical members of the hydrothermal vent community and they are quite often found as epibionts or symbionts of vent fauna like *Rimicaris*, *Calyptogena*, *Bathymodiolus* and *Riftia* (Van Dover, 2000; Dubilier et al., 2008). Within their hosts, they are strongly enriched in number and thus potentially produced natural products can be supposed to be concentrated. Vent metazoa like mussels and tubeworms are

sessile and therefore are easy accessible prey for consumers and also for surface colonization of invertebrate larvae, bacteria and fungi. It was shown for example by Holmström (2002) that marine surface associated *Pseudoalteromonas* species produce antifouling agents and thus inhibit the settlement of common biofouling organisms. Hence it can be considered, that also vent specific macrofauna benefits from the production of bioactive secondary metabolites by associated bacteria. Evidence for grazing defense by symbiotic vent bacteria was given by a feeding study of shallow-water fishes. It was shown that the fishes did not deny food that was enriched with H_2S-rich blood of *Riftia.* But quite interestingly they did not eat food that was supplemented with tissues of vent fauna. It was concluded, that chemical compounds produced by associated bacteria might be responsible for the rejection (Klicklighter 2004).

Furthermore it is supposed, that sub-inhibitory concentrations of bioactive low-molecular weight compounds serve as signalling molecules since they were shown to up- and down regulate the expression of transcripts in different bacteria many of which are involved in environmental interactions (Goh et al., 2002). Macrolide-antibiotics, for instance, specifically bind to ribosomal proteins and ribosomal RNA regulating the transcription and protein biosynthesis (Poehlsgaard and Douthwaite, 2003; Poehlsgaard and Douthwaite, 2005). These processes are expected to conduct to the interaction of bacteria with surrounding organisms (Yim et al., 2007). Thus, also marine natural products might function as signalling molecules for cell-cell communication. In hydrothermal ecosystems such molecules might help to connect the complex biosynthesis pathways of independent bacteria (Thornburg et al., 2010). Regarding the oligotrophic deep sea environment, such signalling molecules might be of special importance for the detection of available nutrients or the defence against competitors. Within this study it was shown that many isolated strains of the genus *Micromonospora sp.* obtained from the Eastern Mediterranean deep sea possessed antimicrobial activity towards the Gram-positive test strains. Thus, cell-cell communication as well as growth-inhibition of nutrient competitors might be probable functions of these bioactive compounds.

Final conclusion

By this study a considerable number of bioactive bacterial strains as well as novel antimicrobial and cytotoxic bacterial compounds have been recovered from the deep sea sediments. Indeed it is much too early to designate the deep sea as natural-product “hot spot” but undoubtedly the deep sea harbours an enormous reservoir of microbial diversity and unique bioactive constituents waiting for discovery. Even though further studies are coming up that support the deep sea environment as rich source for antimicrobial and cytotoxic compounds it is still questionable which *in situ* function these compounds possess. Growth-inhibition of nutrient competitors, defence of predators as well as cell-cell communication properties of the metabolites are hypothesized. In this context it has to be mentioned, that a considerable amount of Gram-positive bacteria was recovered from the deep sea environments. Moreover, growth inhibition activity of the bioactive strains was mainly targeted against Gram-positive bacteria. Thus, it might be assumed that against early assumptions Gram-positive bacteria play an active role in the deep-sea microbial community and may be important competitors for nutrients that need to be inhibited in growth. Further experiments have to be carried out to study the interaction between deep-sea bacteria with special emphasis on the influence of elevated hydrostatic pressure on the production of metabolites.

Data obtained by this study revealed furthermore that not solely strains of novel bacterial taxa might be promising producers of novel constituents, but also strains highly related to well known bacteria as it was shown by the *Micromonospora* strains. Thus, it should be kept in mind that searching for novel bacterial natural products does not solely depend on the exploration of novel habitats, new bacterial taxa or taxonomically relevant producers of pharmaceutical compounds (like actinomycetes). Drug research also depends on a diversified screening approach and a detailed look at single strains to explore their metabolic potential.

As shown by molecular approaches, deep sea habitats harbour an enormous bacterial and metabolic diversity. Thus, ongoing culture-dependent and -independent research on deep sea bacteria and their metabolic pathways still is necessary and it appears reasonable to implement deep-sea bacteria also in future drug research.

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Personal contribution to multiple-author manuscripts

The present thesis is based on published manuscripts (**Chapter III, V** and **VI**), submitted manuscripts (**Chapter I**) and manuscripts in preparation (**Chapter II** and **IV**). My personal contribution to the multiple-author manuscripts was as follows:

Chapter I: All sediment and water samples were recovered at research cruise M71/2 in Dezember 2006/January 2007. Cultivation approaches of heterotrophic bacteria were performed directly onboard by A. Gärtner. Pressure-incubation experiments were planned by M. Blümel. Purification of bacterial strains and cultivation-experiments were performed by A. Gärtner under supervision of J. Wiese. Phylogenetic identification and calculations as well as the preparation of the manuscript were done by A. Gärtner in supervision of Prof. Dr. J.F. Imhoff.

Chapter II: All sediment and water samples were recovered at research cruise M71/2 in Dezember 2006/January 2007. Cultivation approaches of heterotrophic bacteria were performed directly onboard by A. Gärtner. Purification of bacterial strains, cultivation-experiments, phylogenetic identification and calculations, preparation of culture extracts and HPLC-analysis as well as the preparation of the manuscript was done by Andrea Gärtner in supervision of Prof. Dr. J.F. Imhoff.

Chapter III: Cultivation of the strain, culture extraction and HPLC –analysis were performed by A. Gärtner in supervision of J. Wiese, B. Ohlendorf and D. Schulz. Structural elucidation and all chemical analysis were performed by B. Ohlendorf and D. Schulz. Test assays of biological bioactivity were performed by H. Zinecker. The manuscript was prepared by A. Gärtner and B. Ohlendorf in supervision of Prof. Dr. J.F. Imhoff.

Chapter IV: Samples of the Logatchev- hydrothermal vent field were recovered on the research cruise M60/3 in 2004. Cultivation approaches, antimicrobial activity tests and selection of bioactive strains were performed in the laboratory by J. Wiese. Phylogenetic identification by 16S rRNA gene sequencing was performed by A. Gärtner. The data were assembled and evaluated by A. Gärtner in supervision of Prof. Dr. J. F. Imhoff.

Chapter V: Strain M41^T was isolated by J.Wiese. A. Gärtner performed all physiological test assays as well as phylogenetic calculations. The manuscript was written by A. Gärtner in supervision of Prof. Dr. J.F. Imhoff.

Chapter VI: Samples of the Logatchev -hydrothermal vent field were recovered on the research cruise MSM04 in 2006. Phylogenetic analysis by 16S rRNA gene sequencing and tree calculation was performed by A. Gärtner. M. Hügler analyzed the functional genes for CO_2 -fixation and sulfur oxidation. The manuscript was written by M. Hügler with contribution of A. Gärtner and supervision of Prof. Dr. J.F. Imhoff.

List of publications

Gärtner, A.,; Ohlendorf, B.; Schulz, D.; Zinecker, H.; Wiese, J. and Imhoff, J.F.
Levantilide A and B, novel macrolides from the deep-sea *Micromonospora sp.* isolate M71-A77 ***Marine Drugs*** **(2011)** published online doi:10.3390/md9010098

Hügler, M.; **Gärtner, A.**; Imhoff, J. F.
Functional genes as markers for sulfur cycling, hydrogen oxidation and metabolic diversity of bacterial communities from the LHF ***FEMS Microbiology Ecology*** **(2010)**

Schroeder, J.P.; **Gärtner, A.;** Waller, U.; Hanel, R.
The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei.* ***Aquaculture*** 305 (2010), pp.1-6

Neulinger, S.; **Gärtner, A.;** Järnegren, J.; Ludvigsen, M.; Lochte, K.; Dullo, W.-C.
Tissue-associated "*Candidatus* Mycoplasma corallicola" and filamentous bacteria on the cold-water coral *Lophelia pertusa* (Scleractinia). ***Applied and Environmental Microbiology*** 75 (2009), nr. 5, pp. 1437-1444

Wiese, J.; Thiel, V. ; **Gärtner, A.** ; Schmaljohann, R. ; Imhoff, J. F.
Kiloniella laminariae, gen. nov., *sp*. nov., a new alphaproteobacterium from the marine macroalga *Laminaria saccharina.* ***International Journal of Systematic and Evolutionary Microbiology*** 59 (2009), pp. 350-356

Gärtner, A. ; Wiese, J.; Imhoff, J. F.
Amphritea atlantica gen. nov., **sp.** nov., a gammaproteobacterium from the Logatchev hydrothermal vent field. ***International Journal of Systematic and Evolutionary Microbiology*** 58 (2008), pp. 34-39

Petersen, S.; Monecke, T.; Augustin, N.; De Benedetti, A. A.; Esposito, A.; **Gärtner, A.;** Gardeler, A.; Gemmell, J. B.; Gibson, H.; He, G.; Hügler, M.; Kayser, A.; Kleeberg, R.; Küver, J. ; Kummer, N.; Lackschewitz, K.; Lappe, F; Perrin, K. M.; Peters, M.; Sharpe, R.; Simpson, K.; Smith, D. ; Wan, B.
Drilling submarine hydrothermal systems in the Tyrrhenian Sea, Italy ***InterRidge News*** 17 (2008), pp. 21-23

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde weder im Ganzen noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und entstand unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft.

Kiel, den 25.01.2011 ______________________________

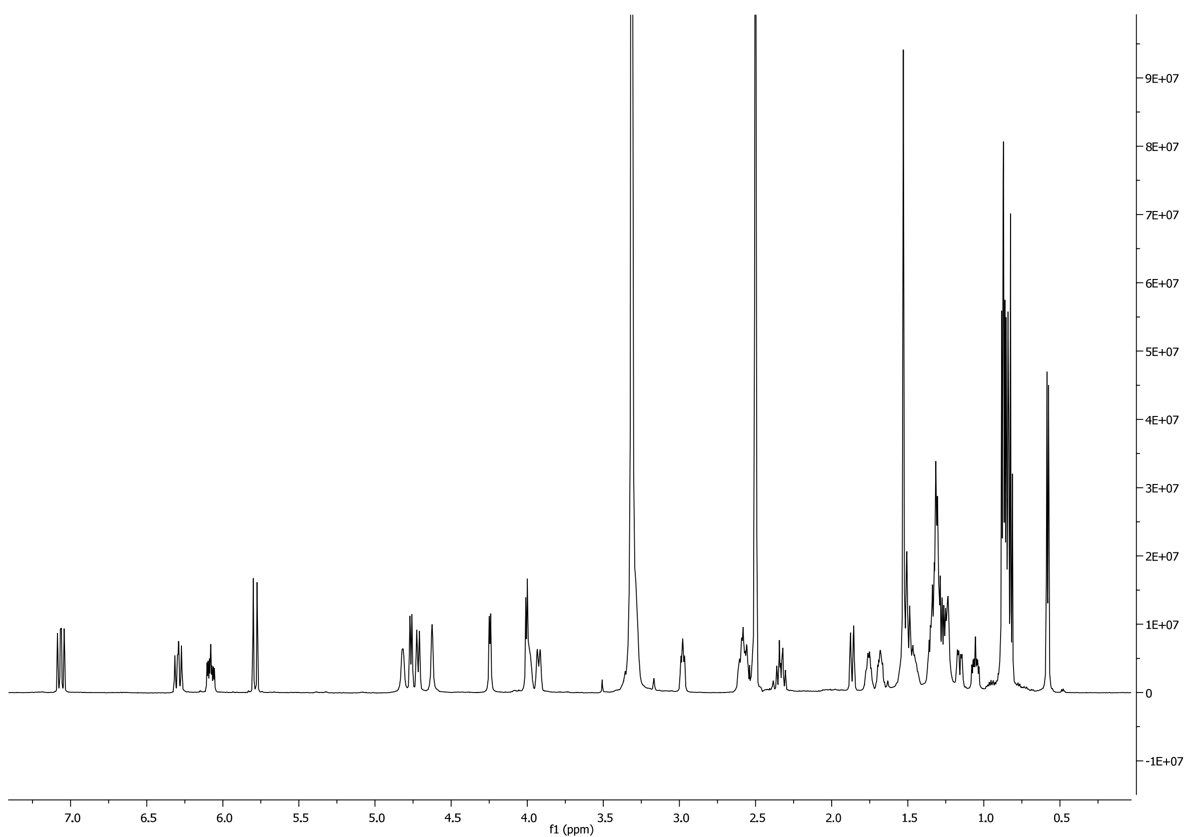
(Andrea Gärtner)

Appendix

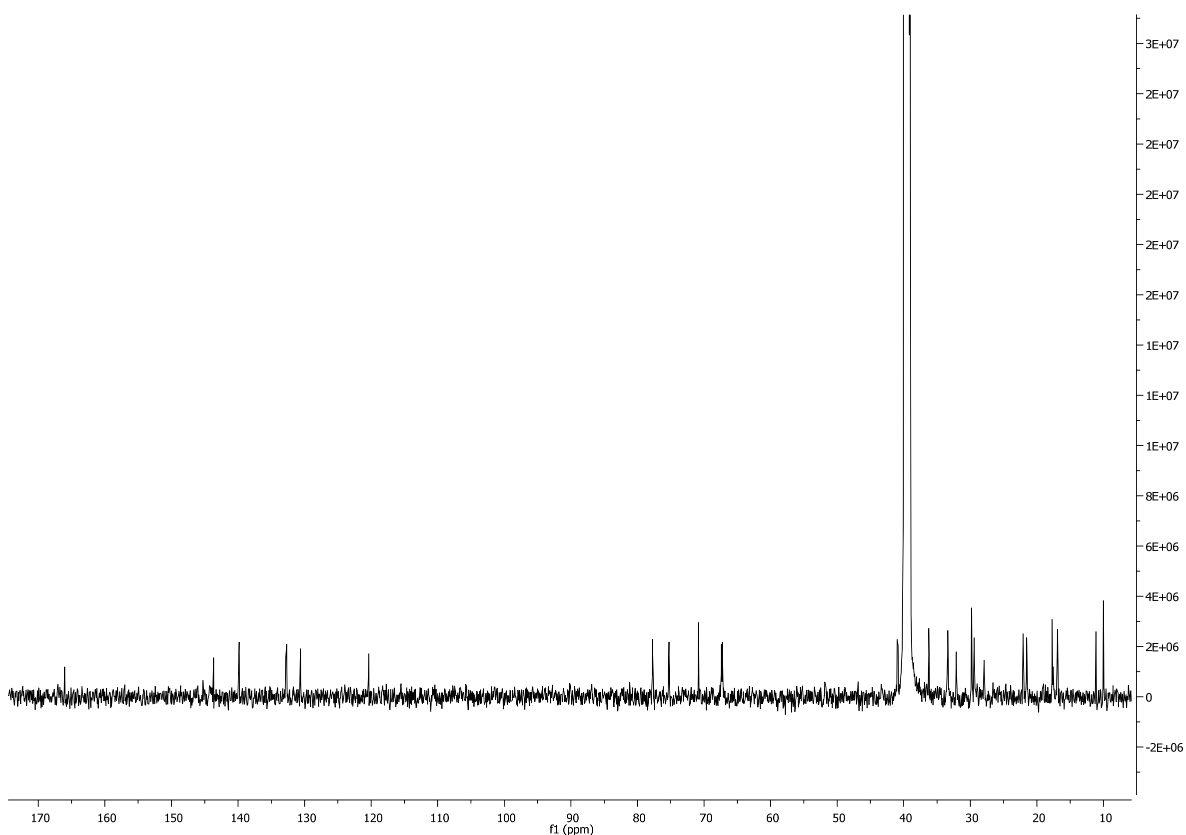
Appendix 1: Results of blastx search. Nrps, pksI and pksII gene sequences related to the sequenced gene fragments of *Micromonospora* strains.

Strain	related sequence (gene, origin & acc. nr.)	AA match	Similarity (%)
	NRPS		
A177	non-ribosomal peptide synthetase, *Micromonospora sp.* ATCC 39149, ZP_04604018	47/88	54
A64	*non-ribosomal peptide synthetase,* *Streptomyces flaveolus* DSM9954, ACY06285.1	45/89	50
A200	*non-ribosomal peptide synthetase,* *Micromonospora sp.* ATCC 39149, ZP_04604018.1	46/84	54
A201	*non-ribosomal peptide synthetase,* *Micromonospora sp.* L5, ZP_06399932.1	46/81	56
A68	*non-ribosomal peptide synthetase,* *Micromonospora sp.* ATCC 39149, ZP_04604018.1	45/85	52
	PKSI		
A200	*type I polyketide synthase ,* *Streptomyces flavogriseus* ATCC 33331, AAR87133.1	27/34	79
	PKSII		
A165	SaqA, *Micromonospora sp.* Tu 6368, ACP19353.1	133/171	77
S51	beta-ketoacyl synthase, *Micromonospora sp* . ATCC 39149, EEP70048.1	130/146	89
A202	beta-ketoacyl synthase, *Micromonospora sp.* ATCC 39149, EEP70048.1	138/171	80
A172	type II ketosynthase, *Micromonospora sp* . 8g107, ACG49888.1	171/200	85
A201	beta-ketoacyl synthase, *Micromonospora sp* . ATCC 39149, EEP70048.1	117/142	82
A176	beta-ketoacyl synthase, *Micromonospora sp.* ATCC 39149, EEP70048.1	123/135	91
A75a	beta-ketoacyl synthase, *Micromonospora sp.* L5, EFC60411.1	79/86	91
A208	type II ketosynthase , *Micromonospora sp* . 8g107, ACG49888.1	141/172	81
A200	type II ketosynthase , *Micromonospora sp.* 8g107, ACG49888.1	129/153	84
A68	type II ketosynthase , *Micromonospora sp.* 8g107, EU868817.1	114/142	80
A177	ketosynthase, *Streptomyces albospinus, BAF43344*	43/52	82
A64	beta-ketoacyl-acyl-carrier-protein synthase II, *Micromonospora sp* . Tu 6368, ACP19353.1	62/86	72

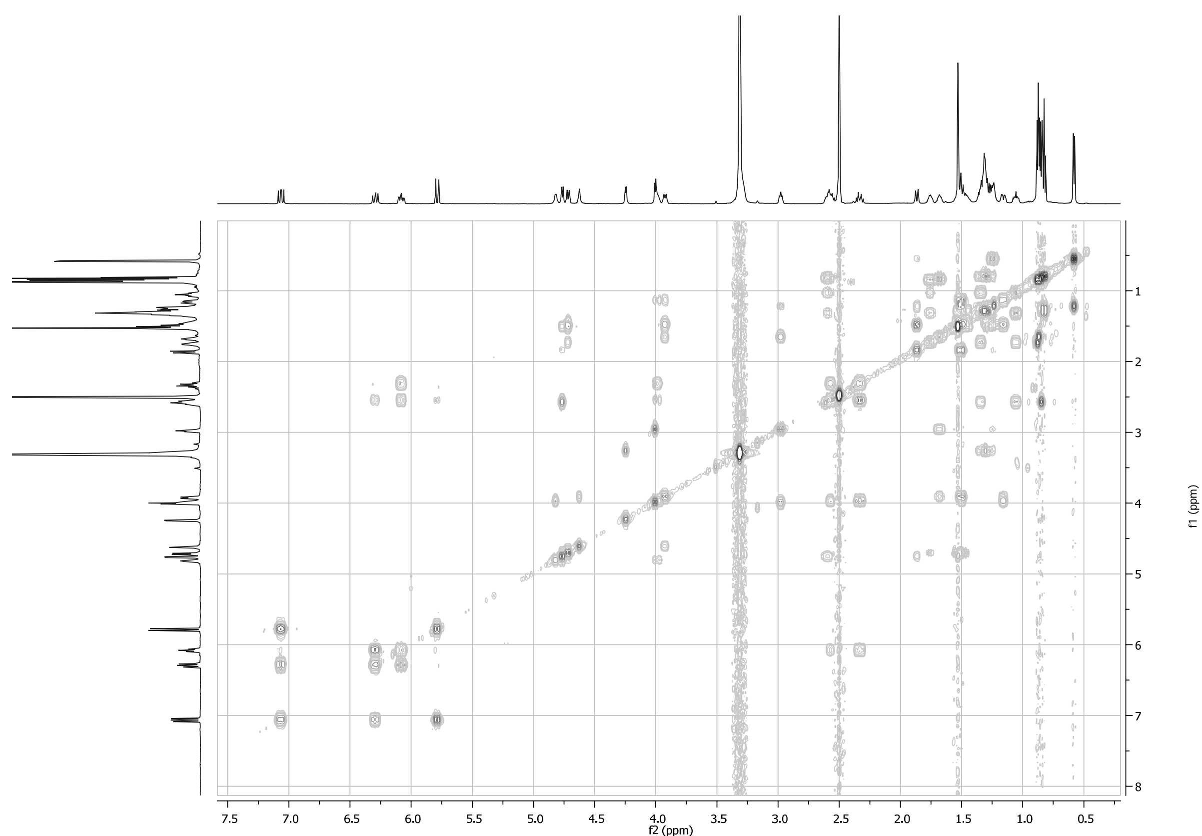
Appendix 2a: ^{1}H-NMR spectrum of levantilide A (**1**) in DMSO-*d*6



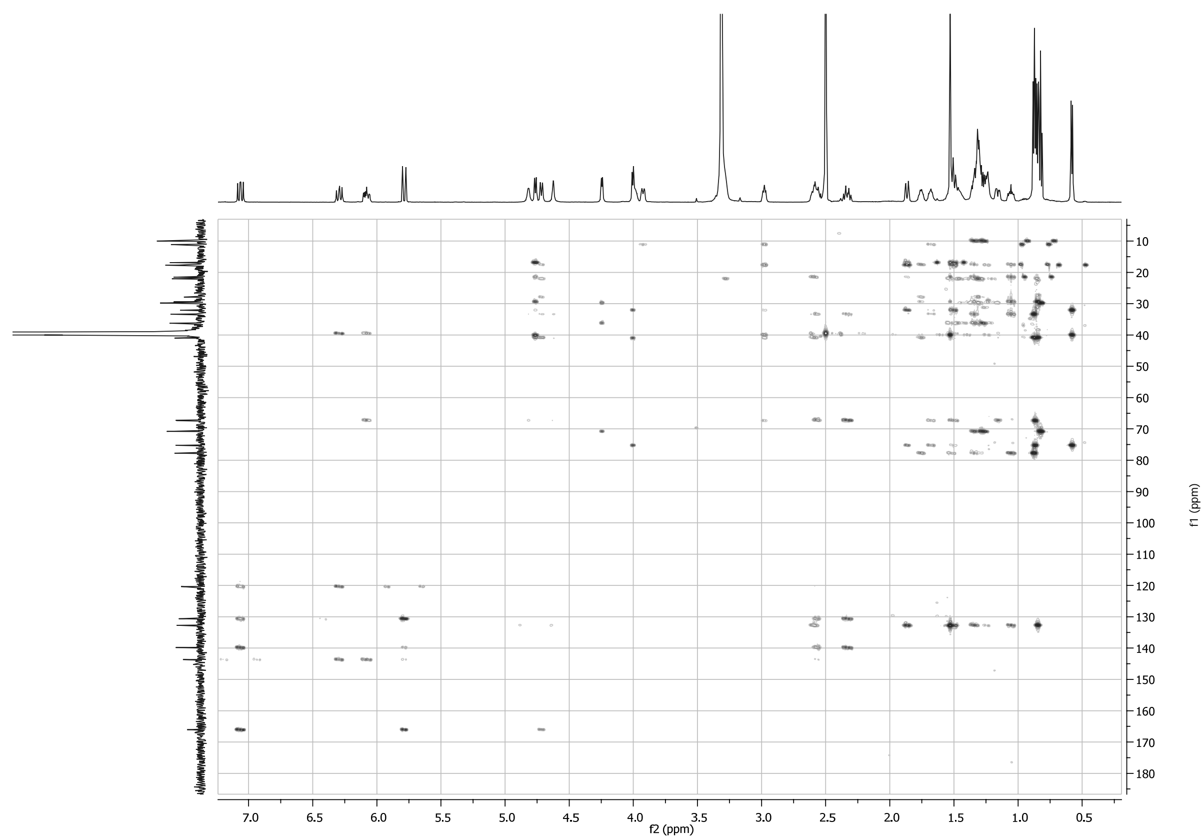
Appendix 2b: ^{13}C-NMR spectrum of levantilide A (**1**) in DMSO-*d*6



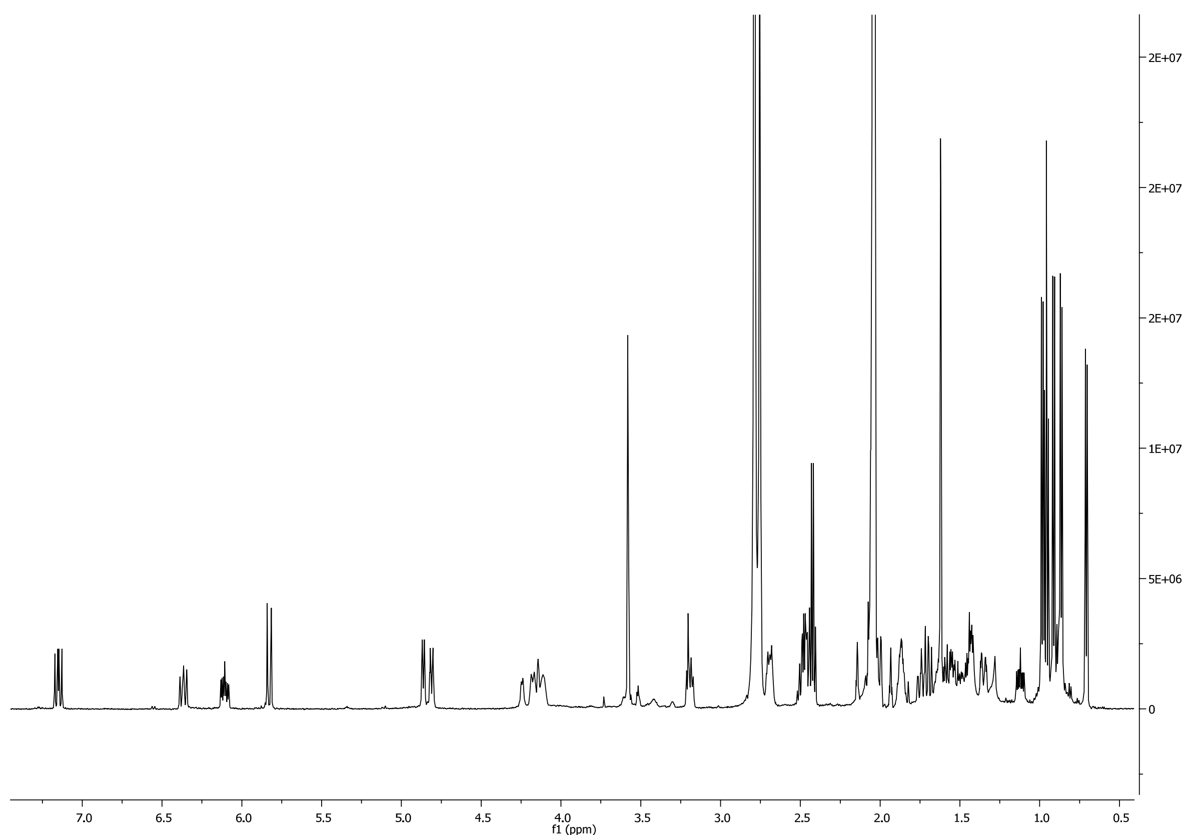
Appendix 2c: ^{1}H-^{1}H COSY spectrum of levantilide A (**1**) in DMSO-*d*6



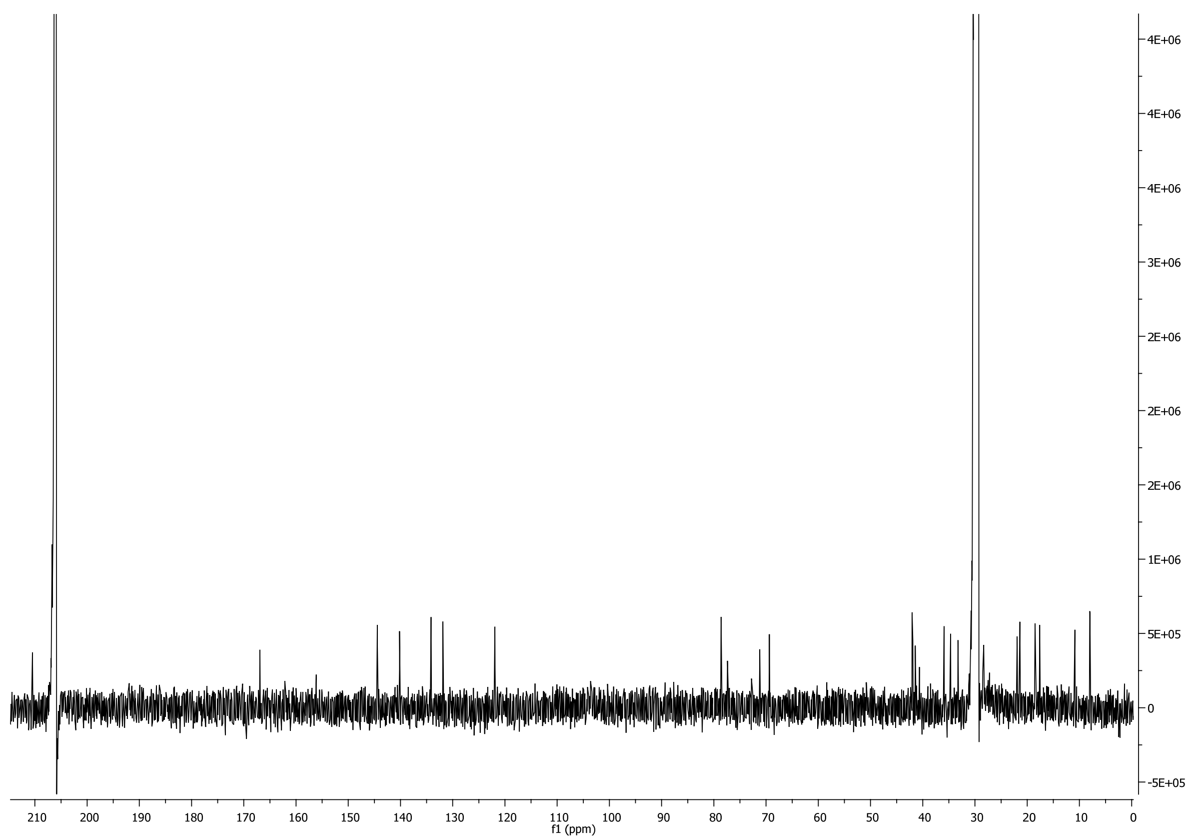
Appendix 2d: ^{1}H-^{13}C HMBC spectrum of levantilide A (**1**) in DMSO-*d*6



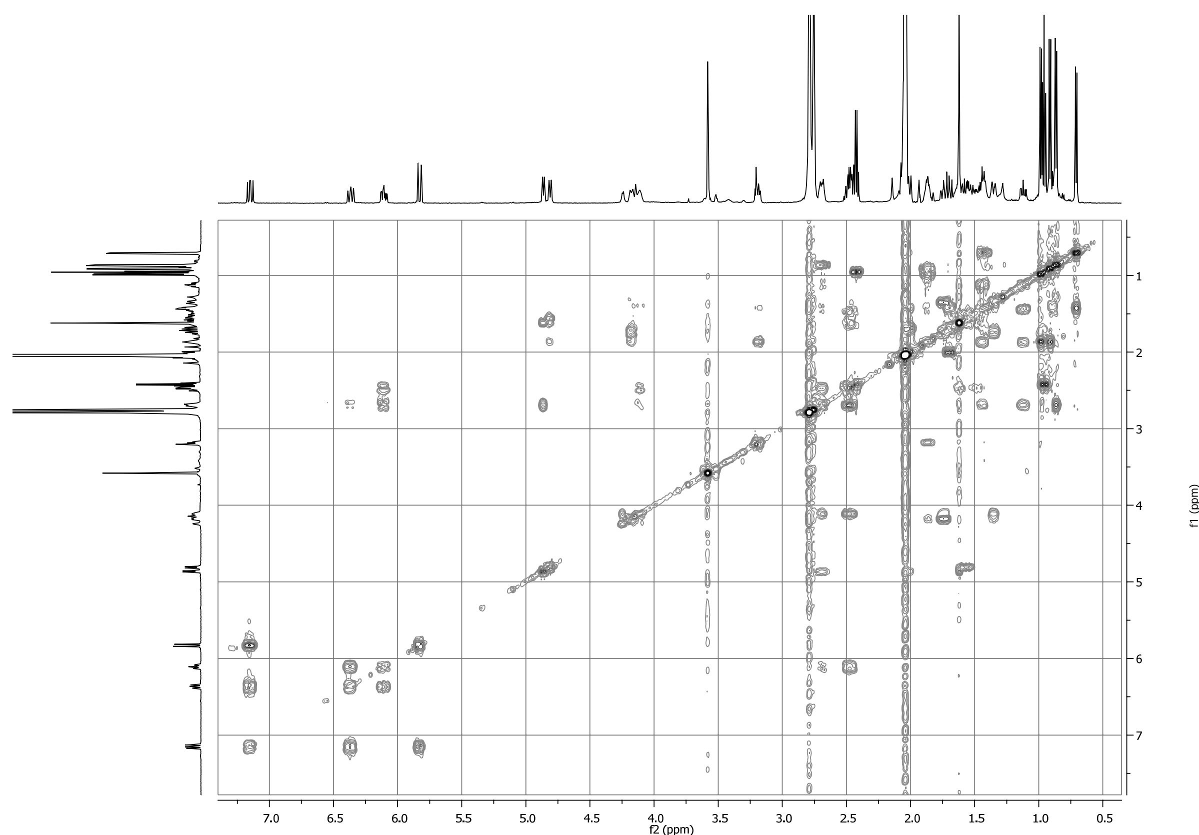
Appendix 2e: ^{1}H-NMR spectrum of levantilide B (**2**) in acetone-*d*6



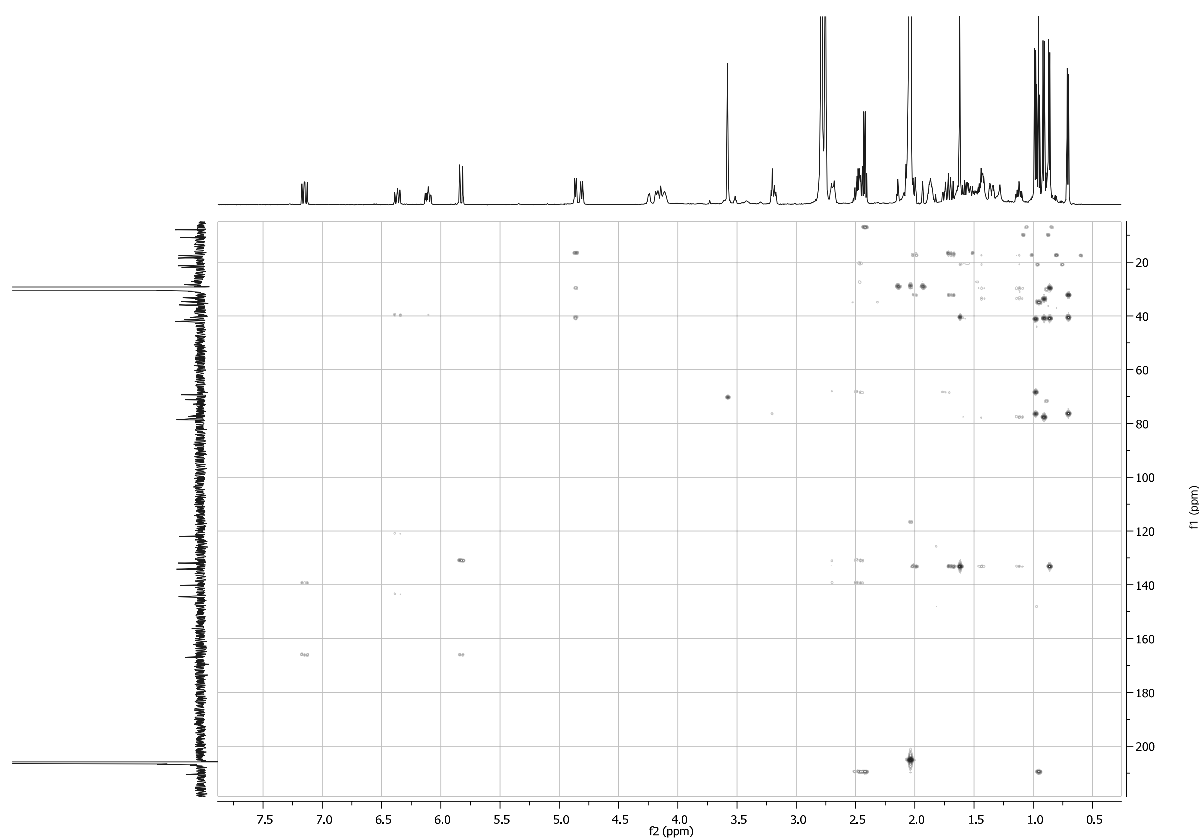
Appendix 2f: ^{13}C-NMR spectrum of levantilide B (**2**) in acetone-*d*6



^{1}H-^{1}H COSY spectrum of levantilide B (**2**) in acetone-*d*6



Appendix 2g: ^{1}H-^{13}C HMBC spectrum of levantilide B (**2**) in acetone-*d*6



Appendix 3: NMR spectroscopic data of levantilide B (**2**) in acetone-*d6*

levantilide B				
	$_{C}$	$_{H}$, *J* [Hz]	COSY	HMBC
1	166.9, C			
2	121.9, CH	5.83, d (15.1)	3	1, 3, 4
3	144.4, CH	7.15, dd (11.0, 15.1)	2, 4	1, 2, 4, 5
4	131.9, CH	6.36, dd (11.0, 15.1)	3, 5	2, 3, 6
5	140.2, CH	6.11, ddd (15.1, 9.8, 4.4)	4, 6	3, 6, 7
6a	40.6, CH_2	2.66, m	5, 7	4, 5, 7, 8
6b		2.46, m	5, 7	4, 5, 7, 8
7	69.4, CH	4.10, m	6	
8a	33.9, CH_2	1.72, m	7, 8, 9	7, 9
8b		1.34, m	7, 8, 9	7, 9
9	69.4, CH	4.17, m	8, 10	30
10	42.3, CH	1.86, m	9, 11, 30	
11	77.4, CH	3.18, m	10, 12	13, 29, 30
12	33.3, CH	1.41, m	13, 29	14, 15
13a	41.4, CH_2	1.99, m	12, 13	11, 12, 14, 15, 28, 29
13b		1.69, m	12, 13	11, 12, 14, 15, 28, 29
14	134.1			
15	134.1, CH	4.87, d (8.4)	16, 28	16, 17, 28
16	30.7, CH	2.69, m	15, 27	14, 15
17a	41.9, CH_2	1.43, m	16, 17, 18	15, 16, 18, 19, 26, 27
17b		1.11, ddd (14.6, 9.0, 5.1)	16, 17, 18	15, 16, 18, 19, 26, 27
18	34.7, CH	1.87, m	17, 19	19
19	78.6, CH	4.81, dt (10.2, 2.5)	18, 20	1, 17, 21
20a	28.3, CH_2	1.57, m	19	16, 21, 23
20b		1.50, m	19	19, 21, 22
21a	21.3, CH_2	1.62, m	22	20, 23
21b		1.47, m	22	20, 23
22	42.1, CH_2	2.45	21	20, 21, 23
23	210.5, C			
24	36.0, CH_2	2.42, q (7.5)	25	23, 25
25	8.0, CH_3	0.96, t (7.5)	24	23, 24
26	18.4, CH_3	0.91, d (7.5)	18	17, 18, 19
27	21.8, CH_3	0.87, d (7.0)	16	15, 16, 17
28	17.6, CH_3	1.63, s	15	13, 15
29	18.4, CH_3	0.71, d (7.0)	12	11, 12, 13
30	11.0, CH_3	0.98, d (7.0)	10	9, 10, 11