Molecular diversity of the shallow water hydrothermal vent (Azores) bacteria, their adaptation and biotechnological potentials

A thesis submitted to Goa University for the award of degree of

Doctor of Philosophy

In

MICROBIOLOGY

By

R. Rajasabapathy

Under the guidance of

Dr. C. Mohandass

Principal Scientist

Biological Oceanography Division

CSIR-National Institute of Oceanography,

Dona Paula – Goa 403004

2015

Declaration

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled "**Molecular diversity of the shallow water hydrothermal vent (Azores) bacteria, their adaptation and biotechnological potentials**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

RAJASABAPATHY

Certificate

Certified that the research work embodied in this thesis entitled “**Molecular diversity of the shallow water hydrothermal vent (Azores) bacteria, their adaptation and biotechnological potentials**” submitted by Mr. R. Rajasabapathy for the award of Doctor of Philosophy degree in Microbiology at Goa University, Goa, is the original work carried out by the candidate himself under my supervision and guidance.

Dr. C. Mohandass
Principal Scientist
Biological Oceanography Division
CSIR-National Institute of Oceanography,
Dona Paula – Goa 403004

Acknowledgements

With heart-felt gratitude, I express my sincere thanks to Dr. C. Mohandass, my research supervisor for encouraging me to choose this topic. I am very grateful to him for valuable guidance and constant support throughout the study. His scientific experience in various fields of research along with effective suggestions has influenced my research throughout.

I express my gratitude to Dr. Ana Colaço and Dr. Raul Bettencourt, Institute of Marine Research (IMAR), Department of Oceanography and Fisheries, University of Azores for their magnanimity and valuable guidance during my InterRidge/ISA fellowship at Portugal.

I wish to thank Dr. S.W.A. Naqvi, Director, CSIR-NIO and Dr. N. Ramaiah, Head, B.O.D for giving me an opportunity to work at CSIR-NIO and providing the necessary facilities.

I owe my deepest gratitude to Dr. C. Ravindran, Scientist, CSIR-NIO, who introduced me to the field of microbial diversity and supported during my initial stage.

I warmly thank Dr. Syed G. Dastager, Scientist, CSIR-NCL for his guidance in bacterial taxonomy and extensive help and discussions during my research work.

I thank Dr. J-H. Yoon (Sungkyunkwan University, South Korea), Dr. Wen-Jun Li, Dr. Qing Liu (Yunnan University, China), Dr. V. Venkata Ramana and Dr. Y. Shouche (NCCS, Pune) for the analysis of Quinones, DNA G+C content, DNA-DNA hybridization and polar lipids.

I am grateful to Mr. Ram M. Meena for his constant support in DNA sequencing throughout my work.

I am thankful to my VC nominee Dr. Sanjeev Ghadi, Professor, Department of Biotechnology, Goa University for his valuable suggestion and encouragement during my work.

I acknowledge Council of Scientific and Industrial Research (CSIR, New Delhi) for Senior Research Fellowship; InterRidge (China) for IR/ISA fellowship to visit University of Azores, Portugal; Department of Science and Technology (DST, New Delhi) for international travel grant to attend IMBC-2013 Conference held at Australia.

I acknowledge Department of Science and Technology, Govt. of India (Indo-Portugal bilateral program), Portuguese Foundation for Science and Technology (FCT) and DRCTC-Regional Government of the Azores.

I am highly thankful to Mr. Areef Sardar, Dr. VK. Banakar and Mr. Sarath for scanning electron microscopy analysis. I take this opportunity to thank Dr. Shanta Nair, Dr. Anaz and Mrs. Vijitha (CSIR-NIO, RC-Kochi) for fatty acid methyl ester analysis.

My sincere thanks to Biological Oceanography staffs Dr. T.G. Jagtap, Dr. Mohan Dhale, Dr. Baban Ingole, Dr. Sanitha Sivadas, Dr. Vishwas Khodse, Dr. Baby Divya, Dr. J. Ravindran, Dr. Samir Damare, Dr. Cathrine Sumathi, Dr. C. Raghukumar, Dr. Rakhee Khandeparker and Dr. Loka Bharathi for their help and support.

I extend my sincere gratitude to Dr. Sarita Nazareth, Dr. S. Bhosale, Dr. S. Garg, Dr. S. K. Dubey, Dr. Irene Furtado and Mrs. Saraswathi from Department of Microbiology, Goa University for their support to carry out this research.

I thank VijayRaj, Satheesh Babu, Ravindra Pawar, Devika Joshi and Varsha for their scientific discussions and valuable company to keep me cheerful and focused. I sincerely appreciate the help and support rendered by my friends Veerasingam, Kesava Priyan, Ranjith, Sam, Periasamy, Viswanathan, Priyanka, Sagar, Geeta, Seema, Karthik, Harish, Sathish, Rashmi, Akhila, Sheba, Naveenan, Subha Anand, Muthukumar and Mangalaa.

I take this opportunity to thank Dr. Ricardo Santos, Director of IMAR, Azores, Portugal, Fredrico Cardigos, Jorge Fontes, João Monteiro and Hugo Parra, Shree Ram Prakya, Dr. Marina, Joana Gaulart, Aida, Eva Martin and Kristel (Department of Oceanography and Fisheries, University of Azores) for their moral support during my stay in Portugal.

My sincere thanks to all my other friends and colleagues whose names I have not mentioned here for want of space.

I have no words to express my deep gratitude to my father, mother and brothers for moral support during my study.

Above all, I thank the almighty God for providing me this opportunity and granting me the capability to proceed successfully.

RAJASABAPATHY

Dedicated to My Beloved Family

Table of contents

Chapters	Title	Page no.
Chapter 1	Introduction	1
Chapter 2	Culture dependent bacterial phylogeny from shallow water hydrothermal vent of Espalamaca (Faial, Azores)	19
Chapter 3	Culture independent bacterial community from shallow water hydrothermal vent of Espalamaca (Faial, Azores)	52
Chapter 4	Metals and element tolerance studies of the bacterial isolates of Espalamaca	80
Chapter 5	Bacterial enzymes from the Espalamaca isolates	103
Chapter 6	Taxonomic characterization of novel bacterial taxa	
6A	*Nioella nitratireducens* gen. nov., sp. nov., a novel member of the family *Rhodobacteraceae* isolated from Espalamaca, Azores	125
6B	*Roseovarius azorensis* sp. nov., isolated from seawater at Espalamaca, Azores	138
6C	*Vitellibacter nionensis* sp. nov., isolated from a shallow water hydrothermal vent in Espalamaca, Azores	152
6D	*Citreicella manganoxidans* sp. nov., a manganese oxidizing bacteria isolated from a shallow water hydrothermal vent in Espalamaca, Azores	166
6E	*Vibrio azorensis* sp. nov., isolated from shallow water hydrothermal vent sediment (Espalamaca, Azores)	178
6F	*Rhizobium azorensis* sp. nov., isolated from a shallow hydrothermal vent (Espalamaca, Azores)	191
Chapter 7	Summary	203
	References	207
	Publications	237

Chapter 1

Introduction

1.1. Marine microbes and their role

Ocean covers more than 70 % of the earth's surface among three major habitats of the biosphere which provides large space for living organisms, most importantly microbes. Majority of the life forms on Earth were likely originated from microbes in the ocean. The word microbes include extensive and diverse communities of viruses, bacteria, protists and fungi with different morphological, ecological and physiological characteristics. They present everywhere in the ocean, from the surface waters of the sea to lower and abyssal depths, from coastal to the offshore, and specialized niches like blue waters of coral reefs to deep-sea hot hydrothermal vents (Das et al. 2006).

Ocean water holds up to one million microorganisms per millilitre and several thousands of microbial types. Overall, the number of microorganisms in marine ecosystem is reported to be 10^{30} cells but very little explored in their diversity. Enormous research activities on the biogeography of marine microorganisms have been carried out, but still many unknowns persist, hence more effort is required to clarify and understand their complexity (Hunter-Cevera et al. 2005).

The marine ecosystem is characterized by the hostile parameters, for instance salinity, high pressure, low temperature and absence of light in the deep-sea; presence of light, low nutrients in open ocean surface waters, etc. Marine heterotrophic bacteria have adapted themselves to withstand in this environment by means of Na^+ requirement for their growth because it is necessary to maintain the osmotic environment to protect the cellular integrity (Das et al. 2006).

Distribution of bacteria is purely based on sea water temperature, salinity, pH, nutrients and other physicochemical constraints. More than 90 % of bacteria in the oceans are

Gram-negative in nature because of their cell wall structure and their adaptation to survive in saline environment. Their cultivable rates are 0.001 – 0.1 % (Ferguson et al. 1984).

In a food chain, bacteria play significant role in both the starting and ending point. They provide first production of particulate food-stuff by converting the dissolved organic matter, and they are responsible for the decisive breakdown of organic matter which leads to the returning of nutrients. Also, their unique metabolisms permit to carry out various functions and they are central catalysts in biogeochemical cycles which are not possible by other organisms (Li and Dickie 1996).

Marine bacteria ruled the Earth for nearly 2 billion years. All of Earth's biogeochemical cycles were established during the age of bacteria (from 3.5 – 1.8 bya). So far, the role of bacteria as nature's recyclers is well-understood, their importance as a food source is less known. Nurtured by groups of dissolved organic carbon, marine bacteria play a fundamental role in marine food webs offering nutrition to small microorganisms. In this manner, they bring back energy in the form of carbon compounds that might otherwise be vanished to the system. This microbial loop, the constituent of marine food web that recycles most of the minerals (biologically important nutrients) and captures energy from dissolved organic matter, correspond to a primary component of marine food webs, particularly in the open ocean. To credit their significance in the world ocean, researchers nowadays often refer to water column bacteria as bacterioplankton (Chamberlin and Dickey 2008).

Since bacteria have the ability to grow rapidly and the relative ease with which they can be manipulated, bacteria are the workhorses for the fields of molecular biology, biochemistry and genetics. By making mutations in bacterial DNA and observing the

resulting phenotypes, researchers can conclude the function of genes, enzymes and metabolic pathways in bacteria, and then apply this knowledge to more complex organisms (Sanatan 2008).

Marine ecosystem consist of Estuaries, Intertidal zone, Continental shelf, Coral reefs, Ocean banks, Pelagic zone, Littoral zone, Seamounts, Hydrothermal vents, Cold seeps, Demersal zone, Kelp forests, Neritic zone, Straits, Oceanic zone and Benthic zone. The focus of the present study will be on bacterial communities from hydrothermal vent ecosystem.

1.2. Hydrothermal vent ecosystem and bacteria

1.2.1. Hydrothermal vents

Hydrothermal vents are associated with sea-floor spreading zones, generally occurs in mid-ocean ridges where two tectonic plates are moving apart and in basins near volcanic island arcs. Magma pockets are the energy engines which creates volcanic activity. The molten rock (800–1,200 °C) beneath the magma discharges lavas onto the sea floor over time periods ranging from <10 years to >50,000 years between eruptions (Hammond 1997). Since the tectonic plate activities takes place, the sea water sinks directly through the crevices in the crust and exposed to the magma chambers. The cold water becomes heated up by the magma and then collects metals and minerals from the molten rocks, and shot up out of an opening into the sea water (Brooks 2006) which forms chemical plume.

Numerous hydrothermal venting sites and faunal assemblages at many mid-ocean ridges and back-arc basins have been explored since from the discovery of hydrothermal vents along the Galapagos Ridge in 1977 (Corliss et al. 1979). These explorations have come

out with clear global biogeography of vent organisms with individual regions in various oceans (Rogers et al. 2012). Many of these hydrothermal vents are reported along the East Pacific Rise, Juan de Fuca, Gorda, Galapagos, Hawaiian and Explorer Ridges, Mid-Atlantic Ridge, Mariana Trough, Okinawa Trough, and Central and Southeast Indian Ridges (Desbruyeres 2006) (Fig. 1.1).

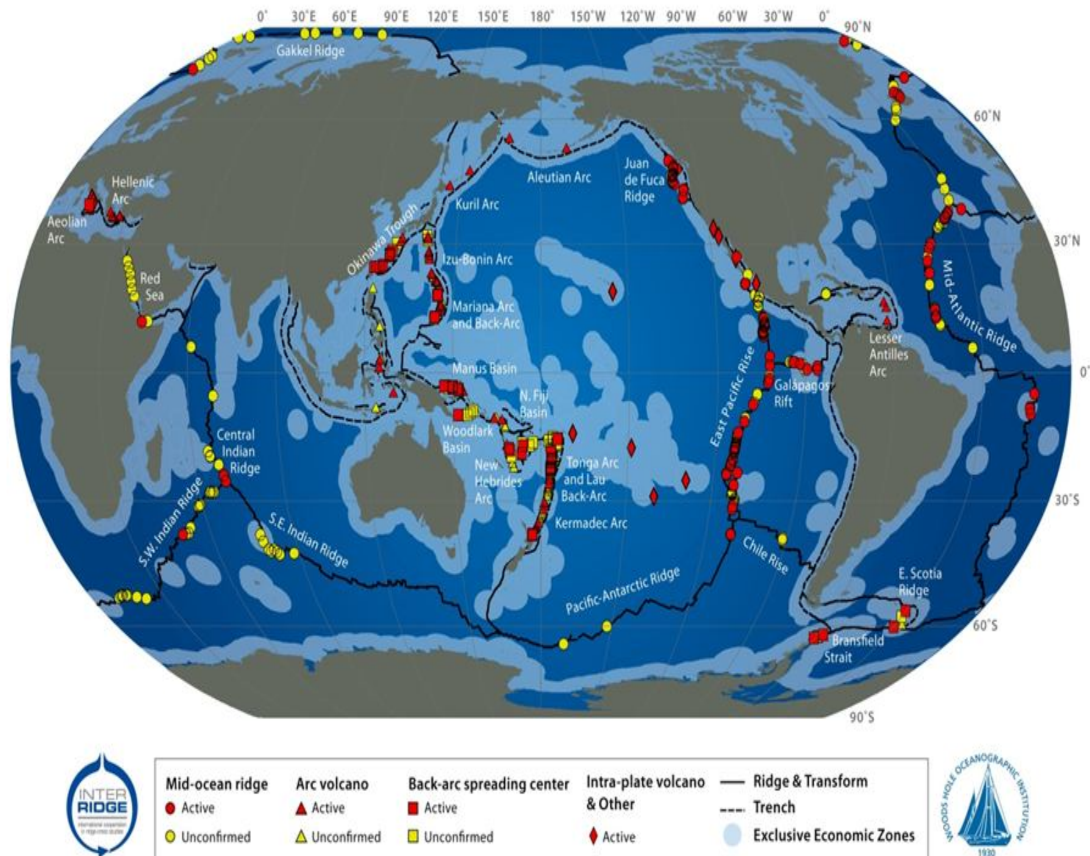


Fig. 1.1. Global distribution of hydrothermal vent fields

Water emerging from the hot regions of some hydrothermal vents will be a supercritical fluid, which have physical properties between those of a gas and a liquid. In contrast to the ambient seawater, the temperature of superheated water in hydrothermal vents is above 400 °C. Most recently, hottest ever measured hydrothermal fluid temperature (464 °C) was reported from the active venting Sisters Peak chimney on the Mid-Atlantic

Ridge (Perner et al. 2014). The depth of these hydrothermal vents varies and maximum recorded in Mid-Atlantic Ridge which is up to 7,700 meters (https://microbewiki.kenyon.edu/index.php/Deep_sea_vent). Based on venting activity, the crevices in the venting ocean floor may enlarge in size and spread from 5-9 cm per year or as fast as 9-16 cm per year (Jones 1985).

Hydrothermal vent sites are far from uniform. Within the individual communities, the distributions of organisms are affected by the environmental gradient created by mixing of vent fluids with ambient sea water. Compared to other deep-sea communities, hydrothermal vents show much higher productivity but the species diversity is less (Van Dover 2000a). Giant tubeworms, bristle worms, yellow mussels, clams, and pink sea urchins are some of the animals found in the distinctive ecological systems that surround the hydrothermal vents. Deep-sea hydrothermal vents provide a sort of micro-niches that are likely habitats for physiologically different thermophilic and hyperthermophilic micro-organisms (Prieur et al. 1995).

Investigation of active hydrothermal vents revealed the records of brightly coloured tubeworm communities and of groups of clams, gastropods and crabs in the volcanically driven hot vents. Nutrient supplements for these macrofauna are supported by several colonies of thermotolerant Archaea/Bacteria that flourish in the absence of sunlight (Baross and Hoffman 1985; Gold 1992; Wirsen et al. 1993; Takai et al. 2001).

1.2.2. Metals and elements in hydrothermal vents

Hydrothermal fluids are enriched with high levels of sulphide (H_2S), hydrogen (H_2), methane (CH_4), manganese (Mn), and other transition metals (Fig. 1.2) like iron, lead, zinc, copper, cobalt and aluminium, whereas levels of oxygen is low (Jones 1985).

Because of these enriched elements and metals, the pH always tends to be in low level (3-5).

Metal compositions and concentrations may vary from deep-sea vent to shallow vents and also vary from region to region. Sarradin et al. (2008) confirmed the presence of Fe, Cu, Zn, Pb, Cd from an active hydrothermal field on the East Pacific Rise (EPR) in which Fe is the predominant metal (5–50 μM) followed by Zn and Cu whereas, Cd and Pb are present at the nM level. Cardigos et al. (2005) reported the presence of Fe (8.8-89.2 μM), Mn (0.5-6.8 μM), Pb (1.9-3.6 nM), Co (30-40 nM) and Cd (5-6 nM) from white and yellow zones in shallow water hydrothermal vents at DJCS (Azores, Portugal). The below picture explain the details of how various metals and elements get involved into the hydrothermal system.

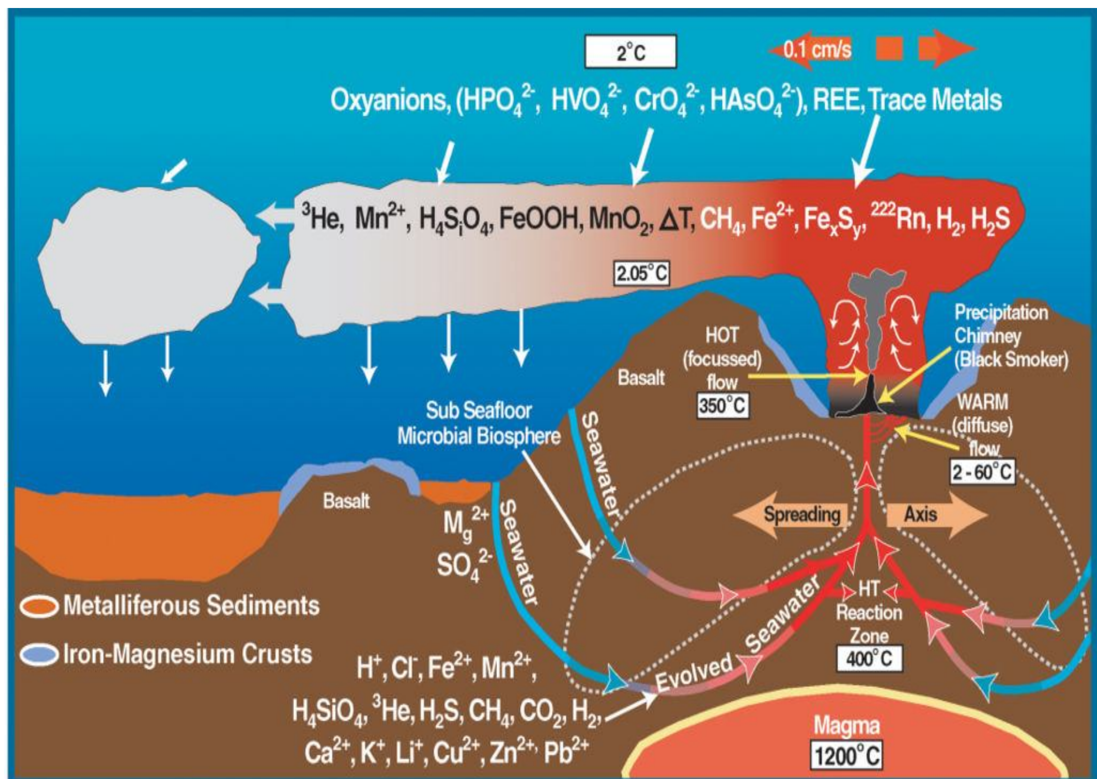


Fig. 1.2. Hydrothermal circulation in a mid-oceanic ridge (MOR) system (Photo courtesy: Wouldloper)

1.2.3. Deep and shallow water hydrothermal vents

The environmental conditions in shallow hydrothermal vent regions vary from those in deep-sea hydrothermal systems and terrestrial hot springs with respect to temperature, water pressure, pH, salinity, sunlight, etc (Hirayama et al. 2007). Shallow water hydrothermal vent ecosystems are widespread that have been previously understudied when compare with deep-sea ecosystems (InterRidge, http://www. interridge.org/).

Deep sea hydrothermal vents are highly productive ecosystems where chemolithoautotrophic microorganisms mediate the transfer of energy from the geothermal source to the higher trophic level (Jannasch and Mottl 1985). Relative to the majority of the deep sea, the areas around shallow submarine hydrothermal vents are biologically much higher productive, swarming complex communities with the energy gained from the chemicals dissolved in the vent fluids.

The shallow-water vents differ from their deep-sea counterparts mainly by the presence of light. At shallow-water vents, photosynthetic organisms such as benthic microalgae and cyanobacteria are present (Sorokin 1991) and thus primary production by photosynthetic organisms can take place (Dando et al. 1995). On the other hand, oxidized sulphur compounds are used by many heterotrophic members of the Archaea and Bacteria as electron acceptors for the anaerobic degradation of organic matter, although some can grow autotrophically.

However, the most commonly understood mode of metabolism thought to dominate the deep-sea hydrothermal vent microbial communities is chemolithoautotrophy, principally through the oxidation of iron compounds and reduced sulphur compounds (Jannasch and Mottl 1985). The habitat for these organisms is the anoxic parts of the hydrothermal

system, and correspondingly many of them are thermophiles or hyperthermophiles (Karl 1995).

Usually there is a high biomass of mostly endemic, but species poor fauna that depends on chemosynthesis based production at deep-sea vent mid-ocean ridges (Tunnicliffe 1991). But contrastingly, shallow water vents tend to have a low biomass of more diverse fauna with few endemic species (Morri et al. 1999; Kamenev et al. 1993; Dando et al. 1995). Only a few examples are known of vent endemic species occurring in shallow water, e.g., a vestimentiferan, *Lamellibrachia satsuma*, in Kagoshima Bay, Japan (Miura et al. 2002) and a crab, *Xenograpsus testudinatus* in Kagoshima Island (8–20 m depth), Taiwan (Jeng et al. 2004).

Hydrogen sulfide, a crucial component for the chemosynthetic processes, provides energy for hydrothermal vent communities, which can have toxic effects on organisms which are not adapted to function at high concentrations. Thermophilic bacteria and archaea which may possibly live at temperatures more than 100 °C are taken a great deal of adaptation to survive in close proximity to hydrothermal vents. More than 500 animals that have been discovered at vent sites which appear to live exclusively with vent communities (Van Dover 2000a).

1.2.4. Bacterial diversity in shallow water hydrothermal vents

Shallow submarine hydrothermal systems exposed to sunlight are expected to harbor more complex microbial communities, because there is in situ primary production by means of chemolithotrophs and phototrophs as well (Hirayama et al. 2007). Several shallow water hydrothermal vents have been investigated worldwide (eg. Dando et al. 1995 ; Hoaki et al. 1995 ; Pichler et al. 1999 ; Amend et al. 2003; Prol-Ledesma et al.

2004 ; Mohandass et al. 2012). However, only limited information is available on microbial community structures in shallow submarine hydrothermal systems.

Hydrothermal vents and cold seeps are some of the extreme environments that strongly select the patterns of species diversity, life strategies, classical ecological pathways, altering food webs and force organisms to find different pathways to survive with the hostile environmental conditions. Shallow water hydrothermal vents create extreme local conditions by naturally releasing free gas and hot water which are strongly variable in space and time, and are often transient. These systems have been reported from several oceans including off the coasts of California, New Zealand, Iceland, Japan, Papua New Guinea and Mexico and from the Mediterranean Sea (Zeppilli and Danovaro 2009).

The abundance of the prokaryotic community and biomass in shallow water hydrothermal vent (Milos Island, Greece) having on average of 1.34×10^8 cells g^{-1} which was equivalent to areas not affected by hydrothermal activity (Giovannelli et al. 2013).

Culturable heterotrophic bacterial population and their composition were investigated in relation to environmental parameters in a shallow water hydrothermal vent off the Island of Vulcano (Gugliandolo and Maugeri 1998). The occurrence of heterotrophic bacteria from the venting seawaters has also been shown by measurements of heterotrophic bacterial activity (Tuttle et al. 1983). Moreover, heterotrophic sulphur oxidizers belonging to the genera *Acinetobacter*, *Pseudomonas*, and *Vibrio* have been described in hydrothermal vent environments (Durand et al. 1994). Heterotrophic members like *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Halomonas*, *Micrococcus*, *Pseudoalteromonas* and *Staphylococcus* were also reported in white and yellow zones of D. João de Castro Seamount (DJCS), Azores (Mohandass et al. 2012).

Teske et al. (2000) investigated the species diversity, phylogenic affiliations and environmental occurrence patterns of thiosulfate oxidizing bacteria from the Galapagos hydrothermal vent samples. The sulphur-oxidizing bacteria (SOB) were investigated from various hydrothermal vents with respect to different depths e.g., *Thiomicrospira* at 8 meter deep (Brinkhoff 1999), *Thiobacilli* and *Achromatium* at less than 30 m deep (Dando et al. 1995) and *Thioploca* at 46 m deep (Dando and Hooper 1997).

Hirayama et al. (2007) explored the microbial communities in a shallow submarine hydrothermal system near Taketomi Island, Japan, using culture-dependent and culture-independent approach. The most abundant culturable microorganisms from the fluid and the mat were autotrophic sulphur oxidizing *Thiomicrospira* spp., thermophilic *Sulfurivirga caldicuralii*, sulfate-reducing *Desulfovibrio* spp., iron-reducing *Deferribacter* sp., and sulphur-reducing *Thermococcus* spp. Whereas, culture independent molecular analyses revealed the dominance of γ-*Proteobacteria*, ε-*Proteobacteria* and δ-*Proteobacteria*.

Davis and Moyer (2008) explored the complete view of microbial diversity in hydrothermal seamounts based on their collection of microbial mats from Axial, Loihi, and volcanoes of the Mariana Arc and backarc. They found either ε-*Proteobacteria* or ζ-*Proteobacteria* at most of the locations. From this point of view, Huber et al. (2010) exclusively studied the abundance and diversity of ε-*Proteobacteria* using 454 tag sequencing approach in 14 low temperature vent fluids from five volcanically active seamounts of the Mariana Arc. ε-*Proteobacteria* constituted the majority of the population along the shallow water hydrothermal vents including Milos Island, with an average contribution of 60 % to the total diversity (Giovannelli et al. 2013). Shallow-sea

hydrothermal system in offshore NE Taiwan was also predominated by ε-*Proteobacteria* (Tang et al. 2013).

Rassa et al. (2009) reported the colonizing microbial populations at Loihi Seamount and which was the first study to show ζ-*Proteobacteria* as the dominant colonizers in a hydrothermal vent ecosystem. Recently, Li et al. (2013a) carried out phylogenetic analyses of 16S rRNA sequences and the aprA functional gene from two low-temperature hydrothermal fields at the Southwest Indian Ridge. He reported ζ-*Proteobacteria*, *Pseudoalteromonas*, *Leptothrix*, and *Pseudomonas* as potential Fe and Mn oxidizers and *Firmicutes*, *Burkholderiaceae*, *Sphingomonadaceae*, and *Caulobacteraceae* as potential Fe and Mn reducers in the low-temperature hydrothermal environments.

Maugeri et al. (2013) revealed the signatures of *Proteobacteria* groups in fluid and sediment samples from submarine vent of Panarea Island (Italy). In their study he concluded that α-*Proteobacteria*, γ-*Proteobacteria*, δ-*Proteobacteria* and ε-*Proteobacteria* dominated the sediment community, whereas β-*Proteobacteria*, α-*Proteobacteria*, γ-*Proteobacteria* and ε-*Proteobacteria* were more abundant in fluid.

1.3. Molecular techniques for bacterial phylogeny

Morphology and physiological properties may usually helps to classify living organisms but the same have always helpless when we consider microbes. Comparisons of the information content of microbial macromolecules, especially nucleic acids and proteins would be more accurate than traditional methods. When two microbes are very closely related, we can expect that the macromolecular sequence of the individual units to be more similar than two unrelated organisms (Munn 2011).

Traditionally bacteria were identified based on its morphological and physiological properties using Bergy's manual systems of classification (based on morphology, Gram stain, spore stain, motility, enzyme activities, and utilization of several substrates as sole carbon and energy sources, etc.). In addition to that, Sasser (1990) formulated a technique to identify bacteria based on cell wall fatty acid methyl ester (FAME) composition. However, these techniques could not able to differentiate closely related species and even genus level in some cases. In the 1970s, Carl Woese and his research team established the use of smaller subunit ribosomal RNA (rRNA) sequencing in order to develop an improved view of microbial diversity.

The smaller subunit rRNA gene has hypervariable regions, where sequences have diverged over evolutionary time (Fig. 1.3). These variable regions are often flanked by designed primers from the sequences of strongly-conserved regions.

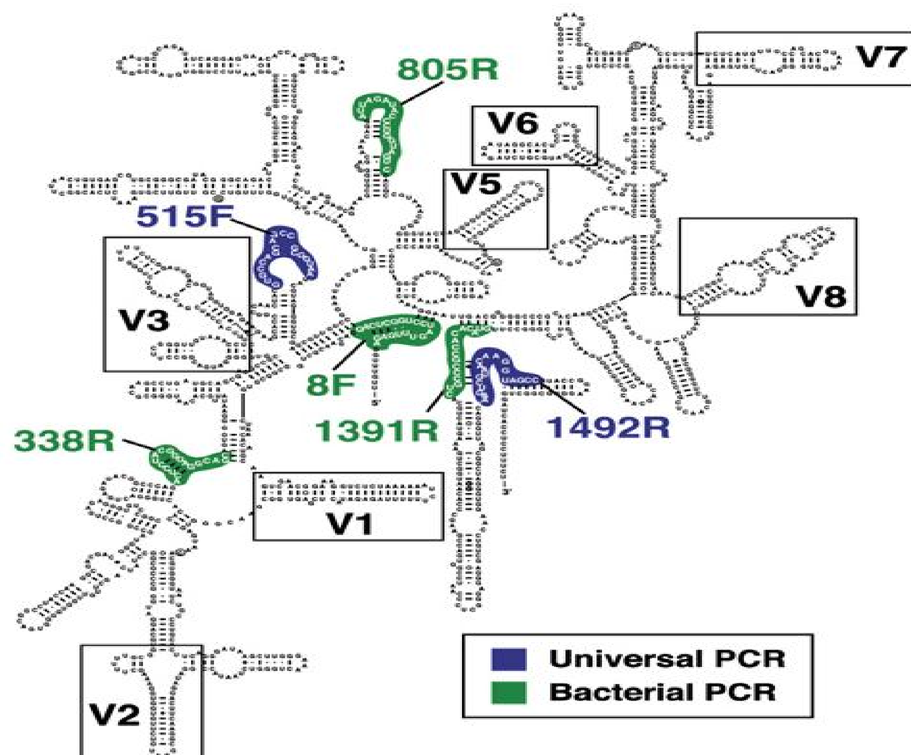


Fig. 1.3. Variable regions in 16S rRNA gene (Photo courtesy Peterson et al. 2008)

Molecular approaches for the investigation of microbial communities, based on the PCR amplification and cloning the genes of 16S and 23S rRNA of the small subunit and larger subunit of the ribosome have led to insights into the community diversity and structure of microbial systems (Bintrim et al. 1997). These molecular techniques have revealed new phylogenetic lineages of microorganisms, several of which serve as the major component in a given microbial community.

Knowledge of microorganisms in the environment had been depended mainly on studies of pure cultures in the laboratory. However, culture dependent analysis may not explore the entire microbial community in a particular ecosystem since only <0.1 % microbes are cultivable. But culture independent approach is required only a gene sequence to identify the organism in terms of its phylogenetic type. For metagenomic studies, nucleic acids can be isolated from environmental samples and amplified with ribosomal RNA genes and microbes can be identified at the phyla, family, genus, even up to species levels by comparing the sequence homology (Fig. 1.4) from rDNA database (Ward 1992).

Since smaller subunit ribosomal gene based identification gives only general diversity of microbes, researchers designed a technique to identify the microbial groups and their functions. Other than the universal 16S rRNA genes, recent studies were authenticated by identifying the functional genes or particular groups of bacteria from a particular environmental sample.

Microbial diversity and their roles in the ecosystem functioning can be revealed by targeting a specific genes involved in a pathway. Some of the genes targeted for analysing the microbial communities are, MmoX/MmoxY genes targeting for soluble methane monooxygenase enzyme (Miguez et al. 1997), PmoA genes targeting for particulate methane monooxygenase enzyme (McDonald and Murrel 1997), mxaF gene

targeting for methanol dehydrogenase (Lau et al. 2013), MnxG genes targeting for Bacterial Manganese(II) Oxidase (Dick et al. 2008a), nifH gene encoding for nitrogenase (Mehta et al. 2003), sox genes targeting for sulphur oxidation (Hugler et al. 2010), CA gene targeting for carbonic anhydrase enzyme (Dobrinski et al. 2010).

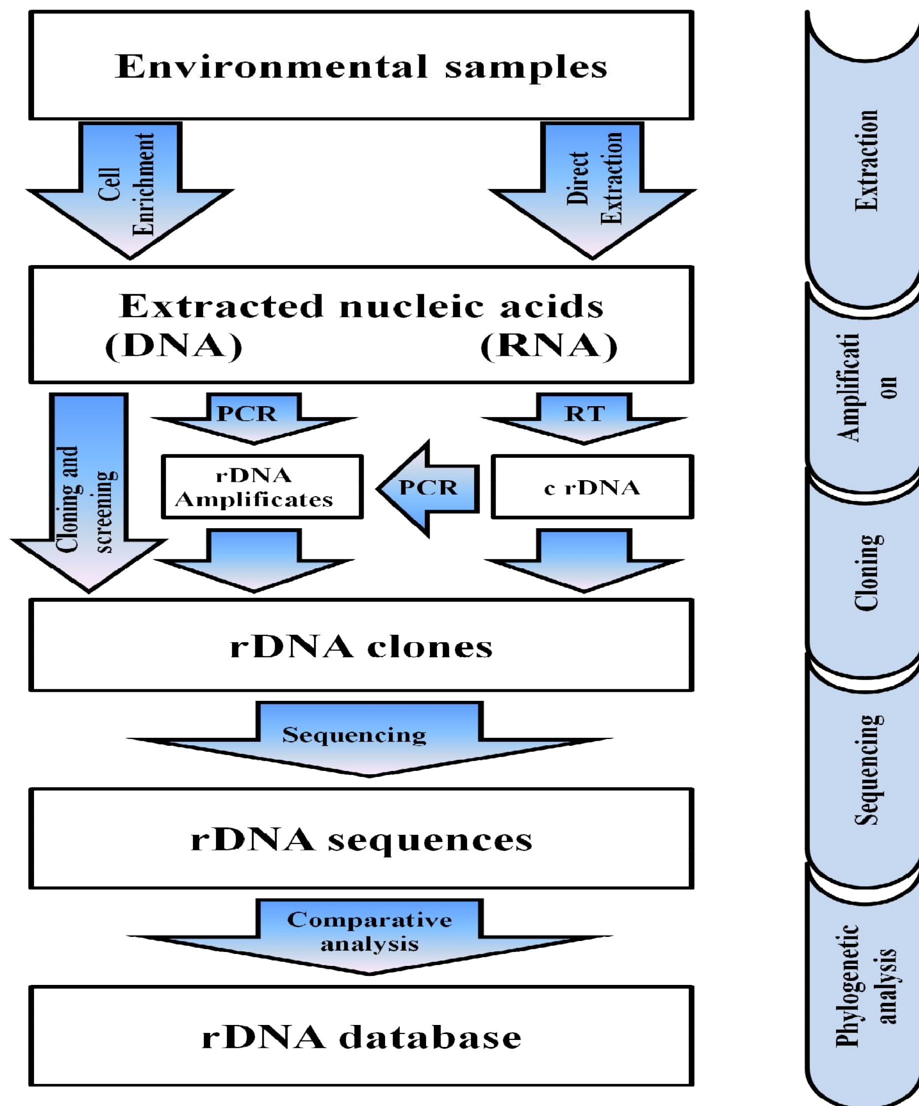


Fig. 1.4. Flow chart showing the microbial community analysis by metagenomic approach (Adopted from Amann et al. 1995)

Recently, powerful sequencing technologies have been developed to investigate the actual microbial diversity, and have been used to study the microbial diversity and richness in environmental samples (Venter et al. 2004). The Illumina sequencing system, allowing to read maximum number of operational taxonomic units (OTUs), has initiated a new era in the study of microbial phylogeny by offering a large number of individual sequence reads and their uniqueness (Bartram et al. 2011).

1.4. Potential applications of hydrothermal vent bacteria

Though many microorganisms already available in public collections, investigation of extremophilic organisms from various extreme environments like hydrothermal vents, cold seeps, and subterranean environments are required either with cultural methods or DNA based molecular approaches to enhance the possibility of finding novel bioactive compounds. Extremophiles are distinctive which are adapted to thrive in ecological niches such as extreme pH, high or low temperatures, high salt concentrations and high pressure. Therefore, biological systems and enzymes can function at temperatures between -5 and 130 °C, pH 0-12, salt concentrations of 3-35 % and pressures up to1000 bar (Bertoldo et al 2002).

Microorganisms inhabiting in extreme environments are often produce polymers and unusual enzymes to survive in high temperatures or in high concentrations of H_2S and heavy metals (Maugeri et al. 2002). Bacterial exopolysaccharides (EPS), especially those from mesophilic *Vibrio* and *Alteromonas* strains isolated from hydrothermal vents, which is currently under evaluation for therapeutic uses (Querellou 2003). The major areas concerned are cardiovascular diseases and tissue regeneration (proangiogenic effects/antithrombotic). Studies conducted by Guezennec (2002) on anticoagulant

activities of the EPS showed that native EPS were deprived of effects whereas sulphated derivatives were active.

Gugliandolo et al. (2012) characterized thermophilic bacilli from Panarea Island (Italy) to identify useful biomolecules for industrial purposes and environmental applications. The study revealed that, many of the bacilli were thermophilic, alkalophilic and haloalkalophilic in nature. Most of the *Bacillus* spp. produced gelatinase, lipase and amylase, and some of them were resistant to mercury.

Erra-Pujada et al. (2001) isolated and purified type II pullulanase from *Thermococcus hydrothermalis* and characterized for pullulanolytic and amylolytic activities. Undoubtedly, pullulanases can be used in tandem with other amylolytic enzymes for the conversion of starch to glucose, maltose or fructose syrups (Saha and Zeikus 1989).

Cornec et al. (1998) investigated on thermostable esterases from hyperthermophilic archaeal and bacterial strains isolated from deep-sea hydrothermal vents. The esterase activity exhibited a half-life of 22 h at 99 °C and of 13 min at 120 °C, and retained its entire initial activity after incubation at 90 °C for 8.5 h without any substrate and/or cofactor. These findings confirm the potential of microbes originated from hydrothermal vents.

Lipases from microbial origin have been used as an important biocatalyst in biomedical applications. Since, their tremendous catalytic action in a variety of organic solvents, they could be used for the synthesis of compounds of pharmaceutical concern. Majority of the isolates from hydrothermal vent region in Aeolian Islands (Italy) showed lipolytic and amylolytic activities (Gugliandolo et al. 1998).

Thermostable enzymes from microbial origin have received huge attention during last two decades. Enzymes that have optimum activity at higher temperatures and pH are widely used in household detergents, food, textile, pulp, paper, chemical and leather processing industries (Podar and Reysenbach 2006).

Apart from the production of thermostable enzymes and EPS, hydrothermal vent microbes play a vital role in metal recovery and detoxification. Further, they are actively participating in the oxidation and reduction reaction. Metal and sulphate reducing bacteria are two biogeochemically important groups having suitable physiology for metal precipitation and immobilization. These microorganisms can interact with heavy metals in a variety of ways to decrease the metal mobility and solubility. Current understanding on these microbes is limited, and research has to be conducted to investigate how these metal and sulphate reducing organisms behave in contaminated sites (Tango and Islam 2002).

Rathgeber et al. (2002) isolated high numbers of Tellurite- and selenite-reducing strains from the seawater samples near hydrothermal vents, bacterial films, and sulfide-rich rocks in Juan de Fuca Ridge in the Pacific Ocean. Growth of these bacterial members in K_2TeO_3 or Na_2SeO_3 amended media resulted in the accumulation of metallic tellurium or selenium. Around 10 bacterial groups (most of them are belong to *Pseudoalteromonas*) could tolerate upto 2,500 μg mL^{-1} of K_2TeO_3, and upto 7,000 μg mL^{-1} of Na_2SeO_3.

Vetrini et al. (2005) isolated several bacteria resistant to mercury from hydrothermal fluids in EPR. Four moderate thermophiles (most of the isolates belonging to the genus *Alcanivorax*), and six mesophiles from the vent plume were resistant to >10 μM Hg(II) and reduced it to elemental mercury [Hg(0)]. These heavy metal resistant and detoxifying hydrothermal vent bacteria may promise in environmental applications.

Understanding the bacterial diversity and its significance over the shallow water hydrothermal vents, the proposed study was executed to find out the bacterial community from a newly identified vent Espalamaca (Azores), North Atlantic Ocean with following objectives:

- Investigation of culturable and non-culturable bacterial diversity from the shallow vent, Azores using molecular analysis
- Metal/elements tolerance (Mn, Fe, Pb and S) and detoxifying potential of vent bacteria
- Investigation on physiological enzymes of the vent isolates and its biotechnological potential
- Comparative studies on bacterial diversity from the vent and non vent site

Culture dependent bacterial phylogeny from shallow water hydrothermal vent of Espalamaca (Faial, Azores)

2.1. Introduction

The hydrothermal vent ecosystem is known for its higher temperature with various gases, elements and metals. Biological productivity at the deep sea hydrothermal vents (>200 m) is not maintained by photosynthetic products, but rather by the chemosynthesis of organic matter by vent microbes, using energy from chemical oxidations to produce organic matter from CO_2 and mineral nutrients (Tunnicliffe 1991; Van Dover 2000b). While, geochemically reactive shallow water hydrothermal vents (<200 m) are exposed to sunlight and their biological production is maintained by photosynthesis as well as chemosynthesis. Shallow hydrothermal vents offer a variety of habitats to metabolically diverse microbes. Though cultivation based methods alone cannot explore the entire microbial community, they do elaborate their metabolic activities in biogeochemical cycles which can be applied in environmental biotechnology.

In general, hydrothermal vent environment represents highly productive ecosystems; the important primary producers in vent food webs are the bacteria that oxidize sulphur, methane, hydrogen, and iron (Kelley et al. 2002; Hugler et al. 2010). Thus hydrothermal vent researchers have focused on the isolation of specialists like thermophilic and chemosynthetic microbes (Sievert et al. 1999; Rusch et al. 2005). But the roles of heterotrophic bacteria which are adapted to metal rich environments have rarely been addressed (Raghukumar et al. 2008; Mohandass et al. 2012). Further, various elements present in the shallow hydrothermal vents are oxidized and utilized by heterotrophic bacterial groups as electron acceptors (Sievert et al. 2000a) although they do not depend only on this for their growth.

Knowledge of hydrothermal vent bacterial community may offer significant information because they react quickly to changes in the concentrations and availability of metals

within their environment. Little is known about how microorganisms from marine hydrothermal environments interact with metals, but their interactions are generally described in one of three ways: the metals are toxic and illicit a response; they are oxidized or reduced to conserve energy in dissimilatory reactions; or they are taken up and utilized in assimilatory reactions (Holden and Adams 2003). Previous studies demonstrated that heterotrophic bacteria not only function as decomposers, but also channel the dissolved organic and inorganic nutrients into higher trophic levels through microbial food-web (Azam et al. 1983; Azam 1998). Since heterotrophic bacteria are highly abundant in the ocean and play a significant role in the biogeochemical cycle of carbon, nitrogen and sulphur (Copley 2002; Karl 2002), it is necessary to study their diversity and adaptations to various elements and metals in the hydrothermal vent ecosystem.

A new shallow hydrothermal vent field was discovered during 2010 at a depth of 35 m which is located close to the Faial Island, just outside the Espalamaca (38°33’N; 28°39’W). Research on various aspects to understand this shallow water hydrothermal vent is going on. This chapter focused on four main issues. One is to know the culture dependent bacterial phylogeny, second to understand the community variation of bacterial diversity between the vent and non-vent, the third to know what made the bacterial species survive in their respective ecosystem and the fourth one to estimate the new/ novel bacterial species.

2.2. Materials and methods

2.2.1. Geological setting

The Azores is an archipelago of nine islands situated in the North Atlantic. These islands spread across an extent of 617 km and are aligned along major tectonic lineaments generally trending WNW-ESE. All islands rise from volcanic edifices sitting on a rugged elevation roughly delineated by the 2000 m depth contour and named the Azores Plateau (Needham and Francheteau 1974). Faial and Pico are two islands located in the central group of the Portuguese archipelago of the Azores (Northeast Atlantic). Both islands are estimated to have emerged during the Pleistocene (800 and 270 ky BP respectively) and are located east of the Mid-Atlantic Ridge. A 5 km wide shelf unites both islands creating a unique shallow water structure in an archipelago where seafloor elsewhere between islands typically exceeds depths of 1000 m (Quartau et al. 2002; Quartau et al. 2003).

A passage which is 6 km-wide in its narrowest section currently separates Faial and Pico. Large expanses of this inter-island shelf are shallower than 100 m and a sill straddling between the Espalamaca head land (Faial Island) to Madalena (Pico Island) bears a maximum depth of 63 m (Tempera 2009).

In Faial Island, the Espalamaca degasification low temperature hydrothermal field has been discovered (Fig. 2.1) in the Faial-Pico channel off the Espalamaca headland (Faial Island, Azores, NE Atlantic). The main venting area, named Espalamaca vent field, extends for a few tens of meters at approximately 35 m depth. The area has been surveyed in detail, during summer 2010, with a multibeam echosounder. Gas emissions can be observed venting out of sediment, as well as through cracked hard ground.

Preliminary analyses of the gaseous discharges from vents suggest that they are mainly composed of CO_2, with low concentrations of methane, no sulphur, temperature as high as 35 °C and pH values of 5.7 (Colaço personal communication). This hydrothermal field is also integrated in a larger protected area designated Baixa do Sul (Canal Faial-Pico) recently classified and integrated the Faial Island Natural Park.

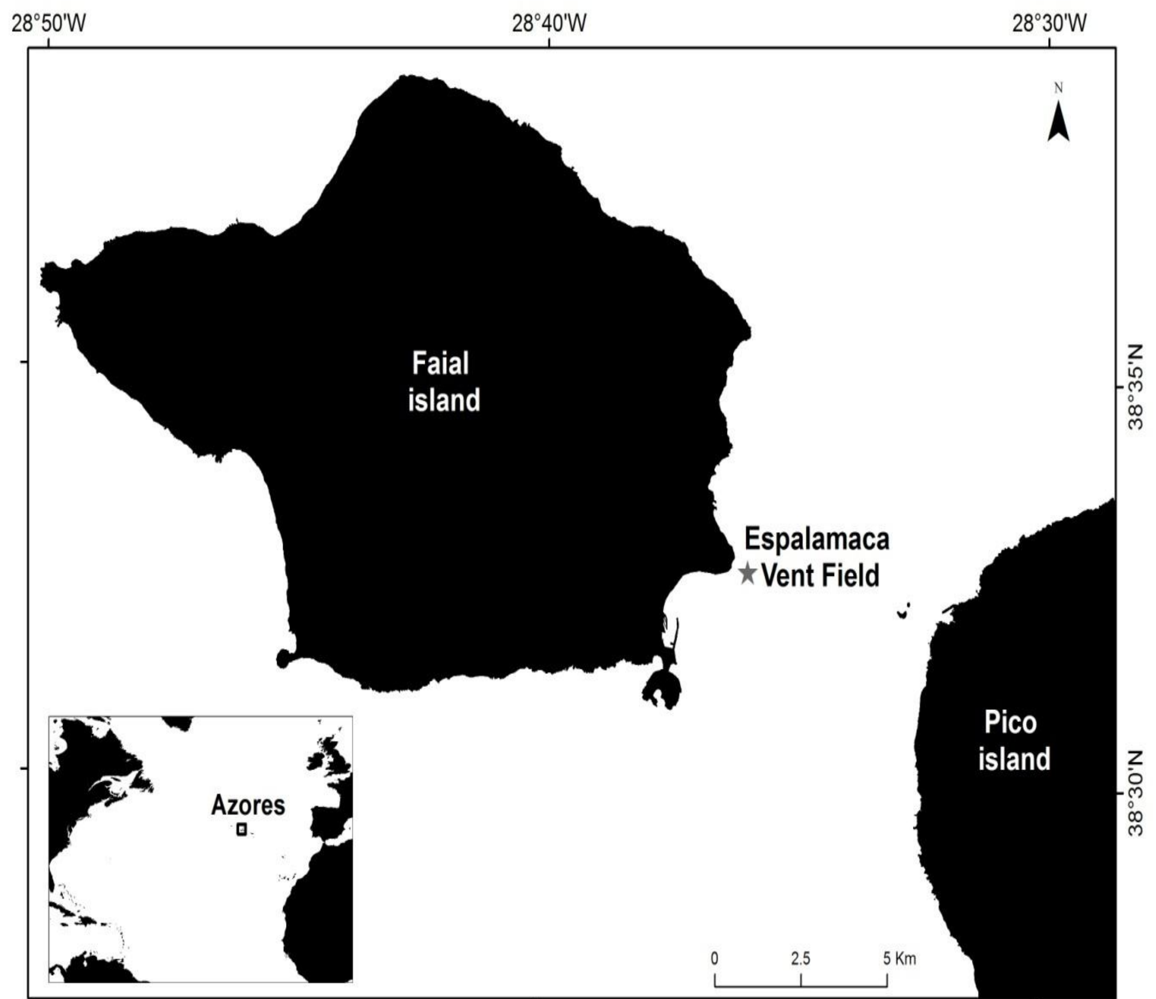


Figure 2.1. Sampling site of the shallow-water hydrothermal vent of Espalamaca in the Azores Islands, Portugal. The asterisk indicates the sampling site, located between Faial and Pico Islands.

2.2.2. Sampling and analysis

Surface, bottom water and sediment samples were collected from venting and non-venting areas at Espalamaca. Sterile polycarbonate tubes were used to collect sediment samples and Niskin samplers were used to collect water samples. Samples were immediately brought to the laboratory, University of Azores and maintained at 4 °C until analysis done. In the vent, the sediments samples were collected from two regions; one from the bubbling area with crevice (VSD) and the other from the bubbling area without crevice (VSG) (Figure 2.2). Samples were collected by scuba diving during October 2010 under Indo Portugal bilateral program and in August 2012 the samples were collected by the Portuguese counterpart and sent to India for analysis. All the samples were transported with ice packs and the analyses were carried out at University of Azores, Portugal and CSIR-National Institute of Oceanography, Goa, India.

2.2.3. Enumeration and isolation of culturable heterotrophic bacteria

One hundred micro litres of serially diluted water and sediment samples were spread plated on the nutrient agar (M001, Himedia) prepared in 50 % sea water (SWNA). pH of the medium was maintained at 5.7 for vent bottom water and sediment samples. Surface water of the vent and all the non vent samples were maintained at a pH of 8.2. All the plates were incubated at 30 ± 2 °C up to 72 h and final counts of colonies were made. Morphologically different bacterial isolates were quadrant streaked several times to obtain pure cultures.

2.2.4. Enumeration and isolation of metals and element tolerant bacteria

To enumerate the bacteria tolerant to manganese and lead, quarter strength nutrient broth

Figure 2.2. Espalamaca hydrothermal vent site. Yellow arrow indicates the presence of crevice from which the bubbles are coming out (a) and white arrow indicated the bubbles coming out without any crevice (b).

prepared in 50 % seawater with 1.8 % agar amended with 1 mM $MnCl_2$ and 1 mM $Pb(NO_3)_2$ respectively were used. Heterotrophic iron bacteria were isolated using 1 mM $FeSO_4$ and 0.02 % (w/v) yeast extract prepared in 50 % seawater (modified slightly from Johnson et al. 2009). Bacteria tolerant to sulphur were isolated using sodium thiosulfate ($Na_2S_2O_3$), i.e. yeast extract 2.0 g, bacteriological agar 18 g, sodium thiosulfate 5.0 g (Pandey et al. 2009) prepared in 50 % sea water. One hundred micro litres of serially diluted seawater and sediment samples were spread plated on the above media and the plates were incubated in dark at 30 ± 2 °C up to 72 h and the colonies were counted. Morphologically different bacterial isolates from each media were quadrant streaked several times on the respective media to obtain pure cultures. Pure cultures obtained were stored at 4 °C for short time and at -80 °C with 30 % glycerol for long term storage.

Methanotrophic populations were also assessed using nitrate mineral salts media (NMS) as described by Whittenbury et al. (1970). The NMS medium composed of separately autoclaved group A (g L^{-1}: $CaCl_2.2H_2O$ – 0.554, KNO_3 – 0.25, $FeSO_4.7H_2O$ – 0.1, $CuSO_4.5H_2O$ – 0.05, $ZnSO_4.7H_2O$ – 0.05, $MnCl_2.4H_2O$ – 0.05, $CoCl_2.6H_2O$ – 0.05, $Na_2MoO_4.2H_2O$ – 0.5 in 500 mL seawater) and group B (g L^{-1}: $Na_2HPO_4.2H_2O$ – 0.3, KH_2PO_4 – 0.002 in 500 mL distilled water) aseptically mixed together and plated. Serially diluted seawater and sediment samples (100 µL) were spread plated on NMS medium. The plates were incubated under air/CH_4 condition over a period of 7 days in dark. Methanotrophic colonies were counted after the incubation period.

Colonies which appeared on various isolation media were characterised based on their size, shape, colour, elevation and texture etc., were assigned with respective strain numbers and stored at 4 °C for further analysis.

2.2.5. 16S rRNA gene sequencing and phylogenetic analysis

Bacterial cultures stored at 4 °C were taken out and grown overnight on the above mentioned (Section 2.2.3 and 2.2.4) respective liquid media. Cells were centrifuged at 8000 rpm for 10 min and Genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. For 16S rRNA gene amplification, eubacterial primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') were used (Lane 1991). PCR amplification was performed in 50 µL reaction volume containing 5 µL of 10X reaction buffer, 5 µL of 15 mM $MgCl_2$, 4 µL of 2.5 mM dNTP, 2 µL of each primer (10 pmol $µL^{-1}$), 1 µL of template (25–50 ng), and 0.5 µL of Taq DNA polymerase (5 U $µL^{-1}$, Genei) and made up with sterile double-distilled H_2O. PCR profile consisted of initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 60 s, 53 °C for 60 s, 72 °C for 90 s and a final extension of 7 min at 72 °C.

The presence of genomic DNA and PCR products was confirmed with 1.0 % agarose gel electrophoresis in TAE buffer (1.0X Tris Acetate EDTA buffer). Briefly, 2-5 µL of DNA samples were mixed with 6X loading buffer in the ratio of 1:5 and loaded into the 1.0 % agarose gel. The electrophoresis was programmed at 80 V for 50 min and the DNA fragments were visualized under a UV transilluminator (Advanced American Biotechnology, USA).

The PCR products were gel-purified using a Gel Extraction Kit or purified with PCR cleanup kit (Sigma) according to the manufacturer's instructions. The purified PCR products were sequenced on automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) with the bacterial primers 27F, 518F and 1492R. The sequences thus obtained were combined to get nucleotide sequences of 16S rRNA gene

using DNAbaser software (version 3.5.3). The PINTAIL 1.0 program (Ashelford et al. 2005) was used for chimera checking and no differences were detected from our sequences. The acquired nearly-complete sequences were subjected to BLASTn on the National Center for Biotechnology Information (NCBI) and EzTaxon 2.1 server (Kim et al. 2012a) to identify the sequences with the highest similarity. 16S rDNA sequence similarity levels of ≥99 % were considered as a same species, whereas phylotypes clustered in a particular genus with <99 % sequence similarity was considered as potential novel species. Multiple and pairwise sequence alignment were performed using Clustal X (Thompson et al. 1997). Neighbour-joining (Saitou and Nei 1987) algorithm was used to reconstruct phylogenetic trees using MEGA 5 (Tamura et al. 2011). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1,000 replications.

2.2.6. Accession numbers for bacterial 16S rRNA gene sequences

The sequences obtained from this study were submitted to GenBank with accession numbers from KC534142 to KC534459.

2.2.7. Statistical analysis

Rarefaction analysis was performed by plotting the number of phylotypes/ OTUs observed against the total number of isolates using EcoSim700 (Gotelli and Entsminger 2004) to estimate the representation of phylotypes. Good's coverage of bacterial isolates was calculated using the formula $C = [(1-(n1/N)]*100$ where C is the homologous coverage, n1 is the number of OTUs appearing only once, and N is the total number of isolates observed. Shannon and Chao I indices were calculated using online program (http://fastgroup.sdsu.edu/cal_tools.htm).

2.3. Results

2.3.1. Bacterial retrievability

Total heterotrophic bacterial populations which appeared on SWNA were one order higher in venting sites when compared to the nonvent area (Table 2.1). The Mn amended media help to retrieve double the counts of heterotrophic bacteria from the nonvent water samples (5.55×10^4 and 2.38×10^4 CFU mL^{-1}). Media with Pb were almost equal to SWNA (2.38×10^4 CFU mL^{-1}) from nonvent surface water (2.45×10^4 CFU mL^{-1}). In the vents especially in bottom waters, the Mn added media resulted low counts compared to SWNA. The ratio of retrieval rates in Mn and Pb amended media to SWNA exhibited 13.38 % and 11.54 % respectively. Whereas the Fe amended media in the VSG sediment showed much higher population than the heterotrophic counts. The ratio of Fe media to SWNA in VSG was 346.1 %, supposed to be the highest bacterial retrievability (5.33×10^5 CFU g^{-1}) than any other metals/elements implemented. In case of thiosulfate, the ratio of Ts amended media to SWNA was found to be 30–60 % in all the samples. Methanotrophs were retrieved one order less when compared to other groups except in vent sediments (10^5 CFU g^{-1}) (Table 2.1).

Addition of metals in the isolation media will attract the metal tolerant bacteria since the hydrothermal vent regions are rich with various metals and elements. At the same time growth of the bacteria should not be hampered by low or high concentrations of metals used in the medium. Keeping the above things in mind, we have chosen 1 mM concentration for two reasons. One is based on earlier literature. Studies conducted by Fernandes et al. (2005) on Mn oxidizing bacteria reveals that 1 mM concentrations could retrieve maximum number of organisms and increasing beyond leads to lesser retrieval

rates. Second to salvage potential metal tolerant bacteria, this could be used further for removal of heavy metals.

Table 2.1. Abundance of culturable bacteria on various isolation media from shallow water hydrothermal vent and non-vent site at Espalamaca

	Vent site				Non-vent site		
Media	Surface water*	Bottom water*	Crevice sediment§	Non-crevice sediment§	Surface water*	Bottom water*	South sediment§
SWNA	1.22×10^5	7.62×10^4	8.27×10^5	1.54×10^5	2.38×10^4	2.65×10^4	3.70×10^4
dSWNA+Mn	9.00×10^4	1.02×10^4	4.73×10^5	1.15×10^5	5.55×10^4	4.80×10^4	3.06×10^4
dSWNA+Pb	8.25×10^4	8.80×10^3	5.70×10^5	7.43×10^4	2.45×10^4	1.85×10^4	1.00×10^4
dSWNA+Ts	3.65×10^4	2.40×10^4	3.70×10^5	6.00×10^4	1.30×10^4	8.50×10^3	2.20×10^4
dSWYE+Fe	ND	ND	2.71×10^5	5.33×10^5	ND	ND	2.70×10^4
NMS (CH_4)	6.70×10^3	1.22×10^3	1.97×10^5	1.74×10^5	5.10×10^3	2.40×10^3	7.03×10^3
			Proportions to SWNA (%)				
dSWNA+Mn	73.77	13.38	57.19	74.67	233.1	181.1	82.07
dSWNA+Pb	67.62	11.54	68.92	48.24	102.9	69.81	27.02
dSWNA+Ts	29.91	31.49	44.74	38.96	54.62	32.07	59.45
dSWYE+Fe	ND	ND	32.76	346.1	ND	ND	72.97

SWNA – Nutrient agar in 50 % seawater; dSWNA+Mn –25 % nutrient agar in 50 % seawater with 1 mM $MnCl_2$; dSWNA+Pb –25 % nutrient agar in 50 % seawater with 1 mM $Pb(NO_3)_2$; dSWNA+Ts – 25 % nutrient agar in 50 % seawater with 0.5 % $Na_2S_2O_3$; dSWYE+Fe – 0.02 % yeast extract in 50 % seawater with 1 mM $FeSO_4$; NMS (CH_4) – nitrate mineral salts media for methanotrophs; ND – Not detectable; *CFU mL^{-1}; §CFU g^{-1}

Based on morphological characteristics, a total of 318 bacterial colonies which appeared on various isolation media mentioned above were selected for 16S rRNA gene sequencing analysis. The analysis results indicated a total of 113 phylotypes from this study and their colony characteristics were given in Table 2.2.

2.3.2. 16S rRNA gene based diversity

Highly diversified bacterial phylotypes spanned nearly 30 families and six phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, α-*Proteobacteria*, β-*Proteobacteria* and γ-*Proteobacteria*. γ-*Proteobacteria* dominated with 68.7 % (152/221) in the vent and 62.8 % (61/97) from the nonvent; α-*Proteobacteria*, 16.7 % (37/221) of the vent isolates and 29.9 % (29/97) of the nonvent; *Firmicutes* 3.2 % (7/221) of the vent isolates and 4.1 % (4/97) of nonvent; β-*Proteobacteria* 0.45 % (1/221) of the vent and 3.1 % (3/97) of the nonvent. *Bacteroidetes* (10 %) and *Actinobacteria* (0.9 %) were only retrieved from vent samples. The details of the phylotypes and novel taxa obtained are given in Figure 2.3.

2.3.3. Diversity of vent bacteria

A total of 113 phylotypes were obtained from 318 sequences in which 95 phylotypes were from the vent. γ-*Proteobacteria* was found to be the dominant phyla with its members like *Alcanivorax*, *Amphritea*, *Halomonas*, *Marinobacter*, *Pseudoalteromonas*, *Vibrio*, etc., and covered 53 OTUs which belong to 16 genera. *Vibrio*, established with 13 different species (Fig. 2.4), was found to be the dominant genus and all the *Vibrio* species were retrieved from the vent sediments. Among the 53 OTUs, 10 were expected to be novel taxa since their identities with the type strain sequences were lower than 99 %. Further the phylogenetic relationship executed with neighbouring sequences expressed distinct variations which were clearly noticeable in the phylogenetic tree (Fig.

Table 2.2. Characteristics of the bacterial phylotypes isolated from Espalamaca

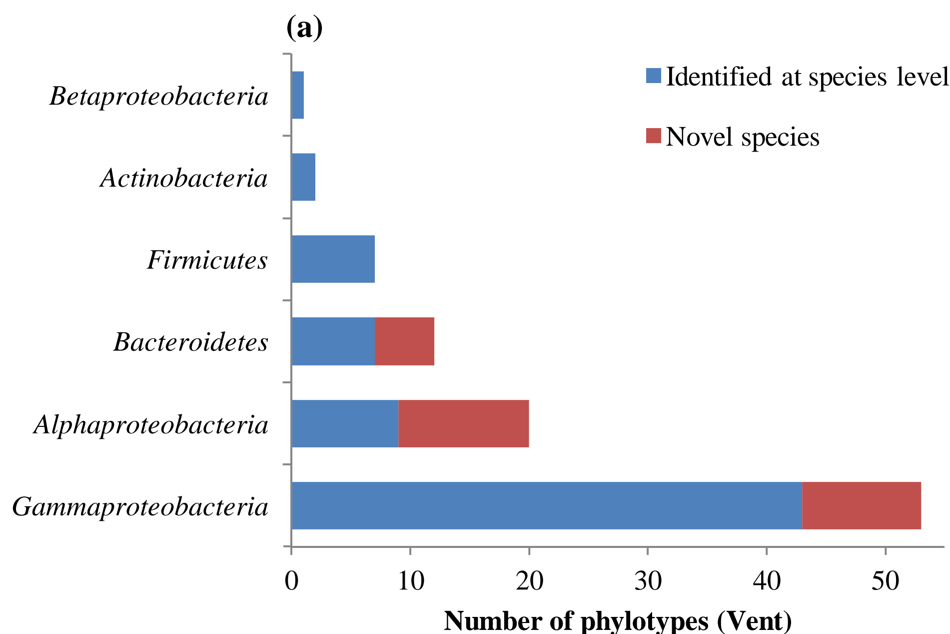
Strains	Gram Reaction	Size	Shape	Colour	Elevation	Margin	texture	Opacity
VBW004	-	6-8	Irregular	White	Umbonate	Entire	Smooth	Transparent
SSW083	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VBW095	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque
VBW098	-	1	Circular	Pink	Raised	Entire	Smooth	Opaque
VSW114	-	0.5	Circular	Pale yellow	Raised	Entire	Rough	Opaque
VBW122	-	0.5	Circular	White	Raised	Entire	Smooth	Opaque
VBW206	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSW210	-	1	Circular	White	Convex	Entire	Smooth	Iridescent
SBW235b	-	1.5	Circular	Blackish	Raised	Entire	Smooth	Opaque
VSW306	-	0.5	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSW310	-	0.5	Circular	White	Raised	Entire	Smooth	Translucent
VSG724	-	2-4	Irregular	White	Flat	Entire	Watery	Transparent
VSG829	-	1.5	Circular	White	Flat	Entire	Watery	Transparent
VBW240	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSD707	-	0.5	Circular	Pale yellow	Raised	Entire	Smooth	Translucent
VBW088	-	Pinpoint	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSG820	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSG922	-	1.5	Circular	White	Raised	Entire	Rough	Opaque
VSW331	-	1	Circular	White	Convex	Entire	Smooth	Opaque
VBW339	-	Pinpoint	Circular	White	Flat	Entire	Smooth	Translucent
VBW123	-	0.5-1	Circular	White	Raised	Wavy	Smooth	Opaque
SSW136	-	0.5	Circular	White	Convex	Entire	Smooth	Opaque
SSW234	-	2	Circular	Brownish	Raised	Entire	Smooth	Opaque
SSW084	-	Pinpoint	Circular	White	Convex	Entire	Smooth	Translucent
VSW109	-	Pinpoint	Circular	Pale yellow	Raised	Entire	Smooth	Iridescent
SSW321	-	Pinpoint	Circular	Pale yellow	Raised	Entire	Smooth	Opaque
VBW011	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSG534	-	4-5	Irregular	White	Raised	Entire	Smooth	Opaque
VSD616	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSG528	-	4	Circular	White	Raised	Entire	Smooth	Opaque
VSG826	-	3	Irregular	White	Raised	Wavy	Smooth	Opaque
SSA928	-	2	Circular	White	Flat	Entire	Rough	Opaque
VSW332	-	2	Circular	White	Raised	Entire	Smooth	Iridescent
VSG927	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque

VSG924	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSD909	-	1	Circular	White	Crateriform	Wavy	Smooth	Opaque
VSG533	-	3	Circular	White	Raised	Wavy	Smooth	Opaque
VSD915	-	2	Circular	White	Umbonate	Entire	Smooth	Opaque
VSD914	-	2	Circular	White	Umbonate	Entire	Smooth	Opaque
VBW118	-	1.5	Circular	White	Umbonate	Wavy	Smooth	Opaque
VSG530	-	4	Circular	White	Raised	Entire	Smooth	Opaque
VSG523	-	4	Circular	White	Raised	Entire	Smooth	Translucent
VSD910	-	0.5	Circular	White	Raised	Entire	Smooth	Opaque
VSD905	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSD904	-	0.5-1	Irregular	White	Flat	Wavy	Watery	Translucent
VSG521	-	5	Circular	White	Raised	Entire	Smooth	Opaque
VSD902	-	2	Circular	White	Raised	Entire	Watery	Opaque
VSD502	-	2	Circular	White	Raised	Entire	Smooth	Opaque*
VSD817	-	20	Circular	White	Raised	Entire	Smooth	Translucent
VSD513	-	4-6	Irregular	White	Raised	Entire	Smooth	Opaque
VSD715	-		Filamentous	White	Raised	Wavy	Smooth	Opaque
VSD706	-		Filamentous	White	Raised	Wavy	Smooth	Opaque
VSD901	-	1-1.5	Circular	White	Raised	Entire	Smooth	Iridescent
SSW087	-	3-5	Circular	White	Umbonate	Entire	Smooth	Translucent
VSG538	-	1	Circular	White	Raised	Entire	Watery	Translucent
VSG718	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSG522	-	4	Circular	White	Raised	Entire	Smooth	Opaque
VBW335	-	2	Circular	White	Raised	Entire	Smooth	Opaque
VSW333	-	1	Circular	White	Raised	Entire	Smooth	Opaque
SSA636	-	4	Circular	White	Raised	Entire	Smooth	Opaque
VSW334	-	1.5	Circular	White	Raised	Entire	Watery	Transparent
VSW111	-	1-3	Irregular	White	Flat	Entire	Smooth	Iridescent
VSW029	-	1.5	Circular	White	Raised	Entire	Smooth	Opaque
VSD709	-	1	Circular	White	Raised	Entire	Smooth	Opaque*
VSW108	-	1	Circular	White	Raised	Entire	Smooth	Translucent
VSG622	-	3	Circular	White	Raised	Entire	Smooth	Transparent
VSG620	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSW212	-	Pinpoint	Circular	Yellow	Raised	Entire	Smooth	Transparent
VSG558	-	1	Circular	White	Raised	Entire	Watery	Transparent
VSD557	-	2	Circular	White	Raised	Entire	Smooth	Translucent
VSD511	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSG525	-	2	Circular	White	Raised	Entire	Smooth	Opaque
VSW026	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSD503	-	3	Circular	White	Raised	Entire	Smooth	Opaque
VSD505	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSD501	-	1.5	Circular	White	Raised	Entire	Smooth	Opaque

SSA547	-	4	Circular	Brownish	Raised	Entire	Smooth	Opaque
SBW227	-	5	Circular	White	Umbonate	Wavy	Smooth	Transparent
SBW226	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Transparent
SSA729	-	3.5	Circular	White	Raised	Entire	Smooth	Opaque
SSA934	-	4-5	Irregular	White	Raised	Entire	Smooth	Opaque
SSA835	-	1.5	Circular	White	Raised	Entire	Smooth	Translucent
SSA728	-	1	Circular	White	Raised	Entire	Smooth	Opaque
SSW096	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSW102	-	0.5	Circular	Yellow	Raised	Entire	Smooth	Opaque
VBW303	-	1.5	Circular	White	Raised	Entire	Smooth	Opaque
VSD907	-	0.5	Circular	White	Raised	Entire	Watery	Translucent
VBW126	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque
VBW014	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSW307	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSW103	-	1	Circular	White	Raised	Entire	Smooth	Opaque
VSW062	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSW105a	-	1	Circular	Brownish	Raised	Entire	Smooth	Opaque
VBW206	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
SSW320	-	0.5	Circular	White	Raised	Entire	Smooth	Opaque
SSW231	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VSW306	-	0.5	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSG725	-	1	Circular	Yellow	Raised	Entire	Rough	Opaque
VBW129	-	1-2	Irregular	White	Umbonate	Wavy	Smooth	Opaque
VSW214	-	Pinpoint	Circular	White	Flat	Entire	Smooth	Transparent
VSW215	-	0.5	Circular	Yellow	Flat	Entire	Smooth	Opaque
VSW209	-	Pinpoint	Circular	White	Raised	Entire	Smooth	Opaque
VBW204	-	1.5	Circular	Yellow	Raised	Entire	Smooth	Opaque
VSD814	-	3	Circular	White	Raised	Entire	Smooth	Opaque
VSG821	-	1	Circular	Yellow	Raised	Entire	Smooth	Opaque
VBW147	+	1	Circular	White	Raised	Entire	Smooth	Iridescent
VSG726	+	1	Circular	Blackish	Raised	Entire	Smooth	Opaque
VSD609	+	5	Circular	Cream	Raised	Entire	Smooth	Opaque
VSD607	+	4	Circular	White	Raised	Entire	Smooth	Opaque
VBW023	+	3	Irregular	White	Umbonate	Entire	Smooth	Transparent
SSA550	+	5	Circular	Cream	Raised	Entire	Smooth	Opaque
VBW089	-	1	Circular	Cream	Raised	Entire	Smooth	Opaque
VBW127	+	Pinpoint	Circular	Yellow	Raised	Entire	Smooth	Opaque
VBW090	+	1.5	Circular	Yellowish	Raised	Entire	Smooth	Opaque

-, Negative; +, Positive; *, Luminescent

2.5a). This may be confirmed with polyphasic taxonomic approaches. The second major phylum of this study was found to be α-*Proteobacteria* which was covering 20 OTUs belonging to 12 genera. The major contributors were *Erythrobacter*, *Hyphomonas*, *Sulfitobacter* and *Thalassospira* each with 3 species. Further, 55 % of the α-*Proteobacteria* obtained from venting area was found to be novel species and their evolutionary relationships with their closest matches are represented in Figure 2.5b. The phyla *Bacteroidetes* contributed 12 phylotypes; *Aequorivita*, *Arenibacter* and *Maribacter* contributed 2 species each and the remaining genera were found to contain only one each. An interesting result of this investigation, *Bacteroidetes* group was only retrieved from the venting site. In addition to this, around 42 % of *Bacteroidetes* comprised of potential novel species and their phylogenetic relationships with closely related taxa are presented in Figure 2.5c. The 16S rRNA genes from *Actinobacteria*, *Firmicutes* and β-*Proteobacteria* were less prominent sequences observed in the venting area with 7, 1 and 2 OTUs respectively.



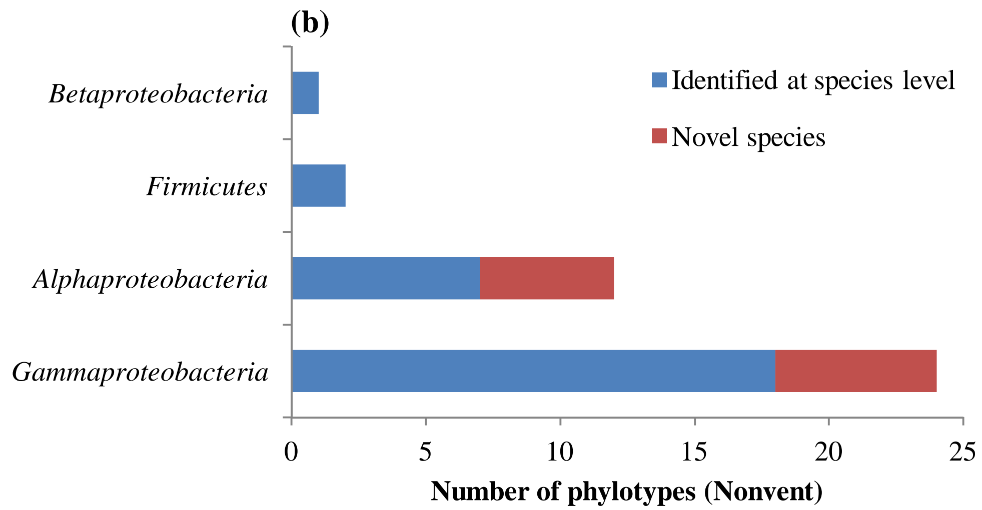


Figure 2.3. Distribution of bacterial Phylotypes (given in numbers) over various phyla from (a) vent and (b) nonvent samples. The set labelled with 'identified at species level' contains phylotypes with ≥99 % 16S rRNA sequence similarity. The set 'novel species' represents phylotypes that belong to novel species considering the threshold level of ≤99 % 16S rRNA gene sequence similarity.

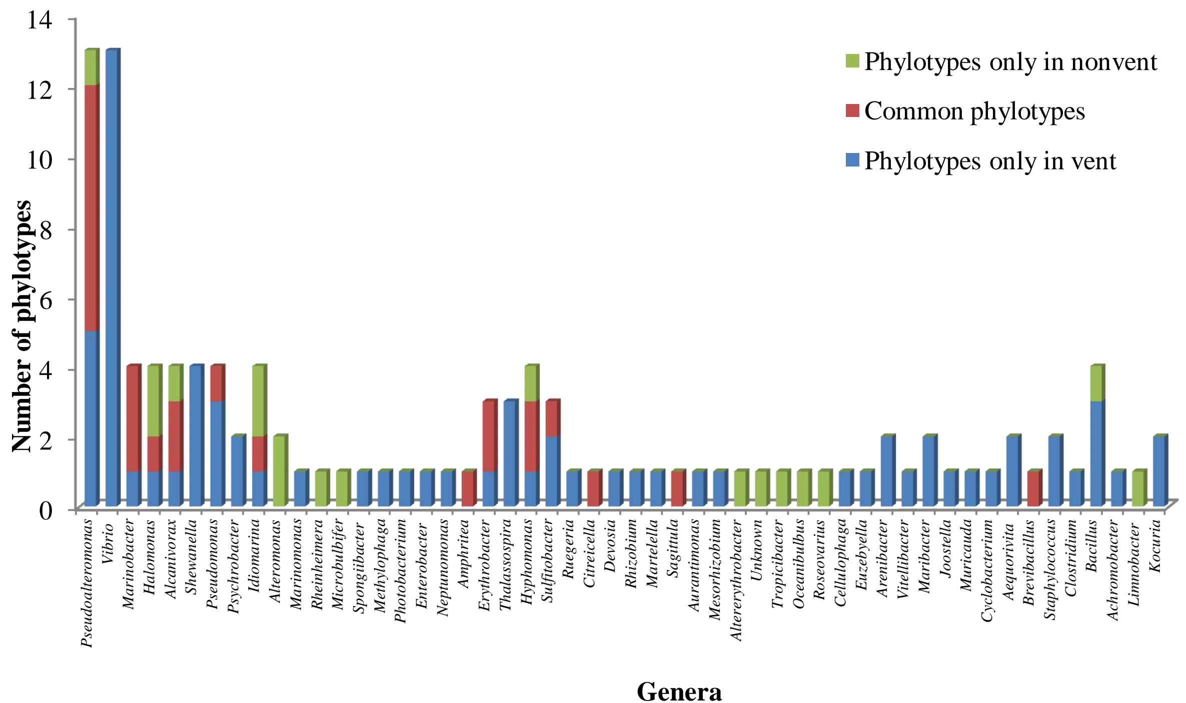
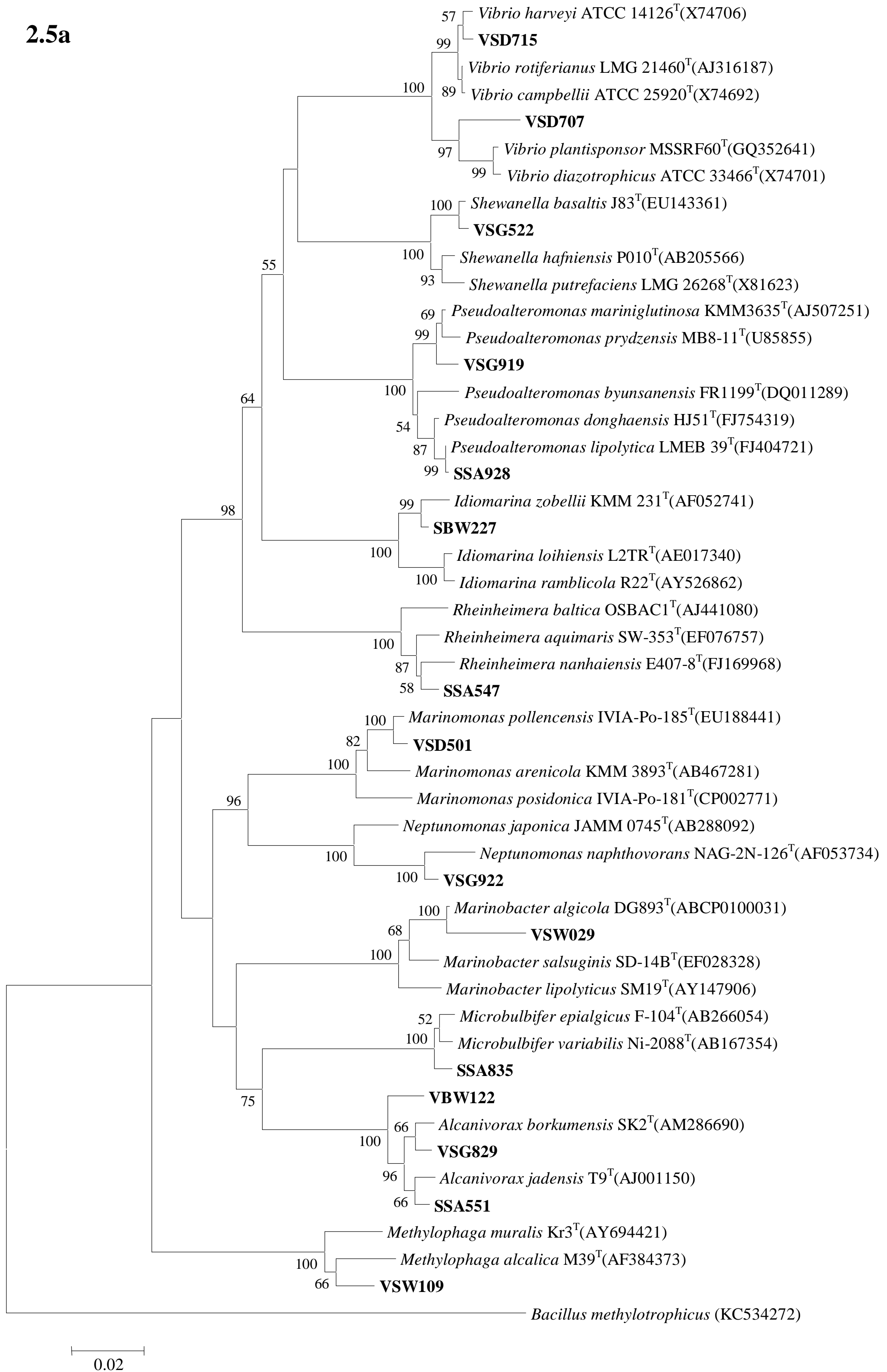
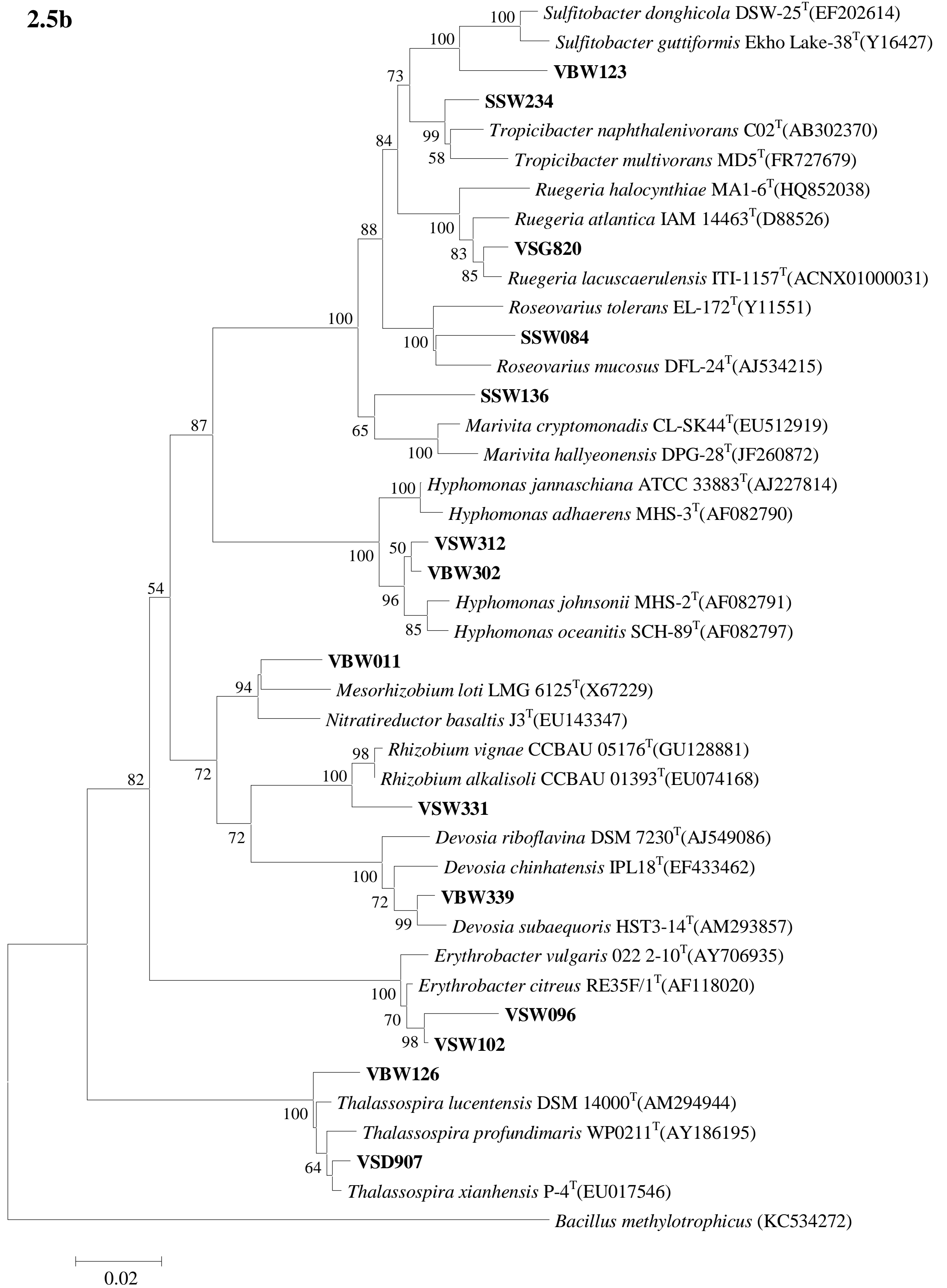
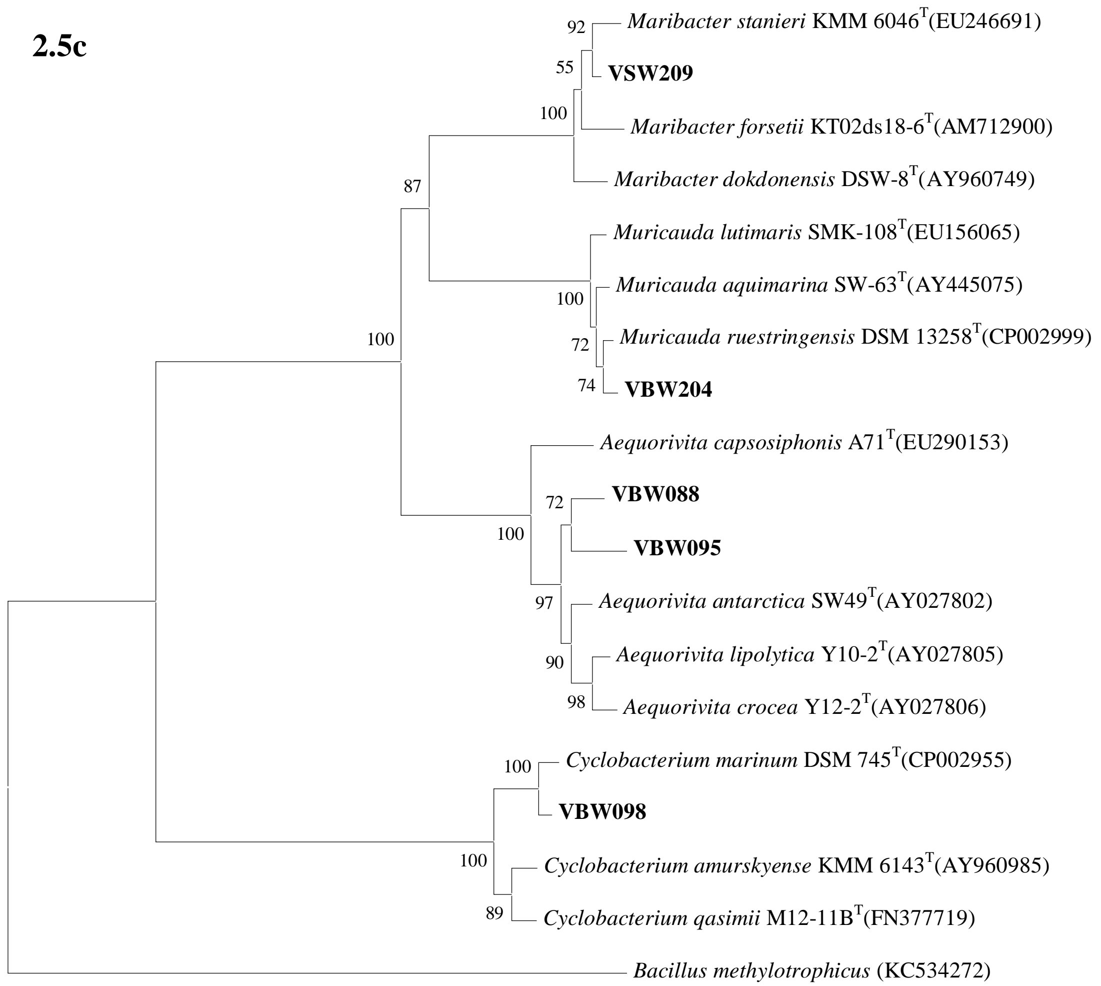


Figure 2.4. Number of vent, nonvent and common phylotypes retrieved from each genus

2.5a




2.5b
Sulfitobacter donghicola DSW-25T(EF202614)
Sulfitobacter guttiformis Ekho Lake-38T(Y16427)
VBW123
SSW234
Tropicibacter naphthalenivorans C02T(AB302370)
Tropicibacter multivorans MD5T(FR727679)
Ruegeria halocynthiae MA1-6T(HQ852038)
Ruegeria atlantica IAM 14463T(D88526)
VSG820
Ruegeria lacuscaerulensis ITI-1157T(ACNX01000031)
Roseovarius tolerans EL-172T(Y11551)
SSW084
Roseovarius mucosus DFL-24T(AJ534215)
SSW136
Marivita cryptomonadis CL-SK44T(EU512919)
Marivita hallyeonensis DPG-28T(JF260872)
Hyphomonas jannaschiana ATCC 33883T(AJ227814)
Hyphomonas adhaerens MHS-3T(AF082790)
VSW312
VBW302
Hyphomonas johnsonii MHS-2T(AF082791)
Hyphomonas oceanitis SCH-89T(AF082797)
VBW011
Mesorhizobium loti LMG 6125T(X67229)
Nitratireductor basaltis J3T(EU143347)
Rhizobium vignae CCBAU 05176T(GU128881)
Rhizobium alkalisoli CCBAU 01393T(EU074168)
VSW331
Devosia riboflavina DSM 7230T(AJ549086)
Devosia chinhatensis IPL18T(EF433462)
VBW339
Devosia subaequoris HST3-14T(AM293857)
Erythrobacter vulgaris 022 2-10T(AY706935)
Erythrobacter citreus RE35F/1T(AF118020)
VSW096
VSW102
VBW126
Thalassospira lucentensis DSM 14000T(AM294944)
Thalassospira profundimaris WP0211T(AY186195)
VSD907
Thalassospira xianhensis P-4T(EU017546)
Bacillus methylotrophicus (KC534272)
0.02


2.5c
Maribacter stanieri KMM 6046^{T}(EU246691)
VSW209
Maribacter forsetii KT02ds18-6^{T}(AM712900)
Maribacter dokdonensis DSW-8^{T}(AY960749)
Muricauda lutimaris SMK-108^{T}(EU156065)
Muricauda aquimarina SW-63^{T}(AY445075)
Muricauda ruestringensis DSM 13258^{T}(CP002999)
VBW204
Aequorivita capsosiphonis A71^{T}(EU290153)
VBW088
VBW095
Aequorivita antarctica SW49^{T}(AY027802)
Aequorivita lipolytica Y10-2^{T}(AY027805)
Aequorivita crocea Y12-2^{T}(AY027806)
Cyclobacterium marinum DSM 745^{T}(CP002955)
VBW098
Cyclobacterium amurskyense KMM 6143^{T}(AY960985)
Cyclobacterium qasimii M12-11B^{T}(FN377719)
Bacillus methylotrophicus (KC534272)
92
55
100
87
100
72
74
100
72
100
97
90
98
100
100
89
0.05

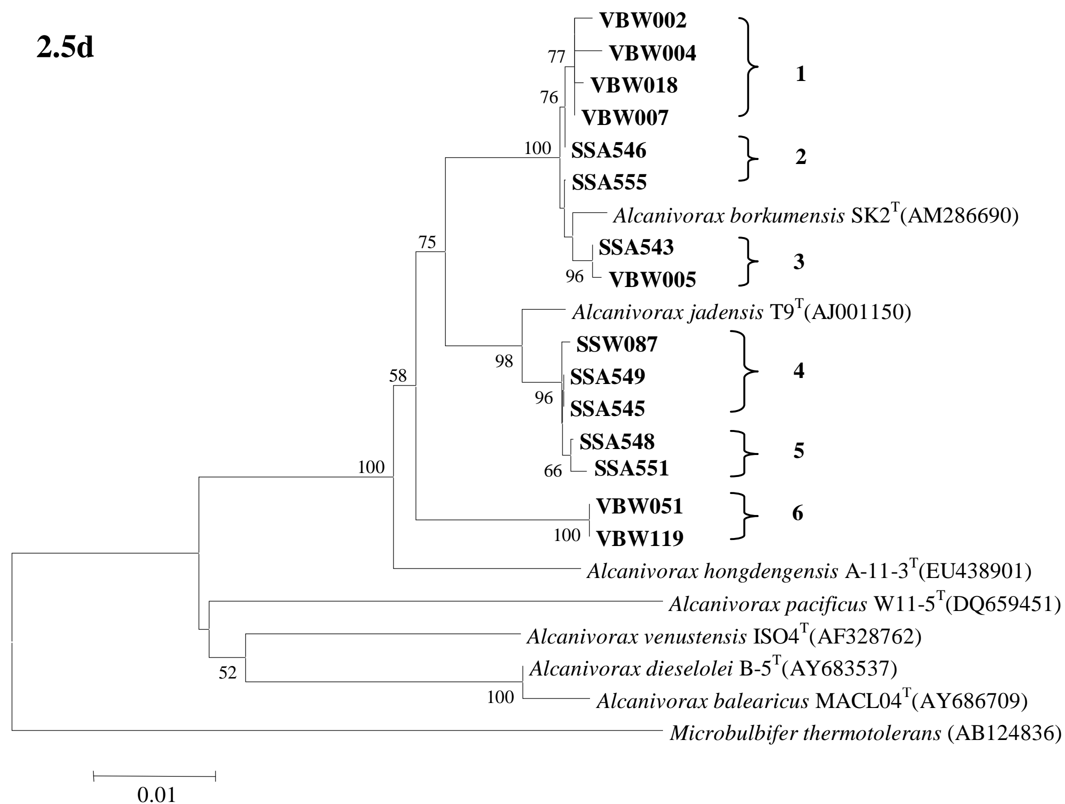


Figure 2.5. Phylogenetic relationships of representative novel taxa obtained from vent and nonvent regions of Espalamaca based on Neighbor-Joining analysis of 16S rRNA gene sequences data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap percentages of >50 % are shown on the nodes. The evolutionary distances were computed using the Maximum Composite Likelihood method. *Bacillus methylotrophicus* (KC534272) was used to assign an out-group for figure a, b & c. *Microbulbifer thermotolerans* (AB124836) was used as out-group for figure d.

(a) Phylogenetic relationships of novel species belong to γ-*Proteobacteria*;

(b) Phylogenetic relationships of novel species belong to α-*Proteobacteria*;

(c) Phylogenetic relationships of novel species belong to *Bacteroidetes*;

(d) Phylogenetic variations of *Alcanivorax* isolates. The numbers from 1 – 6 indicates, 6 possible groups of novel taxa belong to the genus *Alcanivorax*

2.3.4. Diversity of nonvent bacteria

Phylogenetic analysis of nonvent bacteria revealed that the 16S rRNA gene clusters differ greatly compared to the nearby shallow vents. A total of 97 bacterial colonies tested from the nonvent area resulted in 39 phylotypes affiliated to 4 phyla, α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria*, and *Firmicutes*. As in the venting area, γ-*Proteobacteria* was found to be the dominant phyla in the nonventing area. It contributed 24 phylotypes belonging to 10 genera. *Pseudoalteromonas* was found to the dominant genus containing 7 bacterial species, followed by *Marinobacter* with 3 species (Fig. 2.4). Around 25 % of the nonvent γ-*Proteobacteria* belongs to novel taxa. The second most abundant phylogenetic class in the nonvent bacteria was found to be α-*Proteobacteria* with 12 phylotypes belonging to 10 genera. As in the venting area *Hyphomonas* dominated (3 species) followed by *Erythrobacter* (2 species). Nearly 42 % of α-*Proteobacteria* taxa were accounted to be novel including 1 potential new genus. On the other hand, only 2 clusters appeared in *Firmicutes* affiliated to *Bacillus* and *Brevibacillus* whereas the phylum β-*Proteobacteria* contains only one cluster affiliated to *Limnobacter* sp.

2.3.5. Comparative analysis of vent and nonvent bacterial diversity

Overall 221 bacterial colonies from vent samples and 97 bacterial colonies from nonvent samples were selected for the culturable diversity analysis. The numbers of vent bacterial isolates were nearly double compared to nonvent, this was due to an extra sediment sample from the venting site (details are given in materials and methods section) and another reason is comparatively more distinct colonies appeared in the vent than in the non vent. Gene sequence results indicated 113 OTUs (95 OTUs from venting area and 39 OTUs from nonventing area). Though the number of OTUs varied, the rarefaction curve

clearly indicated that the venting area was richer with more number of species than the nonvent. There were 21 phylotypes belonging to 13 genera observed to be common in both the areas i.e. *Alcanivorax*, *Amphritea*, *Brevibacillus*, *Citreicella*, *Erythrobacter*, *Halomonas*, *Hyphomonas*, *Marinobacter*, *Pseudidiomarina*, *Pseudoalteromonas*, *Pseudomonas*, *Sagittula* and *Sulfitobacter*. *Pseudoalteromonas* and *Vibrio* detected to be major genera in this investigation. All Vibrios exclusively came from the vent samples whereas *Pseudoalteromonas* was retrieved in both the areas. Even though γ-*Proteobacteria* followed by α-*Proteobacteria* were supposed to be the dominant phylogenetic classes in the analyzed area, *Bacteroidetes* also contributed significantly in the venting area with 12 different phylotypes. *Actinobacteria* was the least prominent phylum observed from the vent samples with 2 species. In addition to that, phyla *Firmicutes* and β-*Proteobacteria* were common in both the areas.

A total of 33 novel taxa were recovered during this investigation. This number may slightly increase when we perform DNA-DNA hybridization for a few other strains although their 16S rRNA gene sequence similarity was found to be ≥99 %. For example, we came across 3 phylotypes of novel *Alcanivorax*; however, when we constructed a phylogenetic tree for the *Alcanivorax* isolates we were able to get 6 different clusters (Fig. 2.5d).

2.3.6. Statistical analysis

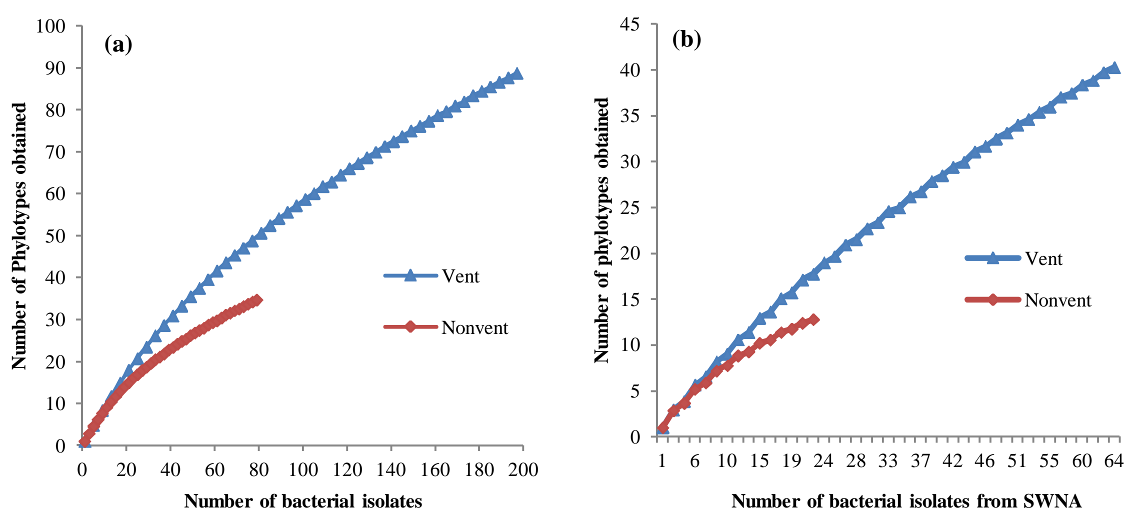
The Shannon index (H′) for venting area was observed to be higher (4.183) than the nonvent ones (3.305). At the same time Chao I richness estimator gave a stronger richness in the venting area (233.61) than in the nonvent (161.9). Coverage values were almost equal in both the sampling areas (74.66 in vent and 77.31 in nonvent) (Table 2.3). These results strongly indicated that the venting area is diversified with a variety of

bacteria while in the nonvent area even though it is located very close to the vent, the bacterial diversity was comparatively low.

Table 2.3. Distribution of bacteria, number of phylotypes obtained, their diversity indices and coverage of OTUs from vent and nonvent

Sampling area	No. of bacterial isolates	No. of Phylotypes	Shannon index (H')	Good's coverage (C)	Chao I
Vent	221	95	4.183	74.66	233.61
Nonvent	97	39	3.305	77.31	161.40

Rarefaction analysis conducted with various phylotypes retrieved from the SWNA and metal amended media revealed high richness in bacterial species from the vent. Only in thiosulfate media the species richness in nonvent were comparable with vent taxa whereas all other media yielded a higher number of phylotypes in the vent samples (Fig. 2.6). On the other hand low retrieval of bacterial isolates was observed in Fe amended medium. In fact the water samples did not give any bacterial colony when 1 mM Fe was added hence we did not study rarefaction analysis for Fe medium.



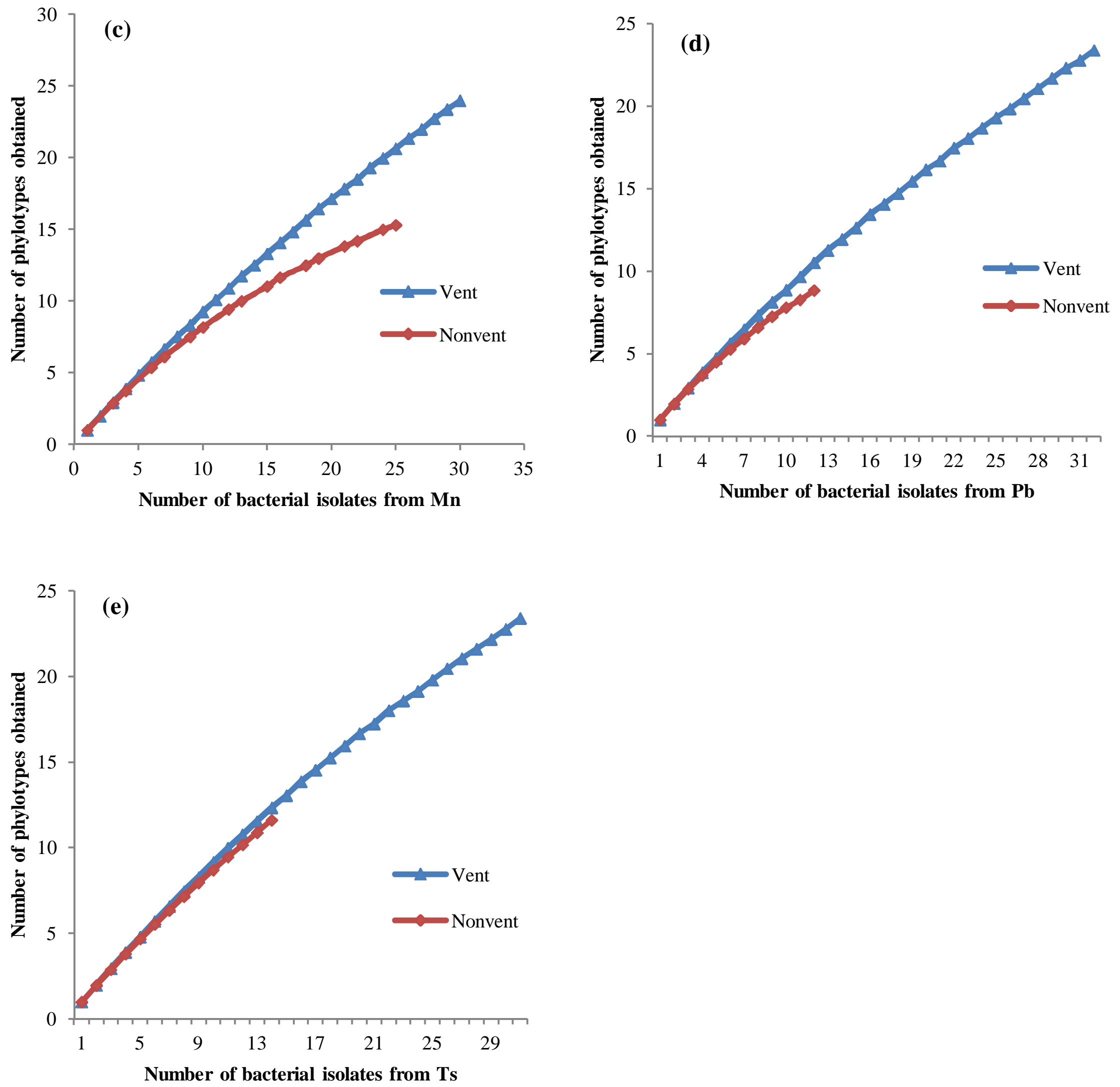


Fig. 2.6. Rarefaction curves of operational taxonomic units based on 16S rRNA gene. Bacterial isolates were grouped into OTUs based on sequence similarity of ≥99 %.

(a) Overall OTUs from vent and nonvent samples

(b) OTUs from the isolation media seawater nutrient agar

(c) OTUs from the isolation media seawater nutrient agar with 1 mM $MnCl_2$

(d) OTUs from the isolation media seawater nutrient agar with 1 mM $Pb(NO_3)_2$

(e) OTUs from the isolation media seawater nutrient agar with 0.5 % $Na_2S_2O_3$.

2.4. Discussion

Bacterial association with hydrothermal systems is generally described in one of 4 ways based on their habitat. First, microbes in the crevices below the surface; second, microbes on the outer surface of sulphide deposits; third, microbes associated with invertebrates and fourth, microbes within the plume of hydrothermal fluid in the overlying seawater. Except the first one, the remaining habitat microbes are generally mesophilic aerobic bacteria and some of them can oxidize Mn and Fe for their respiration (Kelly et al. 2002; Holden and Adams 2003). Hence the exploration of shallow water hydrothermal bacterial communities provides interesting insights on bacterial interactions with hydrothermal vent fluids and adaptations towards various elements.

The main drawback of using basic/normal nutrient media was that it could yield only a moderate level of bacterial diversity. In this investigation we have amended Mn, Fe, Pb and thiosulfate in the diluted nutrient media to overcome this limitation. Use of diluted nutrient media may give way to oligophilic bacteria (Watve et al. 2000). But, studies like this usually recommended with low nutrient and high supply of metals (substrates) so that the bacteria could attack the metals in a better way. Though some researchers have preferred mineral media amended with metals, researchers have also used the diluted nutrient media supplying with metals (Fernandes et al. 2005; Jaysankar et al. 2008) for good retrieval of metal tolerant bacteria. We have used the full strength nutrient media without metals to isolate vast groups of heterotrophic bacteria. The results were obviously interesting because we could retrieve 95 OTUs from vent and 39 OTUs from nonvent area. Innovative culturing methods like preparation of isolation media with the seawater recreates physical and chemical conditions found in the ocean very similarly to

the organically rich media (Suzuki et al. 1997). Different combination of metals in the regular media prepared in seawater really helped to get maximum colonies with very distinct morphology.

The mean values of heterotrophic bacterial numbers in water and sediment samples of Hydrothermal Vent off the Island of Vulcano (Eolian Islands, Italy), were 0.22×10^4 CFU mL^{-1} and 14×10^4 CFU g^{-1} respectively (Gugliandolo and Maugeri 1998) whereas in this study, we could retrieve 9.9×10^4 CFU mL^{-1} and 49×10^4 CFU g^{-1} respectively. This indicates that hydrothermal fluids of Espalamaca have a rich and diverse bacterial load. This higher bacterial abundance may be due to the Azorean vent fluids which have much higher trace elements and gas concentrations than the ambient Atlantic seawater (Colaco et al. 2006) to raise high bacterial numbers.

The 16S rRNA gene sequence data of the Espalamaca bacterial isolates were compared with the public database NCBI GenBank and those with a similarity level of ≥99 % sequence identity were assigned as same species. The standard value of DNA-DNA relatedness for defining a species is less than 70 % (Wayne et al. 1987) which was achievable with ≤97 % of 16S rRNA gene homology (Stackebrandt and Goebel 1994). Although most of our sequence similarity level was ≥97 %, some of the reports represented ≥97.5 % and ≥98 % of sequence identity to assign similar species (Hirayama et al. 2007; Moissl et al. 2007). However, recent studies authenticated that 16S rRNA sequence similarity level of ≥99 % can cover DNA-DNA hybridization values of ≥70 % and less than 99 % match was considered as a novel species (Peeters et al. 2011). Hence, we opted to use cut off level of 99 % for assigning novel taxa. More than half of the vent and nearly 41 % of nonvent α-*Proteobacteria* possessed potential novel taxa respectively. Though γ-*Proteobacteria* was found to be a major phylum comparatively it

yielded less number of novel taxa i.e. 19 % and 25 % from vent and nonvent respectively. *Bacteroidetes*, the phylum found to thrive only in the venting area (Espalamaca), also held 42 % of novel bacteria. Even though the 16S rRNA gene sequence similarity exceeds 99 % and the closest relatives are found to be very few, many literatures reported various novel taxa by doing DNA-DNA hybridization with the closest type strains (Dastager et al. 2011; Qin et al. 2012). From this point of view we have pooled 15 *Alcanivorax* isolates (from this study) to check their phylogenetic relationships with their neighbour species. All of them have close relations with only 2 type strains *A. borkumensis* and *A. jadensis*. From the phylogenetic tree it is evident that there are 6 different clusters (Fig. 2.5d) whereas only 3 phylotypes were observed to contain less than 99 % sequence similarity (Fig. 2.5a). This may be confirmed by DNA-DNA relatedness.

We conducted a simple analysis to explain the effect of more constrained and relaxed cut off points with 98 and 99.5 % similarity respectively. This analysis had shown a total of 13 novel taxa when we used constrained cut off level, whereas the relaxed cut off level could yield 60 novel taxa overall. Even though various cut off rates indicate distinct number of novel species from the study site it is concluded that minimum 13 strains should be novel. However, in the present study we are reporting 33 novel taxa from our study area by sticking with a cut off value of 99 %.

The 16S rRNA gene sequence data of this study concluded the abundance of six different phylogenetic classes from the venting area and four from nonvent area. γ-*Proteobacteria* was found to be the dominant phylum in both the vent (69 %) and nonvent (63 %) regions, but they were diversified with distinct species depending on the area. Phylogenetic analysis of DGGE from the shallow hydrothermal vent of Southern

Tyrrhenian Sea by López-García et al. (2003) and Maugeri et al. (2010) in the Rainbow hydrothermal sediment at MAR libraries also support the dominance of γ-*Proteobacteria* in the vents. Podgorsek et al. (2004) reported the abundance of α- and γ-*Proteobacteria* by culture dependent approach from low-temperature hydrothermal fluids of the North Fiji Basin. Studies from the shallow submarine hydrothermal vent in Taketomi Island, Japan were also supportive of γ-*Proteobacteria* dominancy in the vents (Hirayama et al. 2007). Cho and Giovannoni (2004) revealed that members of γ-*Proteobacteria* have been found not only from the hydrothermal vent systems but also in most of the Oceans.

Bacteroidetes thrive in a variety of marine ecosystems including hydrothermal vents (Sievert et al. 2000b; Kormas et al. 2006). Members of this group are prevalent in marine ecosystems where they play a key role in degradation of organic matters (Gómez-Pereira et al. 2012). In this investigation we could obtain 12 phylotypes, 11 of them belonging to *Flavobacteria* and one reported to be *Sphingobacteria*. This result suggested that the abundance of *Bacteroidetes* depends on the amount of organic matter generated and their absence in the nonvent area could be due to less generation of organic matters compared to venting area of Espalamaca. This was supported by Kirchman (2002) by saying representatives of the class *Flavobacteria* are believed to play a significant role in the degradation of complex organic matter. Current data from the Espalamaca indicate that 42 % of *Bacteroidetes* belongs to the novel taxa and their roles need to be identified. Phyla *Bacteroidetes* are not only present in the warm water vents but also exist in the cold ecosystem like Antarctica with large number of novel taxa (Peeters et al. 2011).

Members of *Actinobacteria* form a relatively small fraction at hydrothermal vents compared to non-thermal environments (Thornburg et al. 2010). However, in this study we could come across 2 OTUs of *Actinobacteria* from the vents and none from the

nonvent regions. Members of ε-*Proteobacteria*, distinguished as characteristics of deep and shallow hydrothermal systems (Thornburg et al. 2010; Campbell et al. 2006), were not observed in this investigation. Although culture dependent bacterial diversity approach did not show the culturability of ε-*Proteobacteria*, culture independent molecular analysis revealed the abundance of such groups in hydrothermal vent systems (Hirayama et al. 2007). On the other side, 77 % of bacterial isolates were reported to be *Firmicutes* in the shallow water hydrothermal vent of DJCS Azores (Mohandass et al. 2012); however, in the present study we could retrieve only 7 OTUs out of 113. This may be due to inconsistency in the concentration of elements and gases present over the study area.

Out of the total 113 OTUs, 21 OTUs were observed to be common in vent and nonvent. In the remaining 92 OTUs, 74 belong to vent and 18 to the nonvent. Here we report the number of phyla obtained from this study area is very high and only one third of the genera were reported previously by the earlier researches from various shallow water hydrothermal vents, including *Alteromonas*, *Aurantimonas*, *Bacillus*, *Erythrobacter*, *Flexibacter*, *Halomonas*, *Hyphomonas*, *Idiomarina*, *Marinobacter*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Staphylococcus*, *Sulfitobacter*, *Thalassospira* and *Vibrio* (Gugliandolo and Maugeri 1998; Sievert et al. 2000b; Templeton et al. 2005; Sudek et al. 2009; Mohandass et al. 2012). Each of the studies explored different kinds of bacterial community with some common species. But in the current study most of the bacteria were not reported earlier and are being reported for first time in shallow water vents; at the same time these groups were reported in various marine environments. Some of the bacteria which were reported in other shallow vents like *Alcaligenes*, *Brevibacterium*, *Colwellia*, *Halobacillus*, *Halothiobacillus*, *Micrococcus*, *Rhodomicrobium*, *Sulfurivirga*, *Thioalkalivibrio*, *Thiomicrospira*,

Xanthomonas, etc. (Moyer et al. 1995; Hirayama et al. 2007; Maugeri et al. 2009; Mohandass et al. 2012) were not found in the Espalamaca vent. This might be due to the bacterial preferences in accordance to their habitat choice or on the physico-chemical and nutritional requirements.

Members of *Vibrio* and *Pseudoalteromonas* were observed to be predominant in this investigation. This result was coincident with Hirayama et al. (2007), where aerobic heterotrophs isolated from hydrothermal system of Taketomi Island, Japan were associated with *Pseudoalteromonas* and *Vibrio*. Though a variety of *Vibrio* species were reported from various marine environments, the present investigation concludes that the abundance of *Vibrio* occurred only in the venting area especially from the vent sediments whereas *Pseudoalteromonas* presence has been recorded in both seawater and sediment samples. The role of heterotrophic sulphur oxidizers belonging to the genera *Acinetobacter*, *Pseudomonas*, and *Vibrio* have been described previously in hydrothermal vent (Durand et al. 1994). Organisms reported for Mn and Fe oxidization by Sudek et al. (2009) from Vailulu'u Seamount was comparable to our investigation because most of their genera were found to be common e.g. *Alteromonas*, *Aurantimonas*, *Halomonas*, *Hyphomonas*, *Idiomarina*, *Marinobacter*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Thalassospira* and *Vibrio.* The interesting thing we noticed from the new vent Espalamaca was that it could harbour more number of phylotypes than the Vailulu'u Seamount. Since most of the isolates were obtained through the metal amended media we could expect better survival over metals/ element. Some of the isolates like *Halomonas* and *Marinobacter* localized with hydrothermal vent, deep-sea and sub-seafloor were already reported for their involvement in metal cycling (Kaye et al. 2011).

Our previous study on cultured bacterial diversity conducted at DJCS, a close by shallow water hydrothermal vent located in the Azorean Island explored 10 different RFLP patterns which belong to *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Halomonas*, *Micrococcus*, *Pseudoalteromonas* and *Staphylococcus* (Mohandass et al. 2012). But in Espalamaca we could obtain only the former 4 genus and the later 3 were certainly not. But in this investigation we could retrieve more number of bacterial isolates to accomplish the maximum culturable diversity compared to DJCS. *Bacteroidetes* and α-*Proteobacteria* members were not observed in the DJCS site, but at the same time *Actinobacteria*, *Firmicutes*, β-*Proteobacteria* and γ-*Proteobacteria* members were common between DJCS and Espalamaca. However, DJCS was rich with gram positive spore producers while Espalamaca is rich with gram negative non-spore producers. This information disclosed that the bacterial populations and diversity varies from one vent to other at the same time some similarities also can be seen.

In conclusion, the diversity of culturable bacteria analysed from the Azorean shallow water vent Espalamaca region gives a unique bacterial phylogeny. In general the bacterial genera which are normally reported from other shallow water vents were minimized but at the same time these vents were overloaded with many uncommon bacteria. Espalamaca has a slightly higher population than the hydrothermal vent off the Island of Vulcano (Eolian Islands, Italy). Bacteriological data on culture dependent organisms from this study brings many new phylotypes in to the existing bacteriological database. Espalamaca diversified with 55 % of the new taxa into a class α-*Proteobacteria* and 19 % to γ-*Proteobacteria*. The phylum *Bacteroidetes* appeared only in the vent samples and was able to bring 42 % of novel phylotypes from this ecosystem. Metal amended media helped to isolate maximum number of bacteria for phylogenetic analysis. The rarefaction and Shannon index clearly indicated that the venting area was

always richer with more species than the nonvent. This area looks a highly potent and promising site for many new taxa which may have various interesting applications in bioremediation especially in metals.

Chapter 3

Culture independent bacterial community from shallow water hydrothermal vent of Espalamaca (Faial, Azores)

3.1. Introduction

Introduction of molecular tools has greatly expanded our understanding on microbial community structure and composition at the distinctive ecosystems like hydrothermal vents (Lentini et al. 2014). For gene sequence based molecular framework, only a gene sequence is required to identify the organism in terms of its phylogenetic type. In brief, DNA can be recovered from the environmental samples and probed for genes with specific functional activities and organisms can be identified at the kingdom, family, genus and even up to species levels based on sequence homology by comparing various portions of DNA coding for ribosomal RNA (Ward 1992).

Cultivation based methods are not well suited for investigation of the general composition of microbial communities in a particular ecosystem (Amann et al. 1995) since cultivable rate of microbes in the Ocean was found to be in the range of 0.001 – 0.1 % (Ferguson et al. 1984). So far culture independent molecular technique using 16S rRNA gene have been successfully studied for investigating the microbial communities in various hydrothermal systems (e.g. Moyer et al. 1995; Polz and Cavanaugh 1995; Sievert et al. 2000b; López-García et al. 2003; Yang et al. 2011).

The Azorean Islands (Portugal) provide accessible sampling sites for studying microorganisms inhabiting in hydrothermal vent ecosystems. D. Joao de Castro seamount (DJCS, located between the islands of Terceira and Sao Miguel) and Espalamaca (located in Faial–Pico channel off the Espalamaca headland) are well documented in the aspect of microbiology (Raghukumar et al. 2008; Mohandass et al. 2012) from the Azores Island. Most recently we investigated the molecular diversity of culture dependent bacteria using 16S rRNA gene sequencing from the vent Espalamaca (Chapter 2). The results indicated that culturable fractions of *Proteobacteria* were

dominant followed by *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Many of those were novel bacteria and they were not having previous records for their existence in such environments.

In this investigation, we present a metagenomic data of 16S rRNA gene clone library of the bacterial communities present in shallow water hydrothermal vent of Espalamaca and its close non-vent site. Data were also compared with few other shallow water hydrothermal vents of similar type.

3.2. Materials and methods

3.2.1. Site description and sample details

Location of the study area and its details are specified in Chapter 2, section 2.2.2. Surface and bottom sea water and sediment samples were collected from the shallow hydrothermal vent and non-vent sites in Espalamaca (38°33'N; 28°39'W) during April 2014 by scuba divers. Around 20 g of sediments collected in a sterile polycarbonate tubes and 5 litres of water samples collected in Niskin samplers. Samples were immediately brought to the laboratory, University of Azores and maintained at -20 °C until DNA extraction.

3.2.2. Genomic DNA Extraction

Seawater samples (2.5 L) were filtered using 0.2 μ cellulose nitrate filters (Sartorius). Genomic DNA was extracted from the filtered papers using E.Z.N.A water DNA kit (D5525, Omega) following the manufacturer's instructions. Genomic DNA from the sediment samples was extracted in 2 steps. In initial extraction we followed the method of Luna et al. (2006) with phenol-chloroform. Briefly, 2.5 g sediment samples were

mixed with 6.75 mL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM Na_2EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1 % Cetyl trimethylammonium bromide) and 50 µL of proteinase K (10 mg mL^{-1}) and horizontally mixed at 225 rpm (30 min, 37 °C). After mixing, 750 µL of 20 % SDS was added, and the samples incubated in a water bath at 65 °C for 2 h, with gentle inversions every 20 min. The supernatant was collected after centrifugation at 6000 g (10 min, room temperature) and transferred into sterile 50 mL Falcon tubes. The sediment pellets were extracted two times more by adding 2.25 mL of the extraction buffer and 0.25 mL of 20 % SDS, vortexing for 10 s, incubating at 65 °C for 10 min, and centrifuging as described before. Supernatants from all these three cycles of extractions were combined and purified with phenol-chloroform. DNA was then precipitated with 100 % chilled ethanol, crude nucleic acids pellets were washed with 70 % ethanol and resuspended in sterile distilled water. In second step, the purification of the above DNA samples was carried out by E.Z.N.A. Soil DNA kit protocol (D5625, Omega).

3.2.3. Agarose gel electrophoresis

The presence of genomic DNA was confirmed with 1.0 % agarose gel electrophoresis with TBE buffer (0.5X Tris Borate EDTA buffer). Briefly, 5 µL of DNA samples were mixed with 6X loading buffer in the ratio of 1:5 and loaded into the 1.0 % agarose gel. The electrophoresis was programmed at 80 V for 50 min and the DNA fragments were visualized using MiniBis Pro gel documentation system (DNR Bio-Imaging systems).

3.2.4. PCR amplification of 16S rRNA gene

Bacterial 16S rRNA genes were amplified using eubacterial primer sets 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')

(Lane 1991). The polymerase chain reaction (PCR) mixture (50 μL final volume) contained template DNA (≈ 100 ng), 10X PCR buffer, 40 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 20 pmol each primer, and 1 U Taq DNA polymerase (Ambion, Life technologies). Amplification profile consisted of an initial denaturation step (94 °C for 5 min) followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. Final extension was kept at 72 °C for 10 min. PCR products were examined by 1.0 % agarose gel electrophoresis with TBE buffer as indicated in section 3.2.3.

PCR products (triplicate for each sample) were pooled and purified with a gel-extraction kit (Promega) according to manufacturer's instructions.

3.2.5. Cloning and library construction

Purified PCR products were cloned into CloneJET PCR Cloning Kit (Thermo Scientific). The cloning reaction consisted of 2 μL of purified PCR product, 10 μL of 2X reaction buffer, 5 μL of sterile distilled water and 1 μL of DNA blunting enzyme. The reaction mixture was incubated at 70 °C for 5 min and chilled on ice. To this reaction mixture, 1 μL of pJET1.2 cloning vector (50 ng μL^{-1}) and 1 μL of T4 DNA ligase were added. This ligation mixture (20 μL) was incubated at 20 °C for 4 h and stored at -20 °C until transformation experiment starts.

The above ligation mixture (20 μL) was added to a vial of competent *Escherichia coli* DH5α cells (200 μL) and incubated on ice for 30 min. The cells were given heat shock at 42 °C for 90 seconds without shaking and immediately transferred to ice. After 5 minutes of incubation on ice, 800 μL of sterile LB broth (Luria-Bertani, Merck) was added to this mixture and shaken horizontally (225 rpm) at 37 °C for 1 h. Various volumes (50, 100

and 200 μL) from the above broth culture were spread plated onto LB agar plates containing ampicillin (50 μg mL^{-1}) and incubated at 37 °C for 16 h to obtain individual bacterial colonies.

Well isolated colonies were inoculated into 1.5 mL of LB broth containing ampicillin (50 μg mL^{-1}) and incubated overnight at 37 °C with shaking (150 rpm). Plasmids were isolated and purified from the clones using Plasmid MiniPrep kit (Invitrogen) following the manufacturer's instruction. The plasmids were subjected to PCR amplification using pJET1.2 primers, for screening the positive clones (clones with inserts).

3.2.6. DNA sequencing and phylogenetic analysis

The purified plasmids from the positive clones were directly sequenced using an automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, CA) with 27F and pJET1.2 primers (Thermo Scientific). Obtained partial sequences were trimmed using DNA baser software version 3.0 and vectors were removed using NCBI online program VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Chimeric sequences were removed using an online tool Decipher (Wright et al. 2012). Nonchimeric sequences were submitted to BLAST search program in NCBI to find the closest neighbour sequences in GenBank and for phylogenetic analysis. To generate taxonomic profiles, the assembled sequences were assigned to taxonomic groups by using RDP (Ribosomal Database Project) classifier. Sequences with >97 % were assigned as same phylotype and aligned using Clustal W sequence alignment program (Thompson et al. 1994). Phylogenetic trees were constructed with neighbour-joining (Saitou and Nei 1987) algorithm using MEGA 5.2 software (Tamura et al. 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985). The sequences were submitted to GenBank with accession numbers KP303396 – KP303589.

3.2.7. Statistical analysis

Shannon and Simpson diversity indices were calculated using online program (http://www.changbioscience.com/genetics/shannon.html). Rarefaction analysis was performed by plotting the number of phylotypes against the total number of clones using EcoSim700 (Gotelli and Entsminger 2004). Good's coverage was calculated using the formula C = [(1-(n1/N)]*100 where C is the homologous coverage, n1 is the number of phylotypes appearing only once, and N is the total number of clones.

3.3. Results

3.3.1. Bacterial diversity from hydrothermal vent region

After chimera removal, we analysed 100 clones from sediments (EVSd), 29 clones from bottom water (EVBw) and 24 clones from surface water (EVSw). Highly diverse bacterial 16S rRNA gene community structures were found from the shallow hydrothermal vent region in Espalamaca. The sequences were affiliated with *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Gemmatimonadetes*, *Ignavibacteriae*, *Planctomycetes*, *Proteobacteria* (includes α, γ, ε, δ, ζ subdivisions), *Spirochaetes* and *Verrucomicrobia*. Relative proportions of the different groups in each clone library were summarized in Figure 3.1.

3.3.1.1. *Proteobacteria*

The phylum *Proteobacteria* was found to be dominant with 59 clones (38 phylotypes) in the hydrothermal vent region which includes α, γ, ε, δ and ζ subdivisions. Among them, half of the bacterial clones were affiliated to γ-*Proteobacteria*, representing 12.5 %, 13.8 % and 22.0 % of clones in the EVSw, EVBw and EVSd libraries respectively. The genus

Colwellia was abundant in the sediment samples with 7 clones. *Marinobacterium* was the only common genus between EVBw and EVSd libraries; otherwise all other phylotypes were sample specific.

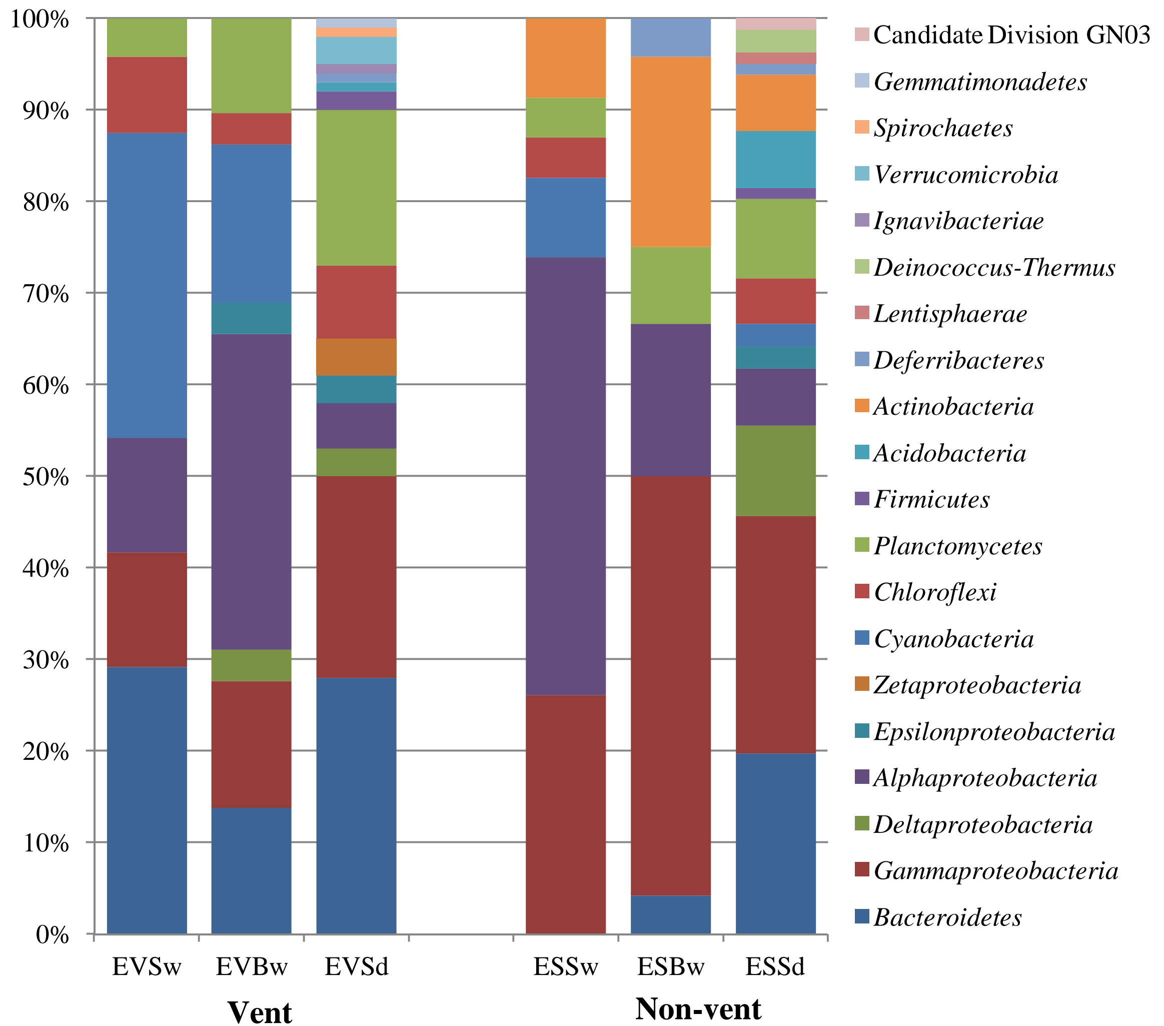


Fig. 3.1. Relative abundance of bacterial phylogenetic groups in sediments and water samples of shallow hydrothermal vent (EVSw, EVBw and EVSd) and non-vent (ESSw, ESBw and ESSd) at Espalamaca, Azores

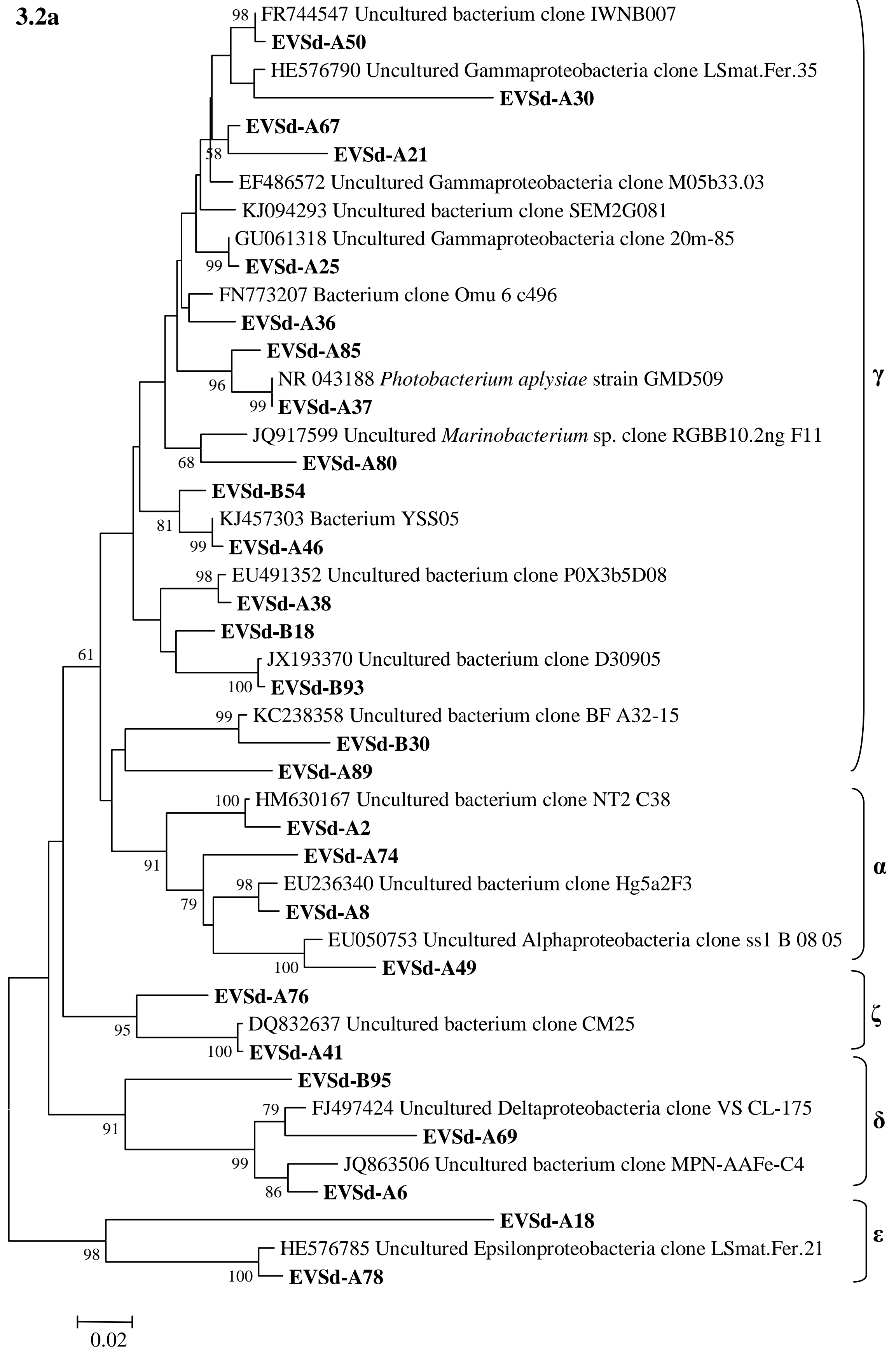
Eighteen clones (12 phylotypes) were affiliated with α-*Proteobacteria*, representing 12.5 %, 34.5 % and 5.0 % of clones in the EVSw, EVBw and EVSd libraries respectively. Highly diverse α-*Proteobacteria* groups were found in EVBw when compared to other

two libraries and other subdivisions of *Proteobacteria*. Eight out of 18 clones were belonging to SAR11 clusters, interestingly all the clones were observed only in seawater libraries, not in vent sediments.

Bacterial sequences belong to ε, δ and ζ subdivisions of *Proteobacteria* were also observed in the study area. Four clones (3 phylotypes) were affiliated with δ-*Proteobacteria* belong to the families *Syntrophobacteraceae* (1 clone) and *Geobacteraceae* (3 clones). Four clones (2 phylotypes) were affiliated to ε-*Proteobacteria* and grouped into the family *Campylobacteraceae*. Only one clone each of δ-*Proteobacteria* and ε-*Proteobacteria* were observed in EVBw, remaining clones are strictly observed in EVSd. Four clones (2 phylotype) belonging to the family *Mariprofundaceae* of ζ-*Proteobacteria* were observed only in the sediment library (EVSd). Detailed phylogenetic position and evolutionary relationships of clone sequences belong to *Proteobacteria* are given in Figure 3.2a.

3.3.1.2. *Bacteroidetes*

Bacteroidetes was the second dominant phyla observed in this study with 39 clones (17 phylotypes). The clones were closely related to the families *Cryomorphaceae*, *Flammeovirgaceae*, *Flavobacteriaceae*, *Porphyromonadaceae* and one unidentified group. *Bacteroidetes* clones were consistent with all the 3 libraries, representing 29.2 %, 13.8 % and 28.0 % of clones in the EVSw, EVBw and EVSd libraries respectively. Four phylotypes from EVBw and 3 phylotypes from EVSw libraries were obtained and none of them were common. Whereas, 15 phylotypes were found in EVSd library in which one phylotype was observed to be common with EVSw and 4 phylotypes were common with EVBw.


3.2a
FR744547 Uncultured bacterium clone IWNB007
EVSd-A50
HE576790 Uncultured Gammaproteobacteria clone LSmat.Fer.35
EVSd-A30
EVSd-A67
EVSd-A21
EF486572 Uncultured Gammaproteobacteria clone M05b33.03
KJ094293 Uncultured bacterium clone SEM2G081
GU061318 Uncultured Gammaproteobacteria clone 20m-85
EVSd-A25
FN773207 Bacterium clone Omu 6 c496
EVSd-A36
EVSd-A85
NR 043188 Photobacterium aplysiae strain GMD509
EVSd-A37
JQ917599 Uncultured Marinobacterium sp. clone RGBB10.2ng F11
EVSd-A80
EVSd-B54
KJ457303 Bacterium YSS05
EVSd-A46
EU491352 Uncultured bacterium clone P0X3b5D08
EVSd-A38
EVSd-B18
JX193370 Uncultured bacterium clone D30905
EVSd-B93
KC238358 Uncultured bacterium clone BF A32-15
EVSd-B30
EVSd-A89
HM630167 Uncultured bacterium clone NT2 C38
EVSd-A2
EVSd-A74
EU236340 Uncultured bacterium clone Hg5a2F3
EVSd-A8
EU050753 Uncultured Alphaproteobacteria clone ss1 B 08 05
EVSd-A49
EVSd-A76
DQ832637 Uncultured bacterium clone CM25
EVSd-A41
EVSd-B95
FJ497424 Uncultured Deltaproteobacteria clone VS CL-175
EVSd-A69
JQ863506 Uncultured bacterium clone MPN-AAFe-C4
EVSd-A6
EVSd-A18
HE576785 Uncultured Epsilonproteobacteria clone LSmat.Fer.21
EVSd-A78
γ
α
ζ
δ
ε
0.02

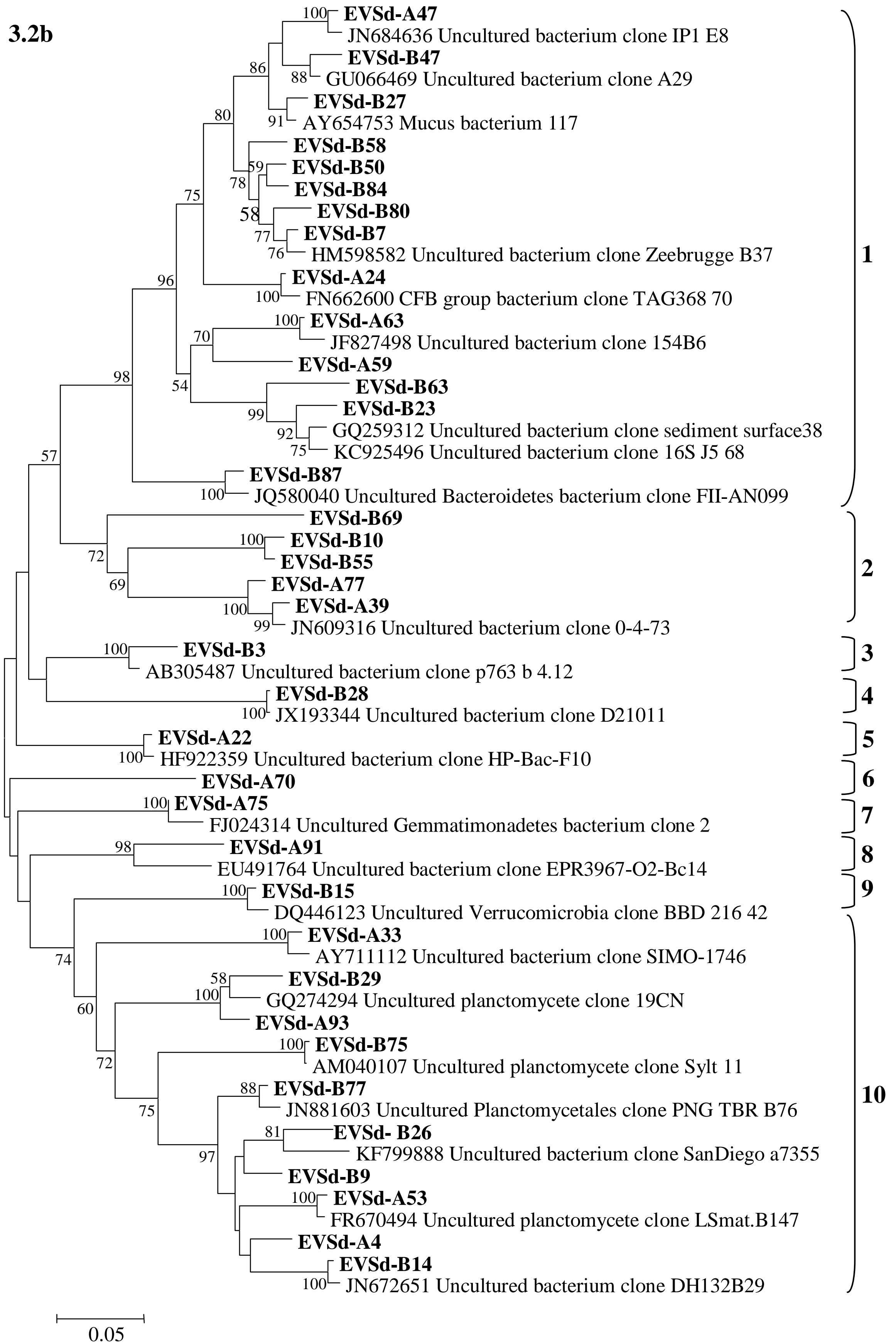

3.2b
EVSd-A47
JN684636 Uncultured bacterium clone IP1 E8
EVSd-B47
GU066469 Uncultured bacterium clone A29
EVSd-B27
AY654753 Mucus bacterium 117
EVSd-B58
EVSd-B50
EVSd-B84
EVSd-B80
EVSd-B7
HM598582 Uncultured bacterium clone Zeebrugge B37
EVSd-A24
FN662600 CFB group bacterium clone TAG368 70
EVSd-A63
JF827498 Uncultured bacterium clone 154B6
EVSd-A59
EVSd-B63
EVSd-B23
GQ259312 Uncultured bacterium clone sediment surface38
KC925496 Uncultured bacterium clone 16S J5 68
EVSd-B87
JQ580040 Uncultured Bacteroidetes bacterium clone FII-AN099
EVSd-B69
EVSd-B10
EVSd-B55
EVSd-A77
EVSd-A39
JN609316 Uncultured bacterium clone 0-4-73
EVSd-B3
AB305487 Uncultured bacterium clone p763 b 4.12
EVSd-B28
JX193344 Uncultured bacterium clone D21011
EVSd-A22
HF922359 Uncultured bacterium clone HP-Bac-F10
EVSd-A70
EVSd-A75
FJ024314 Uncultured Gemmatimonadetes bacterium clone 2
EVSd-A91
EU491764 Uncultured bacterium clone EPR3967-O2-Bc14
EVSd-B15
DQ446123 Uncultured Verrucomicrobia clone BBD 216 42
EVSd-A33
AY711112 Uncultured bacterium clone SIMO-1746
EVSd-B29
GQ274294 Uncultured planctomycete clone 19CN
EVSd-A93
EVSd-B75
AM040107 Uncultured planctomycete clone Sylt 11
EVSd-B77
JN881603 Uncultured Planctomycetales clone PNG TBR B76
EVSd- B26
KF799888 Uncultured bacterium clone SanDiego a7355
EVSd-B9
EVSd-A53
FR670494 Uncultured planctomycete clone LSmat.B147
EVSd-A4
EVSd-B14
JN672651 Uncultured bacterium clone DH132B29
1
2
3
4
5
6
7
8
9
10
0.05

Fig. 3.2. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from hydrothermal vent EVSd library (a, *Proteobacteria*; b, other groups). Bootstrap analysis was performed with 1,000 replications and the values above 50 % are indicated at the nodes. 1, *Bacteroidetes*; 2, *Chloroflexi*; 3, *Ignavibacteriae*; 4, *Acidobacteria*; 5, *Firmicutes*; 6, *Deferribacteres*; 7, *Gemmatimonadetes*; 8, *Spirochaetes*; 9, *Verrucomicrobia*; 10, *Planctomycetes*

3.3.1.3. *Planctomycetes*

Twenty one clones (11 phylotypes) were belong to the phylum *Planctomycetes*, accounted for 4.2 %, 10.3 % and 17.0 % in the EVSw, EVBw and EVSd libraries respectively. Majority of the clones were belonging to the family *Planctomycetaceae* and few of the clones were belonging to the family "*Candidatus* Brocadiaceae". The sequences closely related with the genus *Blastopirellula* were common in all the 3 libraries. On the other hand, sequences closely related to the genus *Pirellula* was found to be common in EVSd and EVBw libraries. Remaining phylotypes were strictly obtained from the sediments (EVSd).

3.3.1.4. *Cyanobacteria* and *Chloroflexi*

A total of 13 clones (5 phylotypes) were affiliated to the phylum *Cyanobacteria*, interestingly they retrieved only from the seawater samples. *Cyanobacteria* accounted for 33.3 % and 17.2 % in the EVSw and EVBw libraries respectively. *Cyanobacteria* were observed to be predominant (33.3 %) in the surface seawater when compared to *Bacteroidetes* (29.2 %) and *Proteobacteria* (25.0 %). All the 5 phylotypes observed in

this group were belonging to 4 different families namely, *Bacillariophyta*, *Cryptomonadaceae*, *Chlorarachniophyceae* and Family II.

There were 11 clones belonging to the phylum *Chloroflexi* in which 6 phylotypes were observed. These clones accounted 8.3 %, 3.4 % and 8.0 % clones in the EVSw, EVBw and EVSd libraries respectively. These 6 phylotypes represents 3 different families, *Anaerolineaceae*, *Ardenticatenaceae* and *Caldilineaceae*.

3.3.1.5. *Firmicutes*, *Verrucomicrobia*, *Acidobacteria* and other minor groups

Three clones (1 phylotype) were belonging to the phylum *Verrucomicrobia* and 2 clones (1 phylotype) were affiliated with the phylum *Firmicutes*. *Acidobacteria*, *Deferribacteres*, *Spirochaetes*, *Gemmatimonadetes* and *Ignavibacteriae* were some of the other phyla observed in this venting region with one clone each. It is noteworthy to mention here that all of these minor groups were observed only in the sediment library (EVSd). Phylogenetic position and evolutionary relationships of clone sequences belong to the phylogenetic groups other than *Proteobacteria* are given in Figure 3.2b.

3.3.2. Bacterial diversity from non-vent region

We analyzed 128 clones from three clone libraries of the non vent samples (81 clones from sediments, 24 clones from bottom water and 23 clones from surface seawater). As like venting site, high diversity in bacterial communities was observed in the non-vent region also. The sequences were affiliated to the phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, Candidate Division GN03, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus-Thermus*, *Firmicutes*, *Lentisphaerae*, *Planctomycetes* and *Proteobacteria* (includes α, γ, ε, δ, subdivisions). Relative proportions of these groups in each clone library were summarized in Figure 3.1.

3.3.2.1. ***Proteobacteria***

The phylum *Proteobacteria* was predominant in the non-vent region with 68 clones (41 phylotypes) which includes α, γ, ε and δ and subdivisions. Among them, more than 50 % of the bacterial clones (22 phylotypes) were affiliated with γ-*Proteobacteria*, representing 26.0 %, 45.8 % and 25.9 % of clones in the ESSw, ESBw and ESSd libraries respectively. *Alteromonadaceae*, *Halomonadaceae*, *Oceanospirillaceae*, *Pseudoalteromonadaceae*, *Hahellaceae*, *Colwelliaceae*, *Ectothiorhodospiraceae*, *Vibrionaceae*, *Piscirickettsiaceae*, *Methylococcaceae* and some unidentified families were observed from the non-vent γ-*Proteobacteria* clones.

Twenty clones (10 phylotypes) were affiliated with α-*Proteobacteria*, contributing 47.8 %, 16.7 % and 6.2 % of clones in the ESSw, ESBw and ESSd libraries respectively. The predominant phyla observed form the surface seawater samples was α-*Proteobacteria* followed by γ-*Proteobacteria*. Majority of the clones (8 clones each) were belonging to the family *Rhodobacteraceae* and SAR11 groups. *Hyphomonadaceae*, *Methylocystaceae* and *Rhodospirillaceae* were some other families observed from this region. Similar to the venting region, SAR 11 groups were observed only from the water samples.

Eight clones (8 phylotypes) were represented the subdivision of δ-*Proteobacteria*. Interestingly, all of them were retrieved from sediment samples contributing 8.6 % clones to the ESSd library. These clones were affiliated with the families, *Syntrophobacteraceae*, *Cystobacterineae*, *Desulfobulbaceae*, *Desulfarculaceae*, *Desulfobacteraceae* and *Campylobacteraceae*. Two clones (1 phylotype) were belonging to the phylum ε-*Proteobacteria* and both the clones were observed in sediment library (ESSd). Phylogenetic relationships of clone sequences belong to *Proteobacteria* are given in Figure 3.3a.

3.3.2.2. *Bacteroidetes*

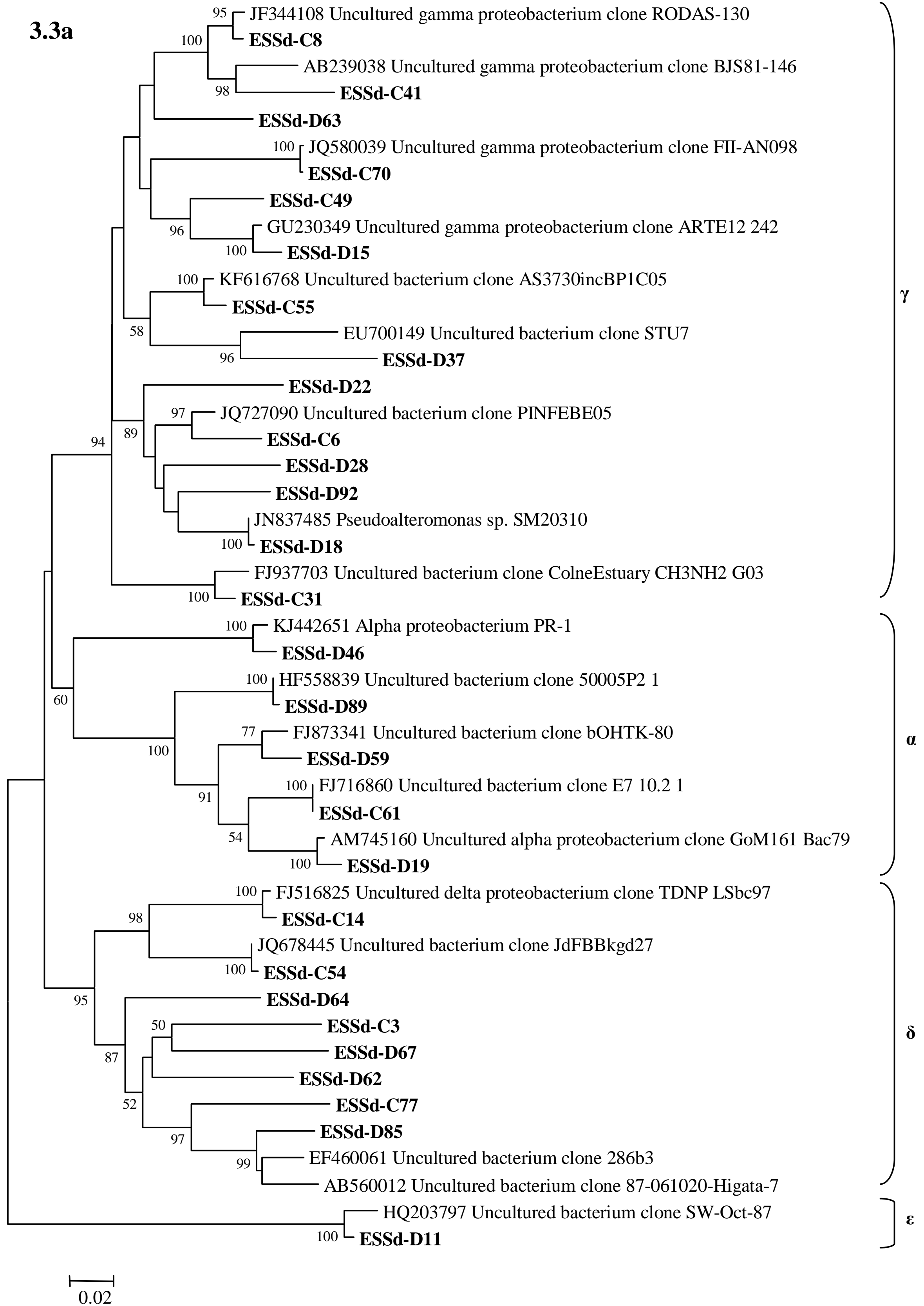
Seventeen clones consisting of 15 phylotypes belong to the phylum *Bacteroidetes*. Only one clone represents ESBw and remaining clones were found in the ESSd library, contributing 4.2 % and 19.8 % respectively. Majority of the clones were grouped into the family *Flavobacteriaceae* followed by *Marinilabiliaceae*, *Cryomorphaceae*, *Saprospiraceae*, *Sphingobacteriaceae*, *Rhodothermaceae* and *Flammeovirgaceae*.

3.3.2.3. *Actinobacteria*

A total of 12 clones (6 phylotypes) were belonging to the phylum *Actinobacteria*, accounting 8.7 %, 20.8 % and 6.2 % clones in the ESSw, ESBw and ESSd libraries respectively. Five clones represented the family *Acidimicrobineae*, four clones represented the family *Actinomycetales* and one clone belong to *Iamiaceae* and *Euzebyales*. *Actinobacteria* was found to be the second dominant in the ESBw library. Interestingly, *Actinobacteria* groups were not recovered from the venting region of Espalamaca.

3.3.2.4. *Planctomycetes*, *Chloroflexi* and *Cyanobacteria*

Ten clones (6 phylotypes) were affiliated with *Planctomycetes*, accounted for 4.3 %, 8.3 % and 8.6 % respectively in the ESSw, ESBw and ESSd libraries. These clones were closely affiliated to the families *Planctomycetaceae*, *Phycisphaeraceae* and "*Candidatus* Brocadiaceae". A total of 5 clones (4 phylotypes) were belonging to the phylum *Chloroflexi*, representing 4.3 and 4.9 % of the clones in the ESSw and ESSd libraries respectively. Two clones each were affiliated to the family *Leptolinea* and the remaining one was unidentified. Four clones (3 phylotypes) were belonging to the phylum *Cyanobacteria*, representing 8.7 and 2.5 % respectively in the ESSw and ESSd libraries.


3.3a
95
JF344108 Uncultured gamma proteobacterium clone RODAS-130
100
ESSd-C8
AB239038 Uncultured gamma proteobacterium clone BJS81-146
98
ESSd-C41
ESSd-D63
100
JQ580039 Uncultured gamma proteobacterium clone FII-AN098
ESSd-C70
ESSd-C49
96
GU230349 Uncultured gamma proteobacterium clone ARTE12 242
100
ESSd-D15
100
KF616768 Uncultured bacterium clone AS3730incBP1C05
ESSd-C55
58
EU700149 Uncultured bacterium clone STU7
96
ESSd-D37
ESSd-D22
97
JQ727090 Uncultured bacterium clone PINFEBE05
94
89
ESSd-C6
ESSd-D28
ESSd-D92
JN837485 Pseudoalteromonas sp. SM20310
100
ESSd-D18
FJ937703 Uncultured bacterium clone ColneEstuary CH3NH2 G03
100
ESSd-C31
100
KJ442651 Alpha proteobacterium PR-1
ESSd-D46
100
HF558839 Uncultured bacterium clone 50005P2 1
60
ESSd-D89
77
FJ873341 Uncultured bacterium clone bOHTK-80
100
ESSd-D59
91
100
FJ716860 Uncultured bacterium clone E7 10.2 1
ESSd-C61
54
AM745160 Uncultured alpha proteobacterium clone GoM161 Bac79
100
ESSd-D19
100
FJ516825 Uncultured delta proteobacterium clone TDNP LSbc97
98
ESSd-C14
JQ678445 Uncultured bacterium clone JdFBBkgd27
100
ESSd-C54
95
ESSd-D64
50
ESSd-C3
87
ESSd-D67
ESSd-D62
52
ESSd-C77
ESSd-D85
97
99
EF460061 Uncultured bacterium clone 286b3
AB560012 Uncultured bacterium clone 87-061020-Higata-7
HQ203797 Uncultured bacterium clone SW-Oct-87
100
ESSd-D11
0.02
γ
α
δ
ε

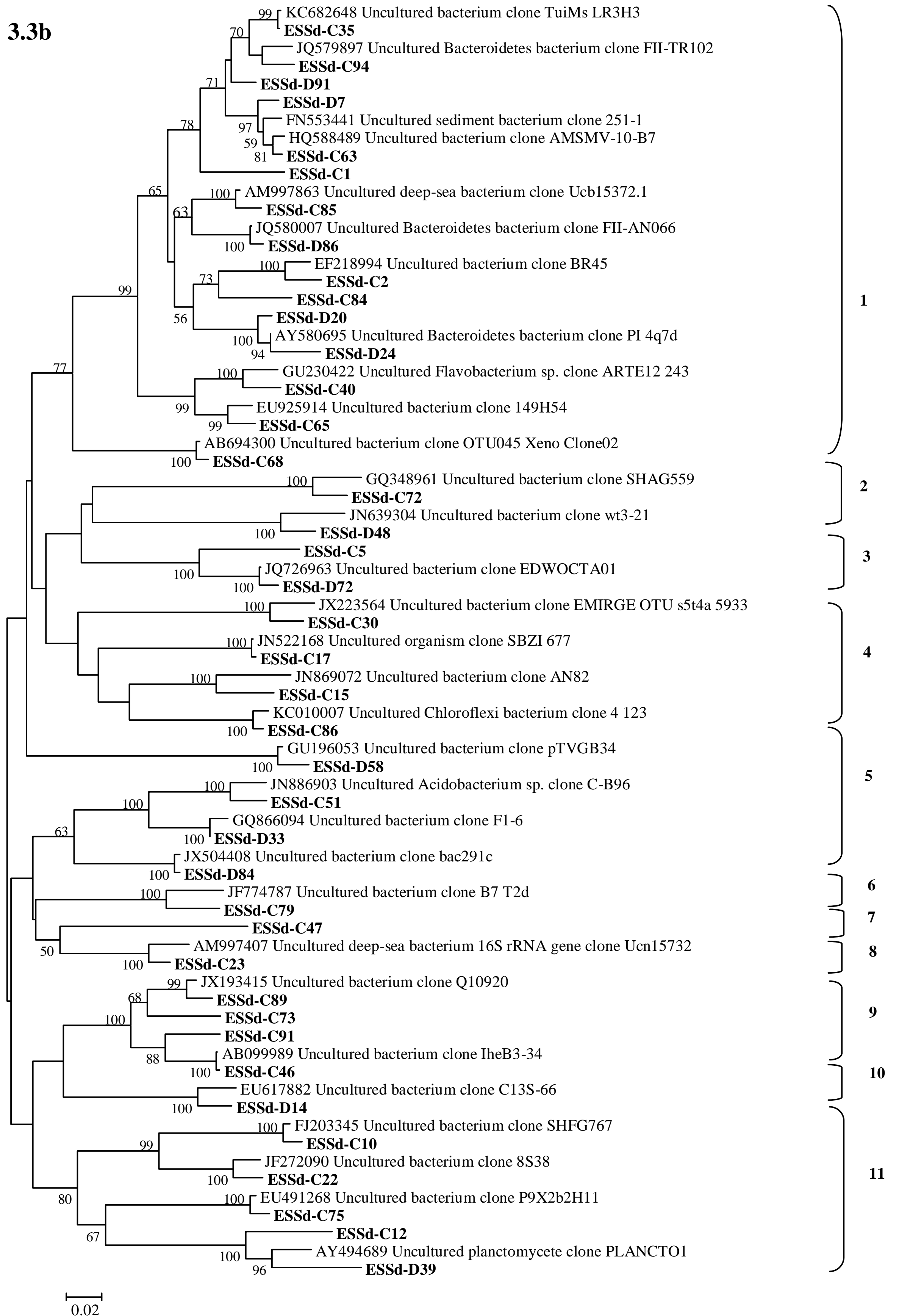

3.3b
KC682648 Uncultured bacterium clone TuiMs LR3H3
ESSd-C35
JQ579897 Uncultured Bacteroidetes bacterium clone FII-TR102
ESSd-C94
ESSd-D91
ESSd-D7
FN553441 Uncultured sediment bacterium clone 251-1
HQ588489 Uncultured bacterium clone AMSMV-10-B7
ESSd-C63
ESSd-C1
AM997863 Uncultured deep-sea bacterium clone Ucb15372.1
ESSd-C85
JQ580007 Uncultured Bacteroidetes bacterium clone FII-AN066
ESSd-D86
EF218994 Uncultured bacterium clone BR45
ESSd-C2
ESSd-C84
ESSd-D20
AY580695 Uncultured Bacteroidetes bacterium clone PI 4q7d
ESSd-D24
GU230422 Uncultured Flavobacterium sp. clone ARTE12 243
ESSd-C40
EU925914 Uncultured bacterium clone 149H54
ESSd-C65
AB694300 Uncultured bacterium clone OTU045 Xeno Clone02
ESSd-C68
GQ348961 Uncultured bacterium clone SHAG559
ESSd-C72
JN639304 Uncultured bacterium clone wt3-21
ESSd-D48
ESSd-C5
JQ726963 Uncultured bacterium clone EDWOCTA01
ESSd-D72
JX223564 Uncultured bacterium clone EMIRGE OTU s5t4a 5933
ESSd-C30
JN522168 Uncultured organism clone SBZI 677
ESSd-C17
JN869072 Uncultured bacterium clone AN82
ESSd-C15
KC010007 Uncultured Chloroflexi bacterium clone 4 123
ESSd-C86
GU196053 Uncultured bacterium clone pTVGB34
ESSd-D58
JN886903 Uncultured Acidobacterium sp. clone C-B96
ESSd-C51
GQ866094 Uncultured bacterium clone F1-6
ESSd-D33
JX504408 Uncultured bacterium clone bac291c
ESSd-D84
JF774787 Uncultured bacterium clone B7 T2d
ESSd-C79
ESSd-C47
AM997407 Uncultured deep-sea bacterium 16S rRNA gene clone Ucn15732
ESSd-C23
JX193415 Uncultured bacterium clone Q10920
ESSd-C89
ESSd-C73
ESSd-C91
AB099989 Uncultured bacterium clone IheB3-34
ESSd-C46
EU617882 Uncultured bacterium clone C13S-66
ESSd-D14
FJ203345 Uncultured bacterium clone SHFG767
ESSd-C10
JF272090 Uncultured bacterium clone 8S38
ESSd-C22
EU491268 Uncultured bacterium clone P9X2b2H11
ESSd-C75
ESSd-C12
AY494689 Uncultured planctomycete clone PLANCTO1
ESSd-D39
1
2
3
4
5
6
7
8
9
10
11
0.02

Fig. 3.3. Neighbour-joining tree showing the evolutionary relationship of phylotypes based on 16S rRNA gene sequences from non-vent ESSd library (a, *Proteobacteria*; b, other groups). Bootstrap analysis was performed with 1,000 replications and the values above 50 % are indicated at the nodes. 1, *Bacteroidetes*; 2, *Deinococcus-Thermus*; 3, *Cyanobacteria*; 4, *Chloroflexi*; 5, *Acidobacteria*; 6, *Deferribacteres*; 7, Candidate Division GN03; 8, *Firmicutes*; 9, *Actinobacteria*; 10, *Lentisphaerae*; 11, *Planctomycetes*

3.3.2.5. *Acidobacteria*, *Firmicutes* and other minor groups

A total of 5 clones were affiliated to *Acidobacteria* with 3 phylotypes. Acidobacterial clones were only recovered from the ESSd library and belong to the groups Gp4, Gp21 and Gp22. Both the clones were originated from ESSd library. Two clones (2 phylotype) were affiliated to *Deinococcus-Thermus*, two clones (1 phylotype) were belonging to *Deferribacteres* and one clone each belongs to the phyla *Lentisphaerae*, *Firmicutes* and Candidate Division GN03. Evolutionary relationships of phylotypes from clone sequences are given in Figure 3.3b.

3.4. Discussion

Studies on microbial communities inhabited in the deep-sea hydrothermal venting regions of Atlantic Ocean have been attempted along the Lost City (Gerasimchuk et al. 2010), Rainbow (Nercessian et al. 2005), Ashadze (Roussel et al. 2011), Logatchev (Reed et al. 2009) and Lucky Strike vent fields (Cŕepeau et al. 2011). However, microbial explorations from the shallow hydrothermal vent counter parts are still poorly explored in the Atlantic Ocean. Present investigation provides a new insight in to the bacterial communities thriving in the shallow water hydrothermal vent of Espalamaca

(Azores, North Atlantic) and how the microbial communities vary from other shallow hydrothermal vent fields.

Culture dependent analysis from the shallow hydrothermal vent of Espalamaca (Chapter 2) revealed that the presence of γ-*Proteobacteria* (68.7 %), α-*Proteobacteria* with (16.7 %), *Bacteroidetes* (10 %), *Firmicutes* (3.2 %), *Actinobacteria* (0.9 %) and β-*Proteobacteria* (0.45 %). Former four phylogenetic groups were also obtained in metagenomic clone library analysis whereas minor groups like *Actinobacteria* and β-*Proteobacteria* were not observed in vent clone libraries. However, many uncultured clones recovered from the same venting regions were belonging to more than 10 phyla. This may be the first report on culture independent microbial community analysis in shallow hydrothermal vent, Azores.

Genomic information of unculturable microbes from metagenomic clone libraries can help in understanding their physiology and their role in the ecosystem (Khandeparker et al. 2014). Most of the 16S rRNA gene sequences obtained from the clone libraries was closely related with the uncultured neighbours in the GenBank database. At the same time, we tried to identify the sequences through RDP database to know closely related organisms. The online tool Decipher helped to find out around 9 chimeric sequences from the clone libraries which were omitted for further analysis.

Bacterial community structure obtained from this investigation was compared with the other shallow water hydrothermal vent regions reported from different Oceans (Table 3.1) to find out the similarities and variations between them. The comparative study revealed that the phylum *Proteobacteria* (especially ε-, γ- and δ-*Proteobacteria*) was consistently present in every hydrothermal vent fields. It is not surprise because the phylum *Proteobacteria* is the most dominant and diverse group of the microbial

assemblage (Gupta et al. 2000). However, *Proteobacteria* subdivisions and their proportions varied from one region to another. Apart from that, the presence of *Bacteroidetes* was observed almost in all the venting regions except in Loihi Seamount (Table 3.1).

Generally ε-*Proteobacteria* groups were predominant in the shallow hydrothermal vents of Loihi Seamount (Moyer et al. 1995), Milos Island (Giovannelli et al. 2013) and Juan de Fuca Ridge (Zhou et al. 2009). It is noteworthy to cite that the ε-*Proteobacteria* group have also been reported to represent a major part of microbial communities at deep-sea hydrothermal vents (Longnecker and Reysenbach 2001; Lopez-Garcia et al. 2003). Results from their investigation revealed that ε-*Proteobacteria* accounted significant part in the domain bacteria, between 40 and 80 % from the 16S rRNA clone libraries. However, microbial communities in the present study revealed that the composition of ε-*Proteobacteria* were only 3 % in total which is contrast to other hydrothermal vents. It is worth mentioning here that the ε-*Proteobacteria* clone sequences were affiliated to the order Campylobacteriales which are generally mesophilic and microaerophilic in nature (Miroshnichenko 2004). Further, one of the phylotype (clones EVBw-F45 and EVSd-A78) belong to the phylum ε-*Proteobacteria* was closely related with the GenBank sequence HE576785, interestingly it was reported from microbial mat in Lucky Strike hydrothermal vent field (Mid-Atlantic Ridge). In addition to that, clones EVBw-F45 and EVSd-A78 are closely related with the genus *Arcobacter* in RDP database which are reported to be the producers of elemental sulphur in filamentous form from the oxidation of sulphide (Wirsen et al. 2002). This information reveals that even though mesophilic ε-*Proteobacteria* groups are present in low level in the study area, their involvements in sulphur oxidation process are more essential especially in these types of environments.

Table 3.1. Bacterial community in Espalamaca vent (present study) compared with few other shallow water hydrothermal vents

Bacterial community	EVSd, Espalamaca	Loihi Seamount	Photic zone, South Tonga Arc	MV1, Milos	MV2, Milos	Juan de Fuca Ridge
α-*Proteobacetria*	5.0	-	8.0	-	-	-
β-*Proteobacetria*	-	-	-	-	-	3.7
ε-*Proteobacetria*	3.0	60.5	17.0	60.0	59.0	52.4
γ-*Proteobacetria*	22.0	33.4	33.0	7.0	20.0	17.1
δ-*Proteobacetria*	2.0	2.1	8.0	5.0	6.0	9.8
ζ-*Proteobacetria*	4.0	-	-	-	-	-
Bacteroidetes	28.0	-	11.0	20.0	6.0	3.7
Cyanobacteria	-	-	0.4	2.0	-	-
Planctomycetes	17.0	-	9.0	-	3.0	-
Chloroflexi	9.0	-	4.9	-	-	-
Acidobacteria	1.0	-	3.0	-	-	-
Actinobacetria	-	-	3.4	-	3.0	-
Firmicutes	2.0	-	0.7	-	-	-
Verrucomicrobia	3.0	-	-	-	-	-
Deferribacteres	1.0	-	0.4	-	-	-
Spirochaetes	1.0	-	1.1	-	-	-
Ignavibacteriae	1.0	-	-	2.0	3.0	-

Deinococcus-Thermus	-	-	-	-	-	6.1
Gemmatimonadetes	1.0	-	-	-	-	-
Others	-	2.1	-	-	-	7.3
Unknown	-	-	-	4.0	-	-
Reference	Present study	Moyer et al. 1995	Murdock et al. 2010	Giovannelli et al. 2013	Giovannelli et al. 2013	Zhou et al. 2009

-, not reported.

γ-*Proteobacetria* groups were predominant in shallow hydrothermal vent of Espalamaca which is coinciding with the South Tonga Arc, South Pacific Ocean (Murdock et al. 2010). α-*Proteobacteria* and γ-*Proteobacteria* are known to form large clusters in all of the marine environments (Fuhrman et al. 1993, Suzuki et al. 1997). Other bacterial groups observed from this study were also in similar pattern to the South Tonga Arc vents, for instance domination of γ-*Proteobacetria* followed by other *Proteobacteria* groups, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi* and even minor groups like *Acidobacteria*, *Verrucomicrobia* and *Deferribacteres*. On the other side, huge variations of bacterial communities were observed between our results and other shallow hydrothermal vent fields indicated (Table 3.1). The present investigation revealed more than 15 phylogenetic groups, which is comparatively higher from the earlier records (Table 3.1).

In Milos and Juan de Fuca Ridge, the numbers of phylogenetic groups were around 7 and in Loihi Seamount, numbers of phylogenetic groups were reported to be 3 excluding few unknown groups. This may perhaps due to less number of clones sequenced. Whereas, in South Tonga Arc vents, they sequenced more number of clones hence the number of phylogenetic groups were more than 10. Even though number of clones sequenced from our EVSd library (n=100) was less than South Tonga Arc vent photic zone, we could detect more phylogenetic groups. Apart from these clone libraries (compared in Table 3.1), recently advanced technologies like illumina sequencing, provides more number of reads (more than 10,00,000) which could explore much entire phylogenetic groups. For instance, Lentini et al. (2014) analysed prokaryotic communities from a shallow hydrothermal site in Eolian Islands (Italy), elucidated by illumina sequencing technology, which resulted about 35 phylogenetic groups.

Bacteria of the SAR11 groups often dominate the marine microbial communities in both the surface and deep waters of the ocean and potentially mediate a large portion of the dissolved organic matter flux (Malmstrom et al. 2004). Though they are not in large proportion, we have observed considerable number of SAR11 clones from Espalamaca waters (7 clones from venting regions and 8 clones from the nonventing region), accounting for 8.3 %, 17.2 %, 21.7 % and 12.5 % of clones from EVSw, EVBw, ESSw and ESBw libraries respectively. The phylogenetic positions of each clones belong to SAR11 groups obtained from this study are given in Figure 3.4. Clones belong to SAR11 clade was not obtained from sediments of both venting and non-vent regions.

The presence of *Cyanobacteria* and *Chloroflexi* groups confirms that these photosynthetic bacteria involved in primary production in the shallow water hydrothermal vents. Few of the clones (EVSd-B10, EVSd-B55, EVSd-B89 and EVSd-B92) belong to *Chloroflexi* were closely related (96.0, 91.0, 91.0 and 97.0 % sequence homology respectively) with a sequence in NCBI database reported from iron oxide sediments in Volcano, Tonga Arc hydrothermal vent (GenBank accession number FJ905709).

Members of *Actinobacteria* appear in small fraction at hydrothermal vents when compared to non-thermal environments (Thornburg et al. 2010). In this investigation, we did not come across with *Actinobacteria* members in the venting region while *Actinobacteria* was found to be the second dominant phyla in ESBw library of non-vent region.

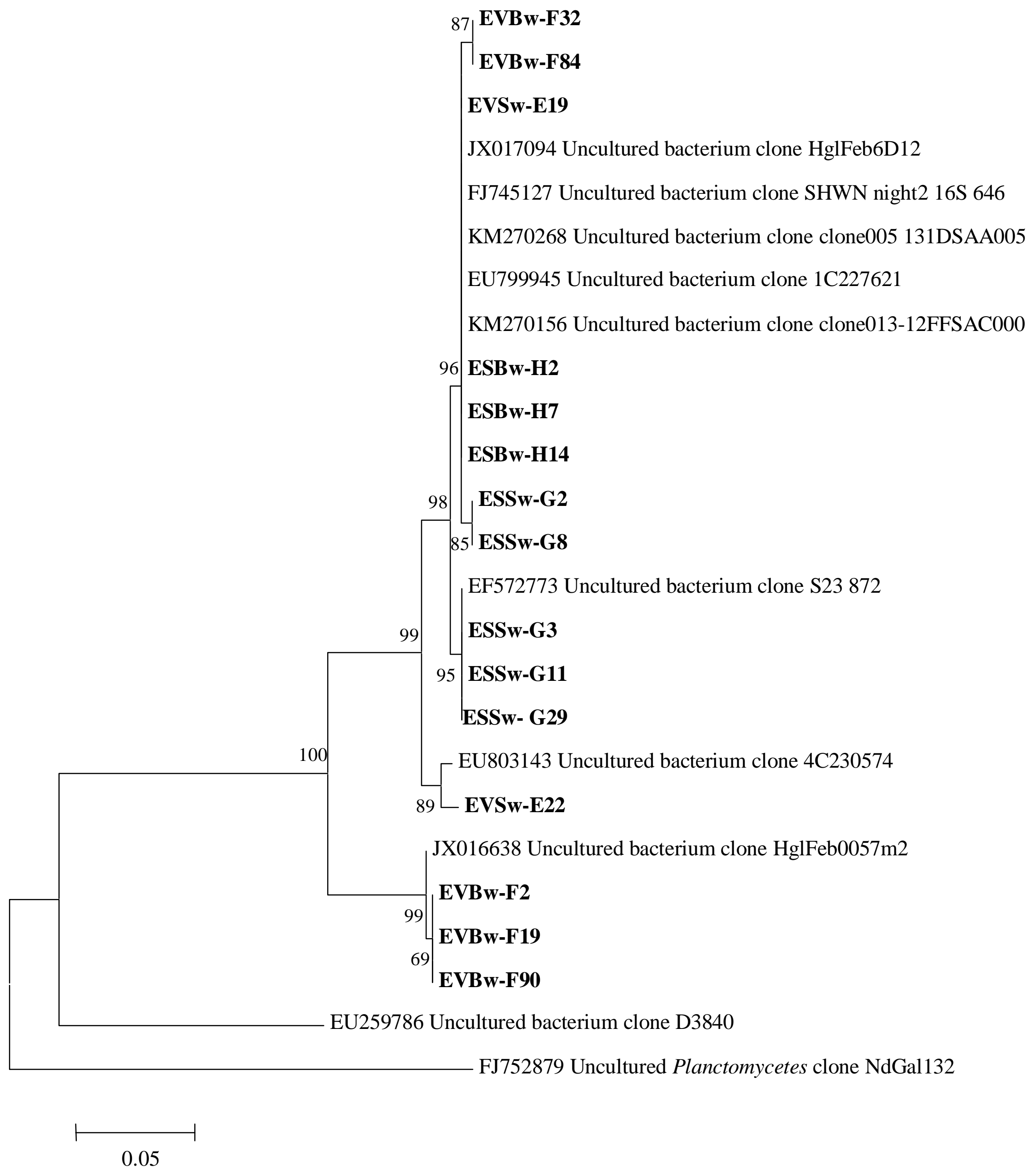


Fig. 3.4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of SAR11 phylotypes from Espalamaca waters. Bootstrap analysis was performed with 1,000 replications and the values above 50 % are indicated at the nodes.

A considerable number of clones (n=21) were observed from the vent libraries were belonging to the phylum *Planctomycetes*. They affiliated with 2 orders namely, *Planctomycetales* and "*Candidatus* Brocadiales" and closely related with the genera *Pirellula*, *Blastopirellula*, *Rhodopirellula* and "*Candidatus* Anammoxoglobus". Interestingly, these groups are reported from the studies conducted by Storesund and Øvreås (2013) on diversity of the phylum *Planctomycetes* in low temperature hydrothermal venting (iron-hydroxide deposits) at the Mohns Ridge, a part of the Arctic Mid Ocean Ridge. Members of *Planctomycetes* are reported to be involved in carbohydrate fermentation and sulphur reduction (Elshahed et al. 2007). In addition, they are the only known organisms able to perform anaerobic ammonium oxidation (anammox), which could be a significant process in these ecosystems (Schmid et al. 2003; Strous et al. 1999).

Shannon diversity index (*H*') for venting region was found to be 2.28, 3.06 and 4.03 respectively in the EVSw, EVBw and EVSd libraries whereas in non-vent region it was 2.40, 2.57 and 4.16 respectively in the ESSw, ESBw and ESSd libraries. Rarefaction curve analysis of vent and non-vent sediment libraries indicated that the nonventing region showed more phylotypes with less clones than the venting site (Fig. 3.5). Overall, a total of 84 phylotypes each were obtained from both the sites and majority of them (84.5 %) were unique to the ecosystem. Only few phylotypes (15.5 % of total) were observed to be common between the vent and non-vent libraries. This massive community variation (within 500 m distance) in metagenomic libraries showed that microbes inhabiting in venting area are entirely different from reference area which may be involved in metal or elemental transformation and oxidation pathways.

We attempted to find out the presence of functional genes from the sediment DNA sample using available primer sets. Interestingly, we could see PCR amplification bands for the genes of methanol dehydrogenase (*mxaF*), carbonic anhydrase and *soxB* (Fig. 3.6). Carbonic anhydrases is a metalloenzyme catalyzing the reversible hydration of carbon dioxide to bicarbonate and play important roles in global carbon cycle (Kumar and Ferry 2014). Methanol dehydrogenase, is highly conserved among distantly related methylotrophic species in α-, β- and γ-*Proteobacteria* (Lau et al. 2013). The presence of *soxB* gene shows that there is active sulphur oxidizing microbial communities in shallow hydrothermal vent.

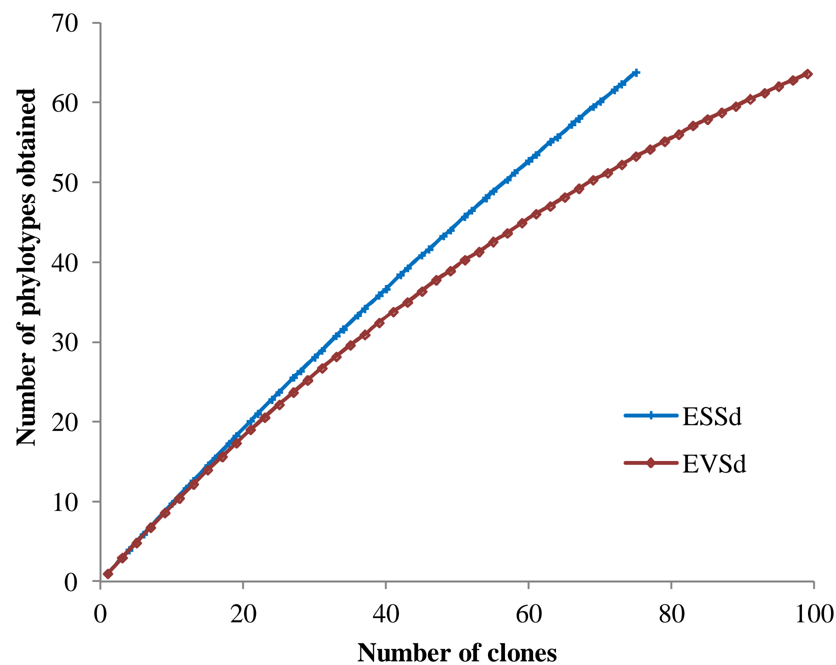


Fig. 3.5. Rarefaction curve of the 16S rRNA gene libraries from vent (EVSd) and non-vent (ESSd) sediments

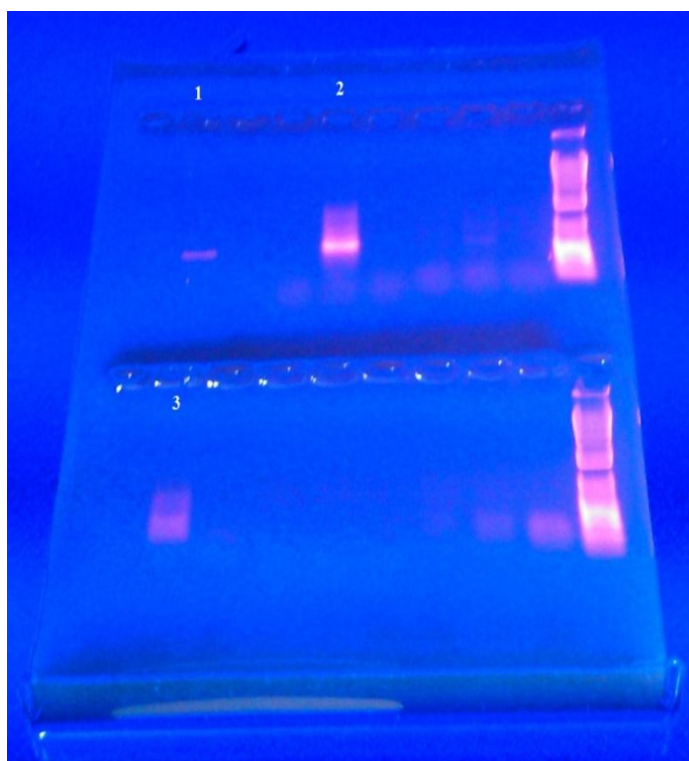


Fig. 3.6. PCR amplification of functional genes (1, methanol dehydrogenase; 2, carbonic anhydrase and 3, soxB genes) from shallow hydrothermal vent sediments in Espalamaca

In conclusion, the Espalamaca hydrothermal vent region harboured distinct bacterial communities when compared with non vent site located just 500 m south to the vent site. Earlier studies reported that ε-*Proteobacetria* was dominant in various hydrothermal vent systems but in Espalamaca γ-*Proteobacetria* was found to be predominant which is coinciding only with the hydrothermal vent in South Tonga Arc. More than 10 phylogenetic groups were obtained with metagenomic analysis in this vent shows supremacy over other vents except South Tonga Arc (Table 3.1). Culture independent analysis brought *Proteobacteria* (ε, δ, ζ subdivisions), *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Gemmatimonadetes*, *Ignavibacteriae*, *Planctomycetes*, *Spirochaetes* and *Verrucomicrobia* which were not able to retrieve in culture dependent

approach. Some of the genera like *Roseovarius*, *Photobacterium*, *Vibrio* and *Pseudoalteromonas* were appeared in both culture dependent and independent methods. Combination of culture dependent and independent analysis concluded that 15 – 20 % of bacterial populations were common between vent and non vent.

Chapter 4

Metals and element tolerance studies of the bacterial isolates of Espalamaca

4.1. Introduction

Deep-sea hydrothermal vents along mid-ocean spreading centers, terrestrial hot springs, marine back-arc basins and shallow water hydrothermal vents are known for its metal-rich biotopes which nourish geothermally dependent microbial population. These microorganisms rapidly respond to the changes in the metal concentration and adapt themselves to withstand extreme conditions (Holden and Adams 2003).

Fe, Mn, Ca, Mg, Pb, Cd, As, Zn, Cu, and Ni are some of the commonly found metals in hydrothermal vent systems. The concentrations of these metals and trace elements in shallow hydrothermal vent fluids are higher when compared to ambient seawater (Aiuppa et al. 2000; Colaco et al. 2006). These metals and sulphur compounds present in the shallow water hydrothermal vents can be oxidized and used as electron acceptors by members of heterotrophic bacteria and archaea (Sievert et al. 2000a). Hence, it is necessary to study the microbial communities inhabiting in hydrothermal vent ecosystems.

Investigation of hydrothermal vent microbes and its physiological properties, like sulphur metabolisms, oxidation of Fe(II) and Mn(II) have explored new lights in the hydrothermal system (Brinkhoff et al. 1999; Emerson and Moyer 2002; Kuever et al. 2002; Edwards et al. 2003; Nakagawa et al. 2004; Tebo et al. 2005; Templeton et al. 2005; Emerson et al. 2007; Sievert et al. 2008). The use of microbial populations exclusively adapted to a specific level of environmentally bioavailable metal will enhance our ability to remediate metal contaminated sites (Roane 1999). On the other hand, microbial communities tolerant to Pb from hydrothermal vents are poorly examined.

The present investigation has been focused to understand the growth and tolerability of various heterotrophic bacteria to Mn, Pb, Fe and thiosulfate. These metals and element expected to be available in significant levels and play a major role in biogeochemical cycles of the hydrothermal vent in Espalamaca.

4.2. Materials and Methods

4.2.1. Isolation and screening of metals and element tolerant bacteria

Metals and element tolerant bacteria from the surface, bottom water and sediment samples were isolated using metal amended diluted nutrient agar and screened for its tolerability using various methods explained in chapter 2, section 2.2.4.

4.2.2. Bacterial tolerance towards Mn, Fe, Pb and Ts

Mn tolerability was tested with vent bacteria using various concentration of $MnCl_2$ in dSWNA medium. The concentrations include 2, 5, 10, 15, 20, 25, 30, 50 and 100 mM. Tolerability of Pb was studied using $Pb(NO_3)_2$ with a concentrations of 1, 2, 5, 7.5 and 10 mM in dSWNA medium. Bacterial tolerance to iron was studied by inoculating the cultures into 2, 5 and 10 mM of $FeSO_4$. Tolerability of vent bacteria studied agaist sulphur by execution of 2, 3, 4, 5, 6 and 7 % of $Na_2S_2O_3$ in dSWNA. Bacterial cultures were streaked on the respective above media and incubated at 30 ± 2 °C for 72 h. Tolerability of the strains were judged by the bacterial growth with respect to the concentration of metals/elements. Media composition and details are explained in chapter 2, section 2.2.4.

4.2.3. Mn(II) oxidation by the vent (Espalamaca) isolates

Mn (II) oxidation was studied by introducing the bacterial isolates into dSWNA media

amended with 5 mM $MnCl_2$ (minimum tolerable concentration for all the isolates). All the plates were incubated in dark for a period of 30 days. After the incubation period, oxidation of soluble Mn(II) in to insoluble Mn(III/IV) oxides were observed as brown or black precipitates in the media plates. Oxidation rates were recorded based on the color intensity in the plates.

4.2.4. Mn(II) oxidation studies by the vent (Espalamaca) isolates in liquid media

The isolate VSW210 which oxidized Mn within 10 days, was introduced into liquid media to study the oxidation rates further. Briefly, 1.0 mL of 1.0 OD (A_{600}) culture was inoculated into diluted nutrient broth amended with 5 mM $MnCl_2$ (final volume of 100 mL in 500 mL conical flask) and incubated statically in dark. Every 5 days, 0.2 mL of sample was taken and added to 1.0 mL of 0.04 % leucoberbelin blue (LBB, prepared in 45 mM acetic acid). This mixture was incubated in dark for 15 min and the oxidation rates were measured photometrically at A_{620} (Krumbein and Altman 1973). To quantify the levels of Mn oxidization in the broth, a $KMnO_4$ Standard curve was generated. Briefly, 0.2 mL of different concentrations of $KMnO_4$ (0, 10, 20, 30, 40 and 50 μM) were added with 1.0 mL of 0.04 % LBB stain and measured photometrically at A_{620}. In LBB assay, 40 μM $KMnO_4$ is equivalent to 100 μM MnO_2 (Okazaki et al. 1997).

4.2.5. 16S rRNA gene sequencing and phylogenetic analysis

Exponentially grown bacterial cells were collected (approximately 2×10^9 cells) by centrifugation and genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. For PCR amplification of 16S rRNA gene, eubacterial primers 27F and 1492R were used (Lane 1991). The 50 μL PCR reaction mixture contained 5 μL of 10X reaction buffer, 5 μL of 15 mM $MgCl_2$, 4 μL of

2.5 mM dNTP, 2 μL of each primer (10 pmol μL^{-1}), 1 μL of template (25–50 ng), and 0.5 μL of Taq DNA polymerase (5 U μL^{-1}, Genei) made up with sterile double-distilled water. PCR profile consisted of initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 60 s, 53 °C for 60 s, 72 °C for 90 s and a final extension of 7 min at 72 °C.

The obtained PCR products were gel-purified using a Gel Extraction Kit or purified with PCR cleanup kit (Sigma) according to the manufacturer's protocol. DNA sequencing was carried out with 3130xl Genetic Analyzer (Applied Biosystems) using bacterial primers 27F, 518F and 1492R. The sequences obtained from three different primers were combined to get nearly completed nucleotide sequences using DNAbaser software (version 3.5.3). The PINTAIL program 1.0 (Ashelford et al. 2005) was used for chimera checking and no differences were detected from our sequences. The sequences were analyzed using NCBI Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon server 2.1 (Kim et al. 2012a) to identify the closest relatives.

4.3. Results and discussion

Bacterial populations in the metal amended media were in the order of 10^4 mL^{-1} in seawater samples and 10^5 g^{-1} in sediment samples. Rarefaction curve analysis indicated that Pb amended medium provided high species richness followed by Ts, Mn and Fe (Fig. 4.1). Phylogenetic analysis of metal and element tolerant bacteria from shallow water hydrothermal vent and non-vent in Espalamaca revealed 4 different phyla. They belong to *Proteobacteria* (α, β, γ), *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. γ-*Proteobacteria* was found to be dominant group retrieved in all the metal amended media i.e, 58 % of the isolates each in Pb, Mn and Ts amended media, and 94 % in Fe amended medium (Fig. 4.2).

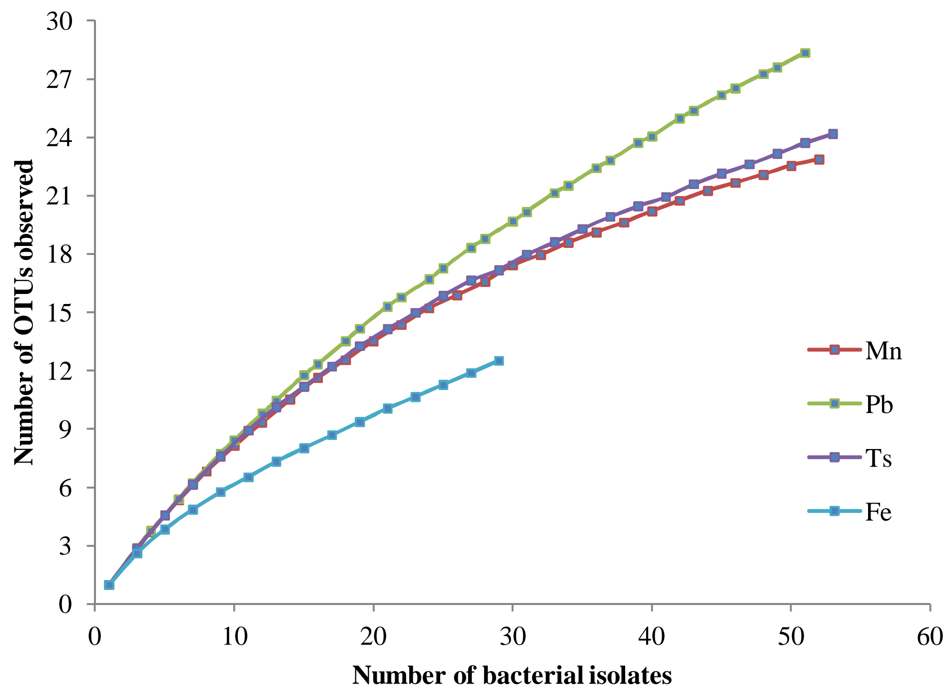


Fig. 4.1. Rarefaction curves of bacterial OTUs based on 16S rRNA gene.

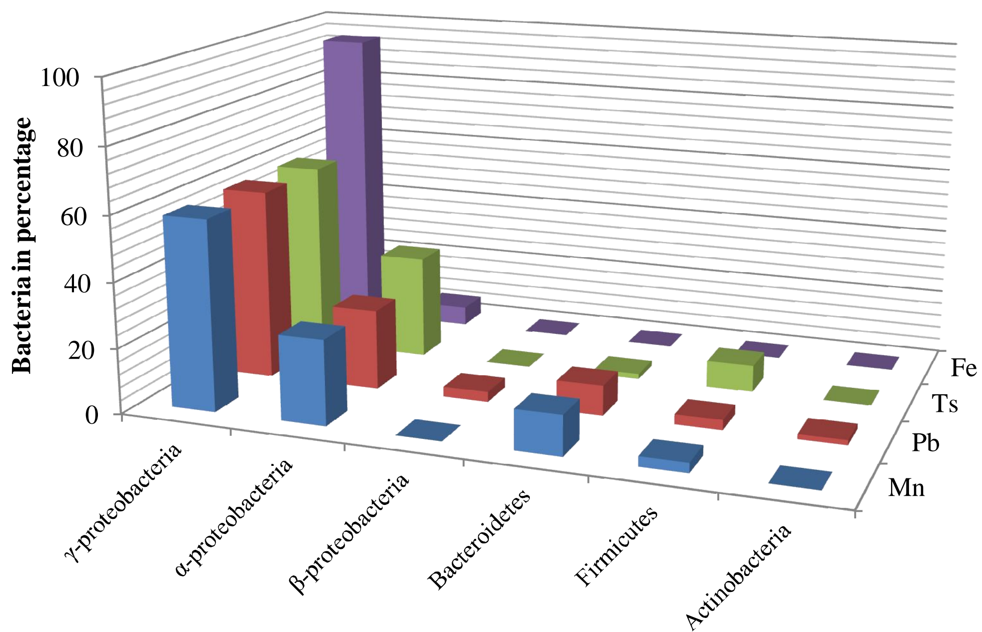


Fig. 4.2. Diversity and distribution of bacterial phylotypes (given in percentage) isolated using Mn, Pb, Fe and thiosulfate

4.3.1. Mn^{2+} tolerance studied with vent isolates

Bacterial strains isolated with 1 mM Mn amended media were further streaked into higher concentrations of Mn^{2+} to test its maximum tolerant level. Most of the tested bacterial isolates were able to grow up to 5 mM Mn^{2+} and hence this concentration was kept as minimum tolerant level for the bacterial community in Espalamaca. Even, 81.5 % of bacterial isolates were capable of growing with 10 mM Mn^{2+} and 64.5 % with 20 mM Mn^{2+} (Table 4.1). When the concentration increased to 30 mM, only 33.8 % of the bacteria were able to survive and almost 88 % of the bacteria did not prefer 50 mM Mn^{2+} while only 12.3 % (n=8) tolerated on that level. Further, 100 mM Mn^{2+} was found to be lethal concentration for all the tested bacterial isolates (Fig. 4.3).

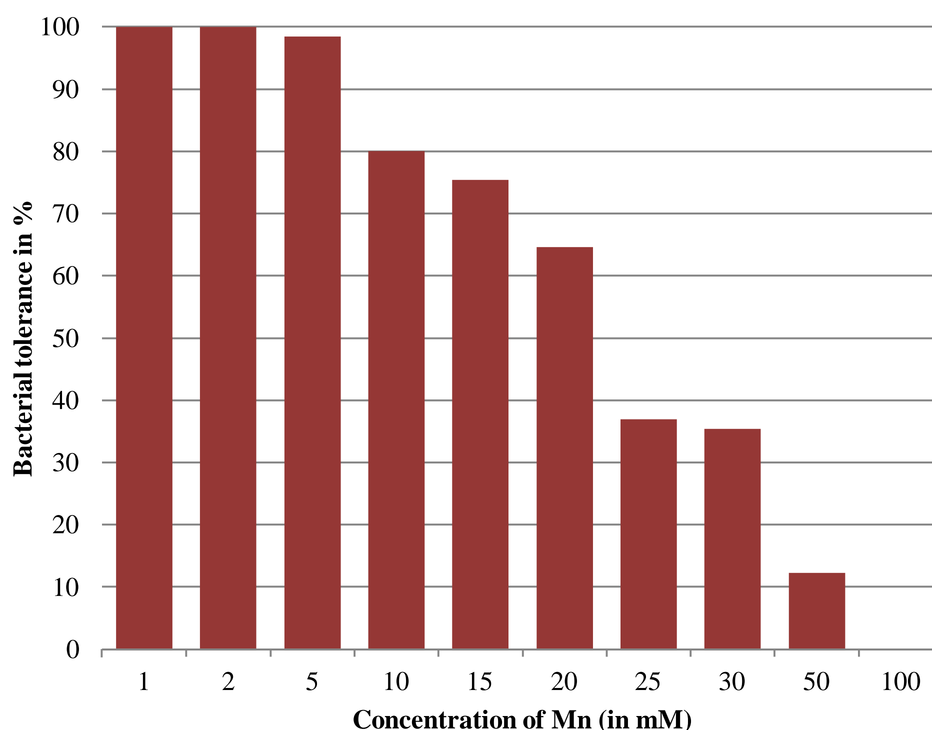


Fig. 4.3. Bacterial tolerance to various concentrations of Manganese

Table 4.1. Bacterial growth on various concentrations of Mn

Strain no.	2 mM	5 mM	10 mM	15 mM	20 mM	25 mM	30 mM	50 mM	100 mM
VBW204	+++	+++	---	---	---	---	---	---	---
VBW206	+++	+++	+++	+++	+++	+++	+++	++-	---
VBW207	+++	+++	+++	+++	+++	+++	+++	+++	---
VBW203	+++	+--	---	---	---	---	---	---	---
VBW240	+++	++-	+--	---	---	---	---	---	---
VBW202	+++	+++	+++	+++	+++	++-	++-	---	---
VSW209	+++	++-	---	---	---	---	---	---	---
VSW210	+++	+++	+++	+++	+++	+++	+++	++-	---
VSW211	+++	+++	+++	+++	+++	+++	---	---	---
VSW213	+++	++-	++-	++-	++-	---	---	---	---
VSW214	++-	++-	++-	++-	+--	---	---	---	---
VSW215	+--	---	---	---	---	---	---	---	---
VSW216	+++	+++	+++	+++	+++	+++	++-	---	---
VSW217	+++	+++	+++	+--	---	---	---	---	---
VSW219	+++	+++	---	---	---	---	---	---	---
SBW220	+++	+++	++-	---	---	---	---	---	---
SBW221	+++	+++	++-	---	---	---	---	---	---
SBW222	+++	+++	---	---	---	---	---	---	---
SBW223	+++	++-	---	---	---	---	---	---	---
SBW224	+--	++-	---	---	---	---	---	---	---
SBW225	++-	++-	++-	++-	++-	---	---	---	---
SBW226	++-	++-	+--	---	---	---	---	---	---
SBW227	+++	+++	+++	+++	+++	---	---	---	---
SBW228	+++	+++	---	---	---	---	---	---	---
SSW229	+++	+++	+++	+++	---	---	---	---	---
SSW230	+++	+++	+++	+++	---	---	---	---	---
SSW231	+++	+++	---	---	---	---	---	---	---
SSW232	+++	+++	+++	+++	+++	+++	+++	---	---
SSW233	+++	+++	+++	+++	---	---	---	---	---
SSW234	+++	+++	+++	+++	---	---	---	---	---
SBW236	+++	+++	---	---	---	---	---	---	---
SBW237	+++	+++	+++	+++	---	---	---	---	---
VSW212	+++	+++	+++	+++	+++	+++	++-	---	---

SBW235a	+++	+++	+++	+++	+++	+++	++-	---	---
SBW235b	+++	+++	+++	+++	+++	+++	++-	---	---
SBW238	++-	++-	---	---	---	---	---	---	---
VSD607	+++	+++	+++	+++	++-	++-	+--	+--	---
VSD608	+++	+++	+++	+++	+++	++-	++-	---	---
VSD609	+++	+++	+++	++-	+--	---	---	---	---
VSD613	+++	+++	+++	+++	+++	++-	++-	---	---
VSD614	+++	+++	+++	+++	+++	+++	+++	++-	---
VSD615	+++	+++	+++	+++	+++	+++	++-	---	---
VSD616	+++	+++	++-	++-	+--	---	---	---	---
VSD617	+++	+++	+++	+++	+++	+++	++-	++-	---
VSG618	+++	+++	+++	+++	+++	+++	+++	+--	---
VSG619	+++	++-	+--	+--	---	---	---	---	---
VSG620	+++	+++	+++	++-	+--	---	---	---	---
VSG621	+++	+++	+++	++-	+--	---	---	---	---
VSG622	+++	+++	++-	++-	++-	++-	+--	---	---
VSG623	+++	+++	+++	++-	+--	+--	+--	---	---
VSG624	+++	++-	++-	++-	+--	---	---	---	---
VSG626	+++	+++	++-	++-	+--	---	---	---	---
VSG630	+++	+++	+++	+++	++-	---	---	---	---
VSG631	+++	+++	++-	++-	+--	---	---	---	---
VSG632	+++	+++	+++	+++	+--	---	---	---	---
VSG633	+++	+++	+++	+++	++-	---	---	---	---
SSA634	+++	+++	+++	+++	+++	++-	+--	+--	---
SSA636	+++	+++	+++	+++	+++	++-	++-	---	---
SSA637	+++	+++	+++	+++	+++	++-	++-	---	---
SSA638	+++	+++	+++	+++	+++	++-	++-	---	---
SSA640	+++	+++	+++	+++	+++	---	---	---	---
SSA642	+++	+++	+++	+++	+++	---	---	---	---
SSA643	+++	+++	++-	++-	++-	++-	++-	---	---
VSG630a	+++	+++	+++	+++	+--	---	---	---	---
VSG627b	+++	+++	++-	++-	++-	---	---	---	---

+++, good growth; ++-, average growth; +--, poor growth; ---, no growth

4.3.2. Oxidation Studies of Mn(II) with vent isolates

Oxidation of Mn(II) was tested with 103 isolates (38 isolates from SWNA and 65 isolates from dSWNA+Mn media). After 30 days of incubation in dark, 45 % of bacteria was found to be oxidizing Mn(II) into insoluble Mn oxides. Excellent oxidation rates were observed in 13 strains; good, average and poor oxidations noticed in 17, 9 and 7 strains respectively (Table 4.2). The experiments extended up to 2 months but not much change noticed after 30 days. Some of the potential isolates like SBW071, VSW210, VSD613 and VSD613 were able to oxidixe Mn by indicating dark brown precipitates in the solid medium within 10 – 12 days. Fernandes et al. (2005) has been reported manganese oxidation from the Indian Ocean strains at the end of 76 days. Whereas some of the Espalamaca isolates could carry out the oxidation within a short incubation time of 10-15 days.

One of the isolate, designated VSW210 was detected to oxidize Mn(II) within 10 days of incubation which was confirmed with LBB staining (Fig. 4.4) and this was taken for further studies. Interestingly, the same strain was tolerant up to 50 mM Mn^{2+} (Table 4.1). The Mn(II) oxidation was started on 15th day by producing 1100 µM of MnO_2 and on 20th day it was observed to be 1739 µM of MnO_2. Growth of VSW210 was nearly 0.2 OD (A_{600}) on 10th day and observed to be increasing 15th and 20th day (Fig. 4.5). Growth was also coincided with Mn(II) oxidation pattern (Fig. 4.5). This experiment was conducted in dark condition, because the rates of Mn(II) oxidation may differ in response to light (Hansel and Francis 2006).

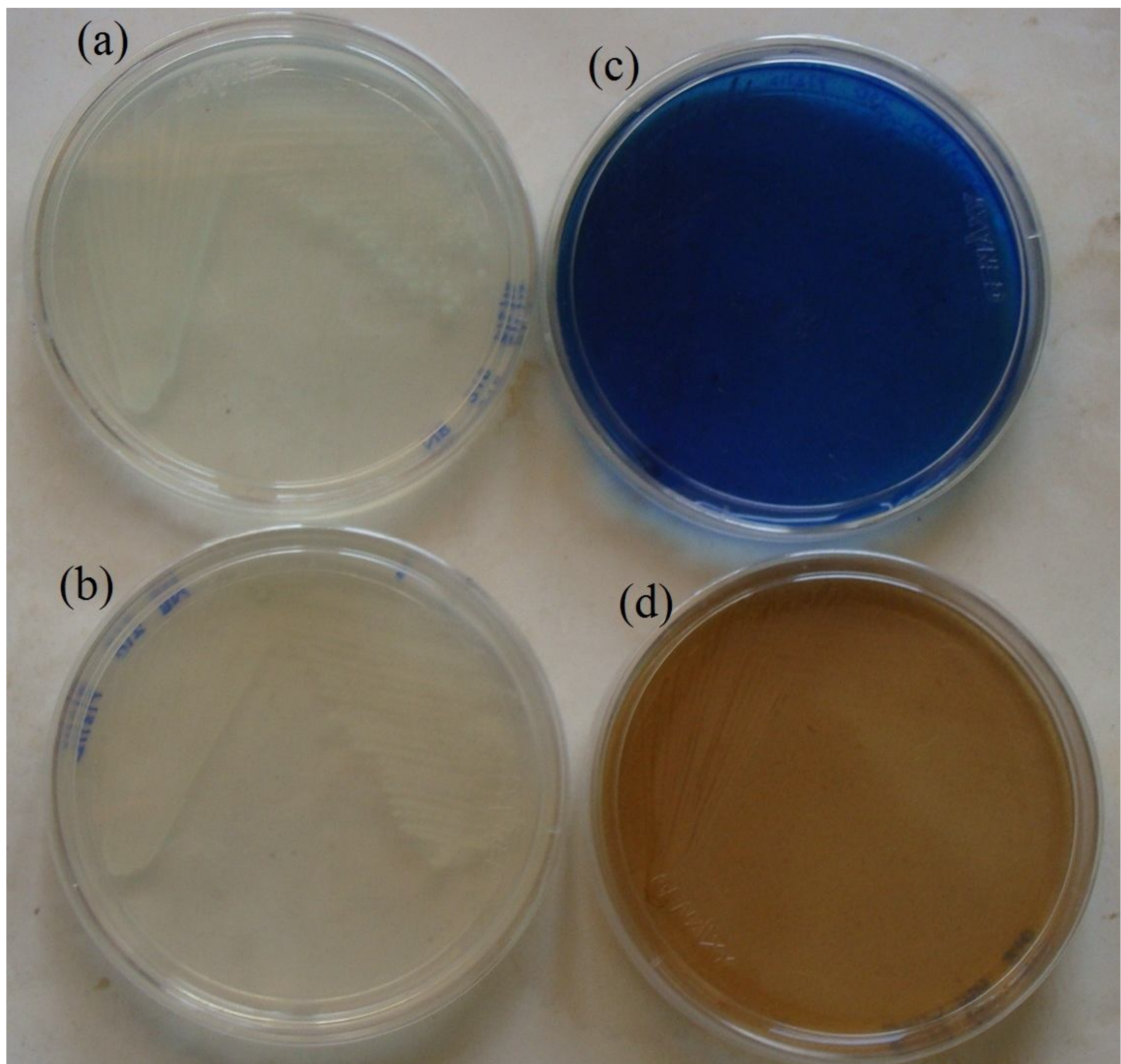


Fig. 4.4. Strain VSW210 oxidized Mn(II) on plates which was detected as brown colour formation and confirmed with LBB staining. Plate (a) and (b) are controls; (c) after addition of LBB stain; (d) Mn(III/IV) oxides as dark brown precipitates before adding LBB

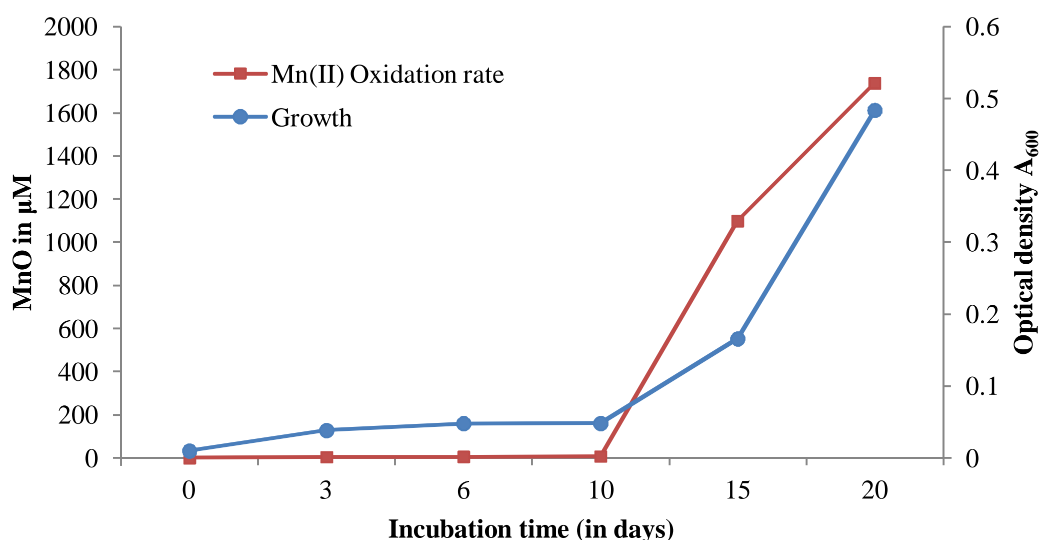


Fig. 4.5. Growth and Mn(II) oxidation rates of potential hydrothermal vent strain VSW210

Bacterially mediated Mn oxidations were studied over wide concentrations (Greene and Madgwick (1991) 25 mM; Mandernack et al (1995) 1 nM – 25 mM; Fernandes et al. (2005) 1 – 10 mM). De Vrind et al. (1986) studied Mn oxidation from *Bacillus* sp. with 0.1-7.0 mM of Mn(II). Studies from Beukes and Schmidt (2012) revealed that 1 mM Mn(II) is optimum for *Acinetobacter* sp. In some of the studies even 0.1 mM was used for Mn(II) oxidation (Table 4.3).

Oxidation of Mn^{2+} may be due to either by Mn^{2+} oxidizing enzymes or some unidentified Mn^{2+} oxidizing factors (Okazaki et al. 1997; Francis et al. 2001). Based on the studies conducted by Adams and Ghiorse (1987) from *Leptothrix*, only least part of the Mn(II) oxidizing activity was depending on the Mn^{2+} oxidizing protein and indeed, it was possible by yet unidentified, Mn^{2+} oxidizing factors. In some extent, few studies explored the genes which are responsible for Mn^{2+} oxidation from *Pseudomonas putida* GB-1 (Brouwers et al. 1999), *Bacillus* sp. SG-1 (vanWaasbergen et al. 1996) and *Leptothrix discophora* SS-1 (Brouwers et al. 2000). Recently, bacterial role in Mn(II)

oxidation was expanded to include an indirect oxidation pathway which involves through extracellular superoxide, particularly *Roseobacter* sp. quickly oxidizes Mn(II) to Mn(III) within both cell cultures and cell-free supernatants through the enzymatic making of superoxide (Learman et al. 2011).

4.3.3. Phylogenetic analysis of Mn(II) oxidizers

We could get a total of 46 Mn(II) oxidizers from the venting and non-venting regions of Espalamaca. Largest number of isolates recovered from shallow vent surface water (n=13) followed by vent sediments (n=9), non-vent bottom water (n=9), non-vent surface water (n=8), vent bottom water (n=6) and one isolate from non-vent sediment. To explore the phylogenetic diversity of Mn(II) oxidizing culturable bacteria from Espalamaca, we prepared nearly full length 16S rRNA gene sequences from all the 46 Mn(II) oxidizers. Overall, 43 bacterial isolates belong to *Proteobacteria* (α, β, γ), 2 isolates belong to *Bacteroidetes* and one isolate of *Actinobacteria.* 16S rRNA gene sequence revealed that majority of the Mn(II) oxidizers belong to the class γ-*Proteobacteria* (n=25) with *Marinobacter* being the most abundant genus (n=12) followed by *Vibrio* (n=6) and *Pseudomonas* (n=3). The second most abundant Mn(II) oxidizers belong to the class of α-*Proteobacteria* (n=16), most commonly *Sagittula* (n=5), *Sulfitobacter* (n=3), *Erythrobacter* (n=2) and *Hyphomonas* (n=2) (Table 4.2). Few of other Mn(II) oxidizing bacteria belong to the phyla *Bacteroidetes* and *Actinobacteria*.

Table 4.2. Mn(II) oxidizing bacterial isolates from Espalamaca and their oxidizing rates

Strain no	GenBank Accession no.	16S rRNA gene sequence based identity	Oxidizing rate of Mn
VBW014	KC534149	*Thalassospira lucentensis*	●●●
VBW026	KC534152	*Marinobacter vinifirmus*	●
VSW029	KC534154	*Marinobacter algicola*	●●
VSW030	KC534155	*Marinobacter algicola*	●●●
VSW034	KC534156	*Sulfitobacter pontiacus*	●●●●
VSW036	KC534157	*Sulfitobacter pontiacus*	●●●●
VSW039	KC534158	*Erythrobacter citreus*	●●●
SSW057	KC534161	*Marinobacter algicola*	●●●
SSW058	KC534162	*Marinobacter algicola*	●●●
VSW062	KC534164	*Sulfitobacter pontiacus*	●●●
SBW071	KC534166	*Hyphomonas johnsonii*	●●●●
SSW074	KC534167	*Marinobacter algicola*	●●●
SBW081	KC534170	*Marinobacter algicola*	●●●●
SSW083	KC534171	*Limnobacter thiooxidans*	●●
SSW084	KC534172	*Roseovarius azorensis*	●●
VBW089	KC534175	*Achromobacter spanius*	●●●
VBW090	KC534176	*Kocuria carniphila*	●●●
VSW096	KC534178	*Erythrobacter citreus*	●●●
VBW206	KC534239	*Hyphomonas oceanitis*	●
VBW207	KC534240	*Sagittula stellata*	●
VSW210	KC534242	*Citreicella marina*	●●●●
VSW211	KC534243	*Pseudomonas sabulinigri*	●
VSW212	KC534244	*Pseudomonas marincola*	●●
VSW214	KC534246	*Joostella marina*	●●●●
VSW216	KC534248	*Pseudomonas sabulinigri*	●●
VSW219	KC534250	*Maribacter dokdonensis*	●
SBW223	KC534254	*Marinobacter algicola*	●●
SBW224	KC534255	*Marinobacter algicola*	●
SBW229	KC534260	*Marinobacter algicola*	●●●

SBW230	KC534261	*Marinobacter algicola*	●●●
SSW232	KC534263	*Sagittula stellata*	●●●●
SSW233	KC534264	*Marinobacter algicola*	●●●●
SSW234	KC534265	*Tropicibacter naphthalenivorans*	●●●●
SBW235a	KC534266	*Sagittula stellata*	●●
SBW235b	KC534267	*Sagittula stellata*	●●●●
SBW237	KC534269	*Sagittula stellata*	●●●●
VSD608	KC534273	*Vibrio campbellii*	●●
VSD613	KC534275	*Vibrio rotiferianus*	●●●●
VSD615	KC534277	*Vibrio owensii*	●●●●
VSD617	KC534279	*Vibrio rotiferianus*	●●●
VSG618	KC534280	*Pseudoalteromonas prydzensis*	●
VSG621	KC534283	*Vibrio gigantis*	●●●
VSG622	KC534284	*Shewanella algidipiscicola*	●●
VSG627b	KC534288	*Shewanella colwelliana*	●●●
VSG633	KC534293	*Vibrio gigantis*	●●●
SSA636	KC534295	*Halomonas neptunia*	●●●

Mn oxidation rates: poor (●), average (●●), good (●●●) and excellent (●●●●)

Table 4.3. Earlier studies compared on Mn(II) oxidization with our bacteria

Genus	Concentrations used for Mn(II) oxidation	References
Citreicella	1, 5 mM	Present study
Pseudomonas	1-10 mM	Fernandes et al. 2005
Acinetobacter	1 mM	Beukes and Schmidt 2012
Erythrobacter	0.1 mM	Francis et al. 2001
Pseudomonas	0.1 mM	Brouwers et al. 1999
Bacillus sp.	0.1-7.0 mM	De Vrind et al. 1986
Bacillus sp.	0.1 mM	Wang et al. 2011
Roseobacter	0.1 mM	Learman et al. 2011
Leptothrix	0.1 mM	Adams and Ghiorse 1987
Aurantimonas	0.1 mM	Dick et al. 2008b

Some of the bacterial genera obtained from this study like *Marinobacter*, *Hyphomonas*, *Shewanella*, *Halomonas*, *Pseudomonas*, *Sulfitobacter*, and *Pseudoalteromonas* were also proven as manganese oxidizers in Vailulu'u Seamount by Sudek et al. (2009) and in Loihi Seamount by Templeton et al. (2005). In addition, a yellow pigmented marine *Erythrobacter* sp. was also reported to be Mn(II) oxidizing bacteria from San Diego Bay (Francis and Tebo 2001). However, some of the other well known Mn oxidizers like *Bacillus* (Rosson and Nealson 1982), *Leptothrix* (Adams and Ghiorse 1987), *Citrobacter* (Douka 1980), *Aurantimonas*, *Pedomicrobium* (Dick et al. 2008b), *Loktanella*, *Vibrio* (Sudek at al. 2009), *Microbulbifer*, *Methylarcula* and *Alteromonas* (Templeton et al. 2005), were not encountered in this investigation. Bacterial strains proved to be good Mn oxidizers obtained from this investigation like, *Citreicella*, *Tropicibacter*, *Limnobacter*, *Achromobacter*, *Joostella*, *Maribacter*, *Kocuria* and *Sagittula* were not reported elsewhere as potential Mn oxidizers and it is believed that these members are the specialists in biogeochemical cycle of Mn in Azorean Island.

4.3.4. Pb tolerance studies with Espalamaca isolates

A total of 65 bacterial strains isolated from Pb amended medium which were further tested in higher concentration of Pb to find out the maximum tolerable level (Table 4.4). Results indicated that 69 % of the isolates can grow in presence of 2 mM lead. When concentration increased to 3 mM, 46 % of the isolates were shown growth. 40 % of the isolates could withstand in 5 mM lead and only 3 % of them were able to grow at 7.5 mM Pb. 10 mM Pb was found to be lethal for all the isolates tested (Fig. 4.6).

Table 4.4. Growth of bacterial isolates towards various concentrations of Pb

Strain no.	1 mM	2 mM	3 mM	5 mM	7.5 mM	10 mM
VSW101	+++	+++	++-	++-	---	---
VSW102	+++	+--	+--	+--	---	---
VSW103	+++	---	---	---	---	---
VSW104	+++	---	---	---	---	---
VSW105a	+++	+++	---	---	---	---
VSW105b	+++	+--	---	---	---	---
VSW106	+++	---	---	---	---	---
VSW108	+++	---	---	---	---	---
VSW109	+++	---	---	---	---	---
VSW110	+++	---	---	---	---	---
VSW111	+++	++-	++-	++-	---	---
VSW112	+++	---	---	---	---	---
VSW113	+++	---	---	---	---	---
VSW114	+++	+--	---	---	---	---
VSW115	+++	+++	++-	++-	---	---
VBW118	+++	---	---	---	---	---
VBW119	+++	++-	+--	+--	---	---
VBW120	+++	---	---	---	---	---
VBW122	+++	+--	---	---	---	---
VBW123	+++	+--	---	---	---	---
VBW126	+++	++-	++-	++-	---	---
VBW127	+++	++-	---	---	---	---
VBW128	+++	+--	+--	+--	---	---
VBW129	+++	---	---	---	---	---
SSW130	+++	++-	---	---	---	---
SSW131	+++	---	---	---	---	---
SSW132	+++	---	---	---	---	---
SSW133	+++	---	---	---	---	---
SSW134	+++	+++	++-	++-	---	---
SSW135	+++	---	---	---	---	---
SSW136	+++	---	---	---	---	---
SSW137	+++	---	---	---	---	---
SBW138	+++	---	---	---	---	---

SBW139	+++	+--	+--	+--	---	---
SBW140	+++	+++	++-	++-	---	---
SBW143	+++	---	---	---	---	---
SBW144	+++	---	---	---	---	---
VBW147	+++	+++	+--	+--	---	---
VSD701	+++	++-	+--	---	---	---
VSD702	+++	++-	++-	+--	---	---
VSD706	+++	++-	++-	+--	---	---
VSD707	+++	++-	++-	---	---	---
VSD708	+++	++-	++-	+--	---	---
VSD709	+++	++-	++-	---	---	---
VSD711	+++	+++	++-	+--	---	---
VSD712	+++	++-	++-	+--	---	---
VSD713a	+++	++-	++-	+--	---	---
VSD713b	+++	++-	---	---	---	---
VSD714	+++	++-	++-	++-	---	---
VSD715	+++	++-	++-	+--	---	---
VSG716	+++	++-	+--	+--	---	---
VSG717	+++	++-	+--	---	---	---
VSG718	+++	+--	---	---	---	---
VSG719	+++	++-	+--	+--	---	---
VSG720	+++	++-	++-	++-	---	---
VSG721	+++	++-	++-	+--	---	---
VSG722	+++	++-	---	---	---	---
VSG724b	+++	++-	+--	+--	---	---
VSG725	+++	++-	---	---	---	---
VSG726	+++	++-	---	---	---	---
SSA727	+++	++-	++-	+--	+--	---
SSA728	+++	+--	---	---	---	---
SSA729	+++	+--	---	---	---	---
SSA730	+++	+--	---	---	---	---
SSA732	+++	++-	++-	+--	+--	---

+++, good growth; ++-, average growth; +--, poor growth; ---, no growth

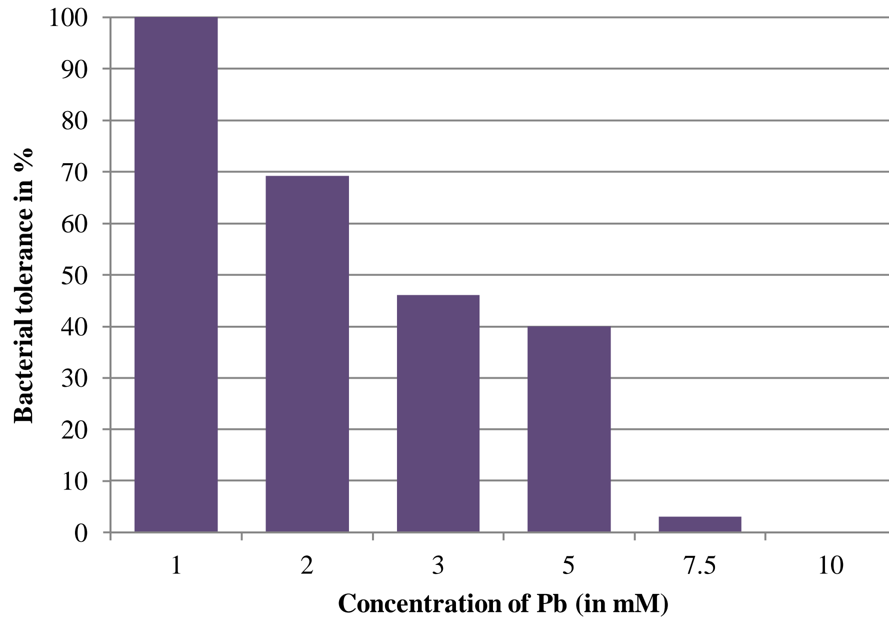


Fig. 4.6. Bacterial tolerance tested with various concentrations of Pb

Strains SSA727 and SSA732 are the only isolates survived in 7.5 mM Pb, which belong to *Pseudoalteromonas flavipulchra* and *P. tetraodonis* respectively. Early reports suggested that some species of *Acinetobacter, Aeromonas, Flavobacterium* and *Pseudomonas* can convert lead nitrate or trimethyl lead acetate to tetramethyl lead (Reisinger et al. 1981, Thayer and Brinckman 1982; Hughes and Poole 1989). *P. marginalis* isolated from metal contaminated soil had a resistance level at 2.5 mM Pb (Roane 1999). Very little work has been done so far on Pb tolerance against these microorganisms.

Gummersheimer and Giblin (2003) reported four distinct bacteria; two were identified as *Corynebacterium* and two belong to the genus *Pseudomonas*, with high resistance to lead (2 mM). Metal resistance of these isolates were encoded by chromosomal DNA. Plasmid encoded lead, zinc, copper and mercury resistance marine bacteria *Ralstonia*

metallidurans CH34 (formarly, *Alcaligenes eutrophus*) were also reported by Diels et al. (1989) and Borremans et al. (2001).

In the present study, two of the Pb tolerant bacterial strains SSW134 and SSW140 belong to *Sagittula stellata* turned almost black after prolonged incubation in presence of 2 and 5 mM Pb. Similarly, Roane (1999) revealed that Pb accumulations were visible as dark granules either inside or surrounding the cells of *P. marginalis* and shown accumulation up to 50 % of the bioavailable lead present in the medium. Similar appearances in the present study were also noticed with *Vibrio*, *Marinobacter*, *Hyphomonas*, *Sagittula* and *Pseudoalteromonas*. This indicates its potential application over detoxification/biosorption of Pb from the industrial wastewaters.

4.3.5. Thiosulfate tolerance studies with vent isolates

Out of 60 strains exposed in higher concentration of thiosulfate, except the strains SSW321 and VSW310; all others could grow up to 7 % thiosulphate. Molecular phylogenetic analysis revealed that genus *Erythrobacter* found to be dominant followed by *Marinobacter* and *Brevibacillus*. In the vent ecosystem oxidations of sulphur compounds are not limited only with sulphur bacteria but also done by the heterotrophic bacteria (Trudinger, 1967; Tuttle et al. 1983; Pandey et al. 2009). Some of the heterotrophic sulphur oxidizing bacteria previously reported are, *Bosea thiooxidans* (Das et al. 1996), *Citreicella thiooxidans* (Sorokin et al. 2005), *Paracoccus pantotrophus* (Rainey et al. 1999), *Silicibacter pomeroyi* (Gonzalez et al. 2003), *Flavobacterium* (Vitolins et al. 1969) and *Comamonas* sp. (Pandey et al. 2009). Our study from the Espalamaca also reveals that some of the heterotrophic bacteria retrieved from this vent could oxidize thiosulphate and grow well.

4.3.6. Iron tolerance studied against vent isolates

Iron bacterial populations in Espalamaca hydrothermal vent sediments were one order higher (10^5 CFU g^{-1}) than the nonvent sediments (10^4 CFU g^{-1}). A total of 34 heterotrophic bacteria were isolated from the iron amended medium (1 mM Fe). Majority of them are belongs to the genera *Pseudoalteromonas* followed by *Amphritea*. Increasing the concentration of Fe to 2 mM was found to be lethal to all the isolates tested. This suggested that 1 mM Fe was the maximum tolerable limit for Espalamaca isolates.

Templeton et al. (2005) reported many heterotrophs which were doing more than one metabolic function (e.g. iron oxidation, siderophore production) in Submarine Basalts at Loihi Seamount and believed that the metal oxidation might not be directly connected to lithoautotrophic growth. As like Loihi Seamount, our studies from shallow hydrothermal vent of Espalamaca in Azores also could occupy many heterotrophs and most of them involved in many works like Fe and Mn oxidation.

4.3.7. Multiple metal/element tolerance observed from the vent isolates

In this study we could obtain many bacterial phylotypes tolerant to one or more metals. Bacterial strains belong to the genera *Pseudoalteromonas*, and *Erythrobacter* were resistant to all the metals (Mn, Pb and Fe) and element (thiosulphate) tested. Some of the *Vibrio* species (Table 4.5) were resistant to all the metals but not to thiosulphate and some of other *Vibrio* species were tolerable with Mn, Pb, Ts. *Alcanivorax*, *Pseudomonas* and *Marinobacter* were some of the other genera resistant to multiple metals (Table 4.5). Characterizations of multiple metal resistant bacteria are of great interest since they promise for bioremediation of toxic heavy metals, including environments that are contaminated by several mixed metals (Jaysankar et al. 2008).

Table 4.5. Multiple metal tolerant phylotypes from Espalamaca

Strains	Identity	metals/element tolerance
VSG719, VSG623, SSA937	*Pseudoalteromonas* sp.	Mn, Pb, Fe
VSG925, VSG921, VSG918, VSD911, VSD906, VSD616, SSA936, SSA929	*Amphritea* sp.	Mn, Fe
SSA932, SSA839, SSA642, SSA833	*Alcanivorax* sp.	Mn, Fe, Ts
VSG926, VSG619, VSG916, VSG828, SSA933, SSA832	*Pseudoalteromonas* sp.	Mn, Fe, Ts
VSG826, VSG722, VSG717, VSG632, VSG630a, VSG630, SSA930, SSA732	*Pseudoalteromonas* sp.	Mn, Fe, Pb, Ts
VSG927, VSG825, SSA830, SSA730, SSA640, SSA727, SSA634	*Pseudoalteromonas* sp.	Mn, Fe, Pb, Ts
VSG924, VBW336	*Marinobacter* sp.	Fe, Ts
VSG920, VSG919, VSD909, VSG829b, VSG631, VSG618, VSG823	*Pseudoalteromonas* sp.	Mn, Fe, Ts
VSG917, VSG721, VSG716, SBW236	*Pseudoalteromonas* sp.	Mn, Pb, Fe
VBW118, VSD908, SBW228	*Pseudomonas* sp.	Mn, Pb, Fe
VSG824, VSD905	*Vibrio* sp.	Fe, Ts
VSD713b, VSG633, VSG626, VSG624, VSG621, VSD903	*Vibrio* sp.	Mn, Pb, Fe
VSD817, VSG720, VSD711, VSD708, VSD608, VSD807, VSD806, VSD714, VSD713a, VSD702	*Vibrio* sp.	Mn, Pb, Ts
VSD816, VSD712, VSD701, VSD617, VSD613, VSD813	*Vibrio* sp.	Mn, Pb, Ts
VSD706, VSD614	*Vibrio* sp.	Mn, Pb
VBW122, VBW119, SSA838b, SSA643, SSA638	*Alcanivorax* sp.	Mn, Pb, Ts
VSG627b, VSG819	*Shewanella* sp.	Mn, Ts

VSW322, VSW317, VSW313, VSW113, VSW112, VSW111, VSW110, SSW319, SSW132, SSW233, SBW230, SBW223, SSW130	*Marinobacter* sp.	Mn, Pb, Ts
VSW108, VSW106, VSW216, VSW316, VSW211	*Pseudomonas* sp.	Mn, Pb, Ts
SSA729, SSA935	*Alteromonas* sp.	Pb, Fe
SSA728, SSA637, SSA831	*Halomonas* sp.	Mn, Pb, Ts
VSG827, VSW315, VBW304, VSW102	*Erythrobacter* sp.	Pb, Ts
VBW303, VSW309, VSW308, VSW217, SSA931, SBW143, SBW139, SBW222, SBW221, SBW220	*Erythrobacter* sp.	Mn, Fe, Pb, Ts
VBW338, VBW337, VSW101, VSW210, SBW344	*Citreicella* sp.	Mn, Pb, Ts
VSW115, VBW207, SBW140, SSW134, SBW237, SBW235b, SBW235a, SSW232	*Sagittula* sp.	Mn, Pb
VSW312b, VSW310, VBW302, SBW138, SBW144, SSW131	*Hyphomonas* sp.	Pb, Ts
VBW129, VBW120, VBW203,	*Arenibacter* sp.	Mn, Pb
VBW128, VBW240	*Vitellibacter* sp.	Mn, Pb
VSW114, VSW104, VSW219	*Maribacter* sp.	Mn, Pb

In conclusion, the venting area in the Espalamaca region is richly diversified with various metal tolerant and oxidizing heterotrophic bacteria. *Proteobacteria* are the key players in Mn oxidation especially γ-*Proteobacteria*. Maximum tolerable level for Mn, Pb and Fe were 50 mM, 7.5 mM and 1 mM respectively. *Citreicella* sp. (VSW210) could oxidize soluble Mn(II) quickly and survive in high concentration of 50 mM Mn. In addition, majority of the bacterial phylotypes isolated from this vent were tolerant to multi-metals. These heterotrophic bacterial isolates may have potential applications in bioremediation where the effluents have a mixer of multi metals.

Chapter 5

Bacterial enzymes from the Espalamaca isolates

5.1. Introduction

The organic matter generated in the marine ecosystem contains high molecular weight compounds, polymeric structures, mainly lipids, proteins, nucleic acids, cellulose, starch, chitin, lignin, etc. (Poremba 1995; Arnosti et al. 1998). These high molecular weight compounds are the source of carbon, nitrogen and energy for heterotrophic bacteria which would be used for respiration and biosynthesis. Heterotrophic members secrete extracellular enzymes to breakdown these complex substances into simple compounds that can be utilized directly by microbes and other organisms (Unanue et al. 1999). Marine hydrothermal vent ecosystem, distinguished by their unusual parameters like high temperature, high concentration of heavy metals, H_2S and hydrocarbons, provide unique opportunity to gain new insights on novel microbes (Gugliandolo et al. 2012) which are able to produce biocatalysts that can cope up with industrial process conditions.

The interest in marine microbial enzymes is related to the ecological attributes of habitat in which marine organisms thrive. Due to the impact of habitation, microorganisms are enabling their biomolecules for their metabolic functions. The resulting enzymatic properties are very important from a biotechnological point of view (Trincone 2011). Though more than 3000 enzymes available to date, many of the available enzymes do not withstand industrial process conditions. Hence, substantial efforts have been assigned for the search of extemophilic enzymes because of increasing industrial demands (Van den Burg 2003). Further, from the literature survey and published patent applications concerning the use of marine enzymes supports the view that these biocatalysts are just waiting to be discovered (Trincone et al. 2011). Some of the enzymes and their applications are given in Table 5.1.

Table 5.1. Some of the microbial enzymes and their applications

Name of the Enzyme	Functions	Sectors in which applied
Protease	Degradation of proteins	Detergent
	Improve digestion of proteins	Animal feed
	Tenderisation	Meat
	Increased area yield	Tanning
Amylase	Degradation of starch	Starch processing
	Conversion of starch to glucose	Starch processing
	Production of high fructose syrup	Starch processing
	Removal of juice starch haze	Fruit/vegetable processing
	Desizing	Textiles
	Process control	Baking
Xylanase	Improve nutrient uptake of poultry	Animal feed
	Removal of lignin bio-bleaching	Paper and pulp
Cellulase	Degradation of cellulose	Detergent
Lipase	Degradation of fats	Detergent
Urease	Removal of urea	Wine

Even though many reports available on enzymatic studies from deep-sea hydrothermal vent microbes, studies from shallow water hydrothermal vents are limited. Lentini et al. (2007) studied the extracellular enzyme activities of thermophilic bacilli isolated from shallow hydrothermal vents located around the Aeolian Islands of Vulcano, Panarea and Lipari (Italy). These isolates were able to hydrolyse gelatin, xylan, dextran and Tween20 and 80, starch, tributyrin and casein. Yavuz et al. (2004) reported that several thermophilic bacterial isolates were able to hydrolyze the substrates Tween 20, Carboxy methyl cellulose (CMC), pectin, casein, xylan and polygalacturonic acid, from geothermal sites in Balcova. Most of these studies were described on Gram positive Bacilli (Demirjian et al. 2001; Maugeri et al. 2001; Gugliandolo et al. 2012). However,

enzymatic profiles of Gram negative *Proteobacteria* strains are less reported especially from shallow hydrothermal vent regions. In this study, we described the enzyme profiles and growth characteristics of 32 bacterial taxa (belong to the phyla *Proteobacteria* and *Bacteroidetes*) isolated from shallow water hydrothermal vent region in Espalamaca (Azores).

5.2. Materials and methods

5.2.1. Screening of vent bacteria for various enzymes

A total of 318 bacterial cultures were isolated from the vent Espalamaca. Among them, 32 bacterial strains showing novelty in 16S rRNA gene sequence were selected for their secretion to various enzymes. Enzymes from the above isolates were looked in to three different categories. First group mainly deal with the oxidoreductases such as catalase, oxidase and nitrate reductase. The second group involves in hydrolytic enzymes such as amylase, lipase, protease, agarose, DNase, etc. The third group of enzymes from API ZYM kit (BioMérieux) which are mainly used in taxonomic identification of bacteria. Bacterial cultures stored in a glycerol vials were taken out aseptically and inoculated on SWNA plates. The enzyme activities were studied by growing them in respective substrates.

5.2.1.1. Enzyme - Oxidoreductases

Catalase

Catalase activity was determined by the addition of a drop of 3 % (v/v) hydrogen peroxide solution on a clean glass slide, to a loopful of bacterial cultures freshly grown

on SWNA. The development of gas bubbles demonstrated the production of catalase enzyme (Smibert and Krieg 1994).

Oxidase

A loopful of bacterial culture was rubbed on oxidase discs containing N, N, N' N'-tetramethyl-p-phenylene diamine dihydrochloride (DD018, Himedia). Formation of deep violet colour within 10 seconds was considered as positive reaction for cytochrome oxidase.

Nitrate reductase

Nitrate reduction test was assessed using Hi25^{TM} Enterobacteriaceae identification kit (Himedia) following the manufacturers' instructions. The formation of pink colour indicates the positive reaction for nitrate reductase.

5.2.1.2. Hydrolytic enzymes

Amylase

The bacterial isolates were spot inoculated on starch-agar plates (SWNA containing 1 % starch) and incubated at 30 ± 2 °C for up to 72 h. After incubation, the plates were flooded with iodine solution (3 % potassium iodide, 0.3 % iodine) and observed for the clear zone around the colony (Mohandass et al. 2005).

Xylanase

The isolates were spot inoculated on xylan-agar plates (SWNA containing 1 % xylan) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the plates were

flooded with 1 % Congo-red and then washed with 1.5 M NaCl. Zone of clearance around the colony indicates the production of xylanase (Khandeparker et al. 2011).

Carboxy methyl cellulose (CMCase)

The bacterial strains were spot inoculated on CMC-agar plates (SWNA containing 1 % CMC) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the plates were flooded with 1 % Congo-red and then washed with 1.5 M NaCl. Zone of clearance around the colony indicates the production of CMCase (Khandeparker et al. 2011).

Gelatinase

The bacterial strains were spot inoculated on gelatin-agar plates (SWNA containing 1 % gelatin) and incubated at 30 ± 2 °C for up to 72 h. After incubation, the plates were flooded with 15 % TCA (Trichloro-Acetic Acid) and observed for clear zones around the colonies (Smibert and Krieg 1994).

Casease

The bacterial strains were spot inoculated on casein-agar plates (SWNA containing 1 % casein) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the plates were flooded with 15 % TCA and observed for clear zones around the colonies (Gerhardt et al. 1981).

Urease

The bacterial strains were spot inoculated on urea agar base medium (M112, Himedia) and incubated at 30 ± 2 °C for up to 72 h. The plates were observed for positive result by appearing pink colour on the medium.

DNase

The bacterial strains were spot inoculated on DNase test agar (M1041, Himedia) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the plates were flooded with 1N HCl and observed for clear zones around the colonies.

Lipase

The bacterial strains were spot inoculated on agar plates (SWNA containing 1 % Tween-20 & SWNA containing 1 % tributyrin) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the formations of an opaque halo around the colonies were considered as lipase producers (Gerhardt et al. 1981).

Esterase

The bacterial strains were spot inoculated on Tween-agar plates (SWNA containing 1 % Tween-80) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, the formations of an opaque halo around the colonies were considered as esterase producers (Smibert and Krieg 1994).

Alginate lyase

The bacterial strains were spot inoculated on alginate-agar plates (SWNA containing 1 % alginic acid) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, clear zones around the colonies were considered as positive reaction (Smibert and Krieg 1994).

Xanthine dehydrogenase

The bacterial strains were spot inoculated on xanthine-agar plates (SWNA containing 0.4

% xanthine) and incubated at 30 ± 2 °C for up to 72 h. After the incubation period, clear zones around the colonies were considered as positive reaction (Smibert and Krieg 1994).

Agarase

The bacterial isolates were spot inoculated on YE-agar plates (yeast extract 0.05 %, peptone 0.25 % in 50 % seawater containing 2 % agar) and incubated at 30 ± 2 °C for up to 72 h. After incubation, the plates were flooded with I-KI solution and observed for the clear zone around the colony (Smibert and Krieg 1994; Temuujina et al. 2012).

5.2.1.3. Enzymes tested using API ZYM stripes

All the bacterial cultures were grown in SWNA and the cell suspensions were prepared with a turbidity equivalent to 5-6 McFarland in sterile distilled water. A volume of 65 µL was inoculated into each cupule of API ZYM strips (BioMérieux). The API ZYM strips were incubated for 5 hours at 32 °C. After the incubation period, one drop of ZYM-A reagent and one drop of ZYM-B reagent (supplied with API ZYM BioMérieux kit) were added to each cupule. Based on the colour development, the enzyme activities were determined following the manufacturer's instructions. The enzymes and substrates provided in API ZYM strips of BioMérieux are given in Table 5.2.

5.2.2. Growth characteristics of bacterial isolates

5.2.2.1. Salinity

Salinity tolerance was investigated in nutrient agar medium prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increment) with and without adding

Table 5.2. Enzymes and the substrates in API ZYM strips (BioMérieux)

No	Enzyme assayed	Substrate
1	Alkaline phosphatase	2-naphthyl phosphate
2	Esterase (C 4)	2-naphthyl butyrate
3	Esterase Lipase (C 8)	2-naphthyl caprylate
4	Lipase (C 14)	2-naphthyl myristate
5	Leucine arylamidase	L-leucyl-2-naphthylamide
6	Valine arylamidase	L-valyl-2-naphthylamide
7	Cystine arylamidase	L-cystyl-2-naphthylamide
8	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide
9	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide
10	Acid phosphatase	2-naphthyl phosphate
11	Naphthol-AS-BI-phospnohydrolase	Naphthol-AS-BI-phosphate
12	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
13	β-galactosidase	2-naphthyl-βD-galactopyranoside
14	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide
15	α-glucosidase	2-naphthyl-αD-glucopyranoside
16	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
17	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
18	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
19	α-fucosidase	2-naphthyl-αL-fucopyranoside

Mg^{2+} and Ca^{2+} (4.53 g L^{-1} $MgCl_2$; 5.94 g L^{-1} $MgSO_4$; 1.3 g L^{-1} $CaCl_2$). Bacterial isolates were streaked on the above medium and growth was measured after 2 days of incubation.

5.2.2.2. Temperature and pH

The pH range for growth was determined by adjusting the pH of the SWNB medium (nutrient broth prepared in 50 % seawater) using various buffers, pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer). Growth at various temperatures 4, 10, 15, 20, 30, 37, 40 and 45 °C was obtained by incubating the SWNB

medium with respective temperatures. Growth was measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation.

5.2.3. 16S rRNA gene sequence based identification

Genomic DNA extraction, PCR amplification, DNA sequencing and 16S rRNA based identification followed by the methods given in section 2.2.5. Corresponding accession numbers of 32 isolates used here are given in Table 5.6.

5.3. Results and discussion

5.3.1. Enzyme profiles

We have characterized 32 bacterial isolates from the Espalamaca region (both from venting and non-vent area) to study their enzymatic profiles. The study results indicated that the Oxidoreductase enzymes such as catalase and oxidase are widespread in the Espalamaca bacterial isolates (Table 5.3). Thirty one isolates produced catalase and 28 isolates were able to produce cytochrome oxidase out of 32 isolates tested, while nitrate reductase enzyme was found in only 15.6 % of the isolates (Fig. 5.1). Nishiyama et al. (1997) reported that oxidase enzymes are widely present in bacteria which acquire an aerobic type of metabolism for the regeneration of NAD in the respiratory system. On the other hand, the enzyme catalase is involved in hydrogen peroxide removal by aerobic microorganisms. The detection of high catalase activity in Espalamaca sediments and waters suggests the presence of bacteria that can alternatively grow with oxygen as an electron donor (Kobayachi et al. 2008). It is remarkable that oxidase and catalase enzymes are reportedly higher in bacterial strains isolated from the other shallow hydrothermal vent in Azores Island (D. Joao de Castro seamount) (Mohandass et al. 2012).

Table 5.3. Oxidoreductases and Hydrolytic enzymes produced by the bacterial strains isolated from Espalamaca, Azores

	Bacterial strains															
Enzymes	VBW 004	VBW 018	SSW 083	VBW 095	VBW 098	VSW 114	VBW 122	VBW 206	VSW 210	SBW 235b	VBW 302	VSW 306	VSW 310	SSA 543	SSA 549	SSA 555
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Nitrate reductase	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Urease	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	+	-	-	+	-	-	-	-	w	-	-	+	-
Amylase	-	-	-	-	w	+	-	-	-	w	-	-	-	-	-	-
DNase	+	+	-	-	-	-	-	-	+	-	+	+	-	+	+	+
Casease	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Lipase	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
Esterase	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Agarase	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-
Xanthine dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(Table 5.3 continued)

Enzymes	Bacterial strains															
	VSG 724	VSG 829	VBW 240	VSD 707	VBW 088	VSG 820	VSG 922	VSW 331	VBW 339	VBW 123	SSW 136	SSW 234	SSW 084	VSW 109	SSW 321	VBW 011
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Oxidase	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reductase	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+	-
Urease	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	w
Gelatinase	-	-	+	-	+	-	-	-	w	+	+	-	-	-	-	-
Amylase	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
DNase	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	+
Casease	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Lipase	w	+	+	+	+	-	+	+	-	+	-	+	-	+	+	-
Esterase	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
Agarase	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
Xanthine dehydrogenase	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-

+, positive; -, negative; w, weak reaction

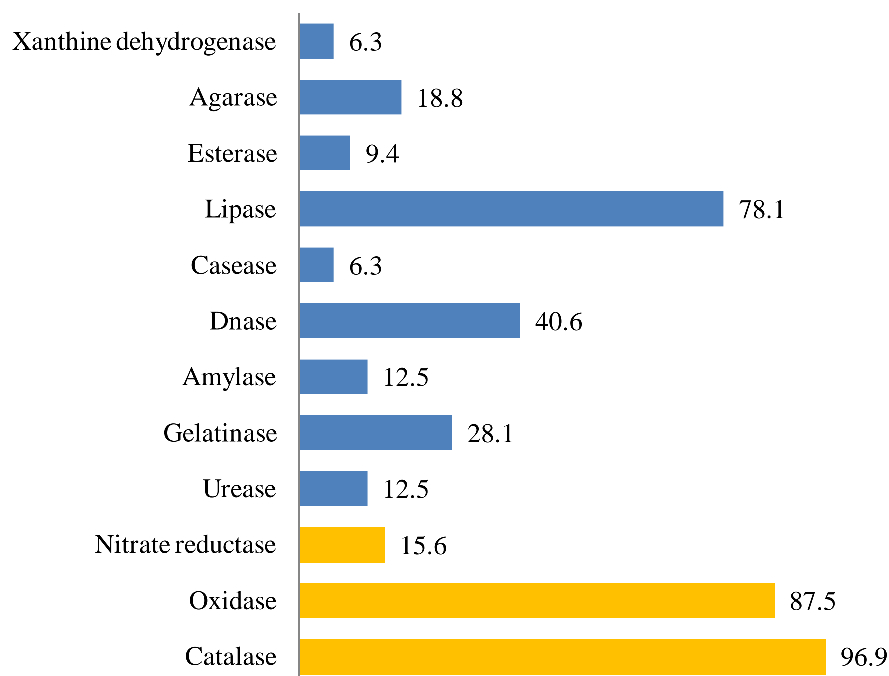


Fig. 5.1. Percentage of bacteria producing hydrolytic (blue) and oxidoreductase (yellow) enzymes

Production and activity of microbial hydrolytic enzymes are depending on the distribution and availability of organic substances (Boetius 1995). Among the hydrolytic enzymes tested, strains producing lipase were the most diverse and abundant functional group in this study area (Fig. 5.1). Higher proportion of lipase activities in this region suggested the water and sediment samples might be enriched with relatively high lipid content. Twenty five isolates (78.1 %) were produced lipase and 40.6 % of the isolates (n=13) were able to hydrolyze DNA. The enzyme DNase is well known for making phosphorous available to the environment by degrading nucleic acids. Further, it is also one of the carbon and nitrogen source for prokaryotic metabolism (Jorgensen et al. 1993; Dell'Anno and Danovaro 2003). Around 28.1 % (n=9) and 6.3 % (n=2) of the isolates were hydrolyzed the substrates gelatin and casein by producing the enzyme protease. Many heterotrophic members in the marine environment are capable of synthesizing

protease enzymes, which hydrolyze the proteins into mono- or oligomers, mainly peptides and amino acids (Mudryk and Podgórska 2006). Six isolates were produced the enzyme agarase and 4 isolates were able to hydrolyze starch and urea by producing amylase and urease respectively. Esterase was produced by 3 isolates and 2 isolates hydrolyzed the substrate xanthine (Table 5.3; Fig. 5.1). VSG820 and SSW083 are the only isolates did not produce any hydrolytic enzymes tested; they belong to the genera *Ruegeria* and *Limnobacter* respectively. Substrates like CMC, alginate, xylan and tributyrin were not hydrolyzed by any of the isolates tested. It is noteworthy to mention that bacterial isolates from nearby hydrothermal vent DJCS produced 55, 45 and 27 % of amylase, casease and lipase respectively (Mohandass et al. 2012).

In API ZYM test, 100 % of the isolates produced leucine arylamidase, enzyme that breaks down amino acid leucine into acetyl acetate and acetyl-coA. Esterase (C 4), esterase lipase (C 8) and Naphthol-AS-BI-phosphohydrolase were produced by 96.8 % of the isolates each. Esterases and lipases are widely distributed within the microbial communities functioning in most of environments where they have important physiological roles (McQueen and Schottel 1987). Apart from that, alkaline phosphatase and acid phosphatase are also prevalent enzymes synthesized by 81.2 % and 78.1 % of the Espalamaca isolates (Table 5.4; Fig. 5.2). Interestingly, Esterase (C4), esterase lipase (C8), alkaline and acid phosphatase activities were more frequently found in *Geobacillus* strains isolated from shallow hydrothermal vents in Aeolian Islands, Italy (Lentini et al. 2007). Phosphatase enzymes are a significant component of most of the marine bacteria and play a major role in recycling of organic phosphorus to avoid phosphorus limitations in marine ecosystem (Hoope 2003). These bacterial taxa have a predominant role in supplying phosphorus to heterotrophic and autotrophic microorganisms (Moutin et al. 2008). High availability of the enzyme phosphatase and moderate level of DNase from

the present study area suggested that these two enzymes play an important role in phosphorus, carbon and nitrogen cycles in the vent Espalamaca.

A total of 7 isolates (21.8 %) produced the enzyme β-glucosidase and 12 isolates (37.5 %) produced valine arylamidase. Other enzymes in the API ZYM stripes are produced by very less number of isolates (Table 5.4; Fig.5. 2). None of the isolates produced the enzymes β-glucuronidase, α-mannosidase and α-fucosidase.

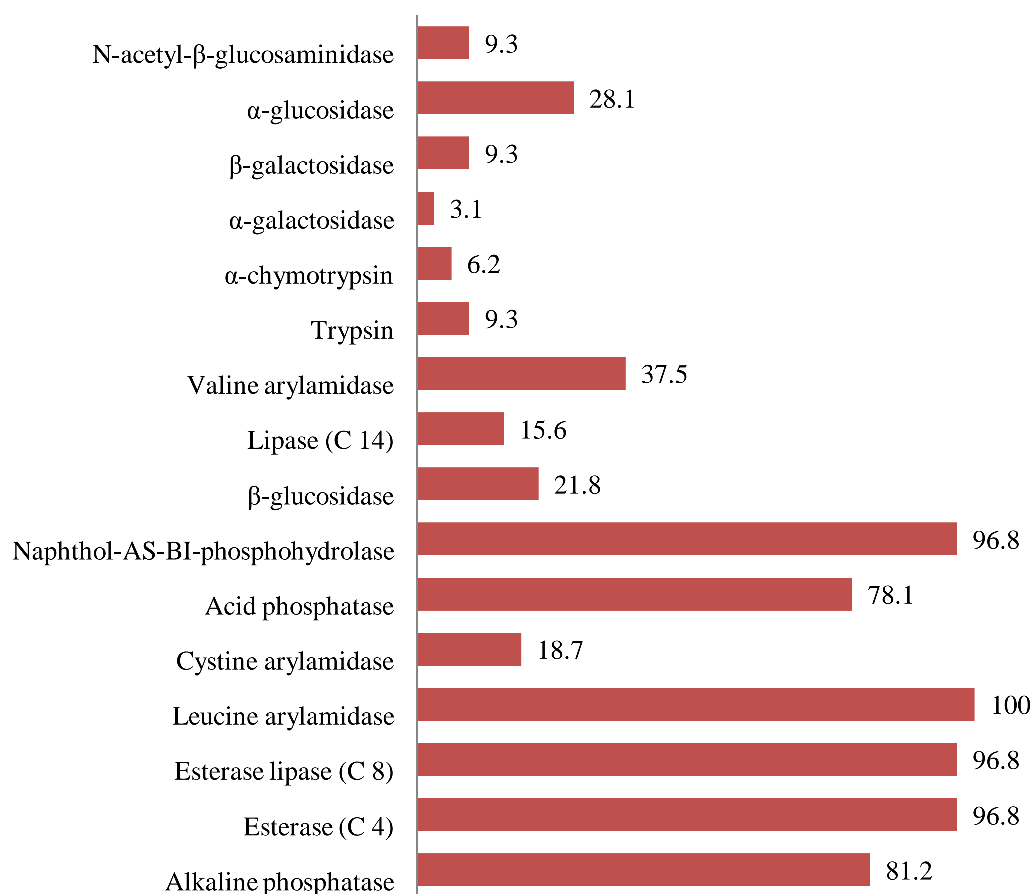


Fig. 5.2. Percentage of bacterial taxa producing enzymes tested in API ZYM

Table 5.4. Enzymes (in API ZYM) produced by bacterial strains from Espalamaca, Azores

	Bacterial strains															
Enzymes	VBW 004	VBW 018	SSW 083	VBW 095	VBW 098	VSW 114	VBW 122	VBW 206	VSW 210	SBW 235b	VBW 302	VSW 306	VSW 310	SSA 543	SSA 549	SSA 555
Alkaline phosphatase	+	+	+	+	+	+	-	+	w	+	+	+	+	+	+	+
Esterase (C 4)	+	+	w	+	+	+	+	w	+	+	+	+	+	-	+	+
Esterase lipase (C 8)	+	+	+	+	+	+	+	w	+	+	+	+	+	w	+	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cystine arylamidase	w	-	-	-	-	-	-	-	-	-	-	-	+	-	w	w
Acid phosphatase	+	+	+	-	+	+	-	+	w	-	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+
β-glucosidase	-	-	-	-	w	-	-	-	-	-	-	-	+	-	-	-
Lipase (C 14)	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Valine arylamidase	-	-	-	+	+	w	-	-	-	-	+	+	-	-	-	-
Trypsin	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
α-chymotrypsin	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
α-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-glucosidase	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

(Table 5.4 Continued)

Enzymes	Bacterial strains															
	VSG 724	VSG 829	VBW 240	VSD 707	VBW 088	VSG 820	VSG 922	VSW 331	VBW 339	VBW 123	SSW 136	SSW 234	SSW 084	VSW 109	SSW 321	VBW 011
Alkaline phosphatase	+	+	+	+	+	+	-	+	-	-	+	w	-	+	-	+
Esterase (C 4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase lipase (C 8)	+	+	+	w	+	+	+	+	+	+	+	+	+	+	+	-
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cystine arylamidase	-	w	-	-	w	-	-	-	-	-	-	-	-	-	-	-
Acid phosphatase	+	+	+	+	+	+	-	+	-	w	+	+	-	+	-	w
Naphthol-AS-BI-phosphohydrolase	+	+	w	w	+	+	+	+	+	+	+	-	+	+	w	+
β-glucosidase	-	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-
Lipase (C 14)	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
Valine arylamidase	-	-	-	-	+	+	-	-	w	-	-	-	-	w	w	w
Trypsin	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-
α-chymotrypsin	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
α-galactosidase	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
β-galactosidase	-	-	-	-	-	w	-	-	+	-	-	w	-	-	-	-
α-glucosidase	-	-	-	-	-	-	-	+	w	-	+	w	-	-	-	+
N-acetyl-β-glucosaminidase	w	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

All strains are negative for β-glucuronidase, α-mannosidase and α-fucosidase. +, positive, -, negative; w, weak reaction;

About 78 % of the bacterial strains were produced ≥10 enzymes. Among them, a strain designated VSW306 belong to the genus *Cellulophaga* synthesized up to 15 enzymes (which includes 10 enzymes from API ZYM). Isolates VSW331, VBW339, SSW234, VSW114, VSW310 and SSA549 belong to the genera *Rhizobium*, *Devosia*, *Tropicibacter*, *Maribacter*, *Hyphomonas* and *Alcanivorax* respectively, were produced 13 enzymes each (Table 5.5). This indicates that the in situ microbiota might have developed the adaptation of genetic and physiological activity for utilizing the high content of organic matters via exoenzyme production (Dang et al. 2009). Some of the above mentioned isolates not only synthesised more than 10 enzymes but also resistant to many metals and element. Presence of these types of organisms in this vent may be an added advantage to play a major role in biogeochemical cycle of the vent Espalamaca.

5.3.2. Phylogenetic analysis of bacterial isolates

Based on the 16S rRNA gene sequences, 32 strains were selected for enzyme studies which belong to two phyla i.e., *Bacteroidetes* and *Proteobacteria* of the α- and γ-*Proteobacteria*. Identification based on closest neighbour retrieved from EzTaxon-e server (Kim et al. 2012a) is placed in Table 5.6.

5.3.3. Growth characteristics of the bacterial isolates

Growth of the isolates was found to occur at a pH range of 6 to 9.5. Optimum pH for growth was varied from strain to strain. Bacterial isolates preferred temperatures between 4 and 40 °C with an optimum of 30 °C for many of the strains. No growth was observed on temperatures of above 40 °C (Table 5.6). It is worth mentioning here that maximum temperature in this hydrothermal vent was observed to be 35 °C, hence the bacterial groups preferred mesophilic conditions for growth (optimum at 25 – 37 °C).

Table 5.5. Multiple enzymes (≥13) produced by the Espalamaca isolates

Strain	Enzymes produced	Number of enzymes
VSW331	Catalase, Oxidase, Urease, Lipase, Agarase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, β-glucosidase, α-glucosidase	13
VBW339	Catalase, Oxidase, Urease, Gelatinase, Xanthine dehydrogenase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Naphthol-AS-BI-phosphohydrolase, β-glucosidase, Valine arylamidase, β-galactosidase, α-glucosidase	13
SSW234	Catalase, Oxidase, Lipase, Agarase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Acid phosphatase, β-glucosidase, α-galactosidase, β-galactosidase, α-glucosidase	13
VSW114	Catalase, Oxidase, Urease, Amylase, Lipase, Agarase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, Valine arylamidase	13
VSW306	Catalase, Gelatinase, Dnase, Lipase, Agarase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, Valine arylamidase, Trypsin, α-glucosidase, N-acetyl-β-glucosaminidase	15
VSW310	Catalase, Oxidase, Nitrate reductase, Lipase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Cystine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, β-glucosidase, α-glucosidase	13
SSA549	Catalase, Oxidase, Gelatinase, Dnase, Lipase, Alkaline phosphatase, Esterase (C 4), Esterase lipase (C 8), Leucine arylamidase, Cystine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, Lipase (C 14)	13

Bacterial isolates obtained from this study were having a wide range of salinity tolerance (0 – 15 % NaCl). Growth of strains VBW004, VBW018 and SSA555 belong to the genera *Alcanivorax* were observed at a maximum concentration of NaCl (15 %) in the present study. Strains VBW302 and SBW235b were able to grow up to 13 % NaCl. Growth of half the isolates from the present study area was found to occur with ≥10 % NaCl. Only few isolates (SSW321, SSW083, VSW114 and VSW306) did not prefer to grow with ≥5 % NaCl, otherwise all the remaining stains were able to grow well with ≥5 % NaCl. The detailed salinity ranges for growth and optimum concentrations are given in Table 5.6. The salinity results suggested that most of the bacteria are belonging to the category of moderate halophiles (5 – 10 % NaCl) and few of them are slight halophiles (2 – 5 % NaCl).

Mesophilic enzymes are the biological catalysts, which perform their role in normal temperatures. Even though the Espalamaca isolates were belong to mesophilic category, the enzymes produced by these isolates may have flexible temperature ranges. For instance, studies conducted by us in similar kind of shallow water hydrothermal vent in D. Joao de Castro Seamount (DJCS, Azores) revealed that mesophilic bacteria are producing enzymes which are very well active at 70 °C (Mohandass et al. 2012). In addition to this, majority of the Espalamaca isolates are well adapted to survive with various heavy metals (chapter 4). The current results on bacterial growth explained, most of the Espalamaca strains preferred to grow well with neutral pH, temperature 30 °C and wide preference of NaCl (0-15 %). Though the enzyme studies carried out here at pH 7.5, 30 °C with 1.5 % NaCl, further characterization on these enzymes with optimal growth characteristics (given in Table 5.6) may have better prospects in enzyme activity and their stability to apply in biotechnological industries such as starch processing, detergent, animal feeds, textiles and tanning.

Table 5.6. Growth characteristics and 16S rRNA gene sequence based identity of bacteria from Azorean Island

Strain no.	Identification by 16S rRNA gene sequence	GenBank Accession no.	pH range (opt)	Temp range (opt) °C	Salinity (opt) %
VBW088	*Vitellibacter nionensis*	KC534174	6-8 (6)	10-37 (30)	1-5 (1-2)
VSG820	*Ruegeria lacuscaerulensis*	KC534405	6-8 (6)	15-37 (25)	2-10 (3-5)
VSG922	*Neptunomonas naphthovorans*	KC534445	6-9 (7-8)	10-40 (30)	2-10 (6-8)
VSW331	*Rhizobium vignae*	KC534384	6-8 (8)	4-40 (30)	0-10 (3-5)
VBW339	*Devosia subaequoris*	KC534392	6-9 (7-8)	4-40 (30)	2-10 (5)
VBW123	*Sulfitobacter donghicola*	KC534320	6-9 (7)	10-40 (37)	3-10 (7)
SSW136	*Nioella nitratireducens*	KC534331	6-8 (6)	10-37 (30)	2-11 (8)
SSW234	*Tropicibacter naphthalenivorans*	KC534265	6-8 (8)	15-37 (30)	1-10 (4)
SSW084	*Roseovarius azorensis*	KC534172	7-9 (7)	15-40 (30)	0.5-7 (4-5)
VSW109	*Maribacter doktonensis*	KC534309	6-8 (8)	10-35 (30)	1-8 (5)
VBW004	*Alcanivorax borkumensis*	KC534144	7-9.5 (7)	10-35 (30)	1-15 (5-7)
VBW018	*Alcanivorax borkumensis*	KC534150	7-9.5 (7)	10-35 (30)	1-15 (5-7)
SSW083	*Limnobacter thiooxidans*	KC534171	6-9.5 (7-8)	15-37 (37)	1-3 (2)
VBW095	*Aequorivita antarctica*	KC534177	6-9 (7)	10-37 (30)	1-6 (3)
VBW098	*Cyclobacterium marinum*	KC534179	6-9.5 (7)	4-40 (30)	2-8 (3)
VSW114	*Maribacter dokdonensis*	KC534314	6-8 (7)	10-35 (30)	2-5 (3)
VBW122	*Alcanivorax jadensis*	KC534319	7-9 (7)	15-37 (30)	1-9 (5)

VBW206	*Hyphomonas oceanitis*	KC534239	6-9.5 (7)	10-30 (30)	1-9 (3)
VSW210	*Citreicella marina*	KC534242	6-9.5 (7)	4-40 (30)	1-10 (6)
SBW235b	*Sagittula stellata*	KC534267	6-9.5 (7)	10-35 (30)	1-13 (3-4)
VBW302	*Hyphomonas johnsonii*	KC534366	6-9.5 (6-7)	10-37 (30)	1-13 (7-8)
VSW306	*Cellulophaga tyrosinoxydans*	KC534369	7-8 (7)	10-35 (30)	1-4 (2)
VSW310	*Hyphomonas johnsonii*	KC534373	6-9 (7)	10-35 (30)	1-6 (3)
SSA543	*Alcanivorax borkumensis*	KC534220	7-9.5 (7)	15-30 (30)	1-9 (5)
SSA549	*Alcanivorax jadensis*	KC534225	7-9.5 (7)	15-37 (30)	1-6 (4)
SSA555	*Alcanivorax borkumensis*	KC534231	7-9.5 (7)	10-40 (37)	1-15 (7-8)
VSG724	*Spongiibacter tropicus*	KC534358	7-9.5 (7)	15-40 (37)	1-6 (4)
VSG829	*Pseudoalteromonas prydzensis*	KC534414	6-9.5 (7)	10-35 (30)	2-15 (8-10)
SSW321	*Porphyrobacter donghaensis*	KC534382	6-9 (7)	15-37 (30)	1-4 (3)
VBW240	*Vitellibacter vladivostokensis*	KC534271	6-8 (7)	10-40 (30)	1-5 (3)
VSD707	*Vibrio plantisponsor*	KC534342	6-9 (6)	4-40 (25)	1-10 (4-5)
VBW011	*Hoeflea suaedae*	KC534148	6-8 (7)	10-40 (30)	0-11 (5-8)

In conclusion, the shallow water hydrothermal vent and surrounding regions in Espalamaca restrain diverse novel microbes which are dynamic in catabolism by producing enormous enzymes. These novel bacteria are mesophilic and moderately halophilic in nature. Catalase, oxidase, esterase, lipases, phosphatase, and leucine arylamidase are the foremost enzymes produced by the vent strains. Highly abundant phosphatase and moderately available protease and DNase from this environment may involve in regeneration of nutrients and play a significant role in P, C and N cycles. Multiple enzymes producing organism from this vent may be of greater help to use in industriesm and environmental applications.

Chapter 6

Taxonomic characterization of novel bacterial taxa

Chapter 6A

Nioella nitratireducens gen. nov., sp. nov., a novel member of the family *Rhodobacteraceae* isolated from Espalamaca, Azores

6A.1. Introduction

The family *Rhodobacteraceae* which belongs to the phylogenetic class α-*Proteobacteria* and comprises of more than 100 recognized genera (http://www.bacterio.net/classifgenerafamilies.html#Rhodobacteraceae) was proposed by Garrity et al. (2005) with *Rhodobacter* as the type genus of this family. Around 55 % of the genera from the family *Rhodobacteraceae* consist of only one species, on the other side *Paracoccus* being the largest, includes 40 species. During a comparative study on bacterial diversity from shallow water hydrothermal vent and non-vent regions of Espalamaca (Azorean Island), a strain designated SSW136^T, was isolated from the surface seawater of the non-vent region at Espalamaca. The main aim of the present investigation was to establish the precise taxonomic position of the strain SSW136^T using polyphasic approach.

6A.2. Materials and Methods

6A.2.1. Isolation and maintenance of the bacterial strain

The strain SSW136^T was obtained from the surface seawater collected from the Espalamaca region during September 2010. Serially diluted samples were spread plated (100 µL) on diluted sea water nutrient agar (Peptone 1.25 g L^{-1}, Beef extract 0.75 g L^{-1} and 1.5 % Agar in 50 % seawater) amended with 1 mM $Pb(NO_3)_2$ and incubated for 3 days at 30 °C. Strain SSW136^T was maintained on seawater nutrient agar (SWNA: Peptone 5.0 g L^{-1}, Beef extract 3.0 g L^{-1}, 1.5 % Agar prepared in 50 % seawater) at 4 °C for short-term maintenance and as a 15 % glycerol suspension at -80 °C for long-term maintenance.

6A.2.2. Morphological, physiological and biochemical characterization

Gram staining was carried out using the standard Gram staining method, and counter checked by KOH test (Cerny 1978). Motility was determined by hanging drop method (Collee et al. 2008) under 1000 × magnification and confirmed by motility agar test. Spore staining was performed using Schaeffer and Fulton's spore stain kit (K006, Himedia). Morphological characteristics of strain SSW136^T were observed by light microscopy (Olympus BX-60) and scanning electron microscopy (Hitachi TM3000). Growth at temperature range of 4, 10, 20, 25, 30, 37, 40 and 50 °C was tested on sea water nutrient broth (SWNB, the compositions are same as SWNA excluding agar). The pH range for growth was determined in SWNB adjusted to pH 4–10 (1 unit increment) by using acetate (pH 4–5), carbonate (pH 9–10) (Lee et al. 2012) and phosphate (pH 6–8) buffer systems (Wang et al. 2012). For pH and temperature experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation. NaCl tolerance tests were examined with different NaCl concentrations from 0 to 15 % (1 % increment) on nutrient agar (NA) prepared with distilled water. Catalase activity was detected by the production of bubbles after the addition of a drop of 3 % H_2O_2 (Smibert and Krieg 1994). Oxidase activity was determined by the oxidation of tetramethyl-*p*-phenylenediamine. Nitrate reduction, Methyl red, Voges-Proskauer tests, determination of indole and H_2S production, were assessed using Hi25™ Enterobacteriaceae identification kit (Himedia). Utilization of various carbon sources was determined using API50CH strips (bioMérieux) according to the manufacturer's instructions with inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer and Hickman-Brenner 2006). Hydrolysis of urea and DNA were determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Xylan, starch and carboxymethyl cellulose hydrolysis were

determined as per the methods provided by Khandeparker et al. (2011). Hydrolysis of casein (1 %), gelatin (1 %), Tweens 20 and 80 (1 %), tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert and Krieg 1994) were tested on SWNA medium. API ZYM kit (bioMérieux) was used to analyse the various substrate utilization according to manufacturer's protocol.

Antibiotic susceptibility tests were performed on SWNA media using antibiotic discs (Himedia) containing the following concentrations (μg per disc unless indicated): ampicillin (25), kanamycin (30), streptomycin (300), chloramphenicol (25), tetracycline (10), ciprofloxacin (30), ceftazidime (30), lincomycin (10), novobiocin (30), neomycin (30), vancomycin (5), amoxycillin (30), cefadroxyl (30), tobramycin (10), chlortetracycline (30), rifampicin (15), amikacin (30), norfloxacin (10), penicillin-G (2 IU), polymyxin-B (50 IU) and bacitracin (8 IU).

6A.2.3. Phylogenetic analyses

Genomic DNA of strain SSW136^T was isolated using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was PCR amplified using universal eubacterial primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane 1991). The PCR products were purified using PCR cleanup kit (Sigma) as per the method provided by the manufacturer. Sequencing of the amplified 16S rRNA gene was performed by using the automated 3130xl DNA analyzer (Applied Biosystems). The sequence alignment was carried out using Clustal W sequence alignment program (Thompson et al. 1994). The 16S rRNA gene sequences of the neighbouring taxa were obtained from the GenBank database. Phylogenetic trees were established with neighbour-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971) algorithms using MEGA 5 software

(Tamura et al. 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985).

DNA-DNA hybridization was performed between strain SSW136^T and *Oceanicola litoreus* M-M22^T by the method proposed by Ezaki et al. (1989). Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA-DNA relatedness values.

6A.2.4. Chemotaxonomy

Cell biomass of the strain SSW136^T for the analysis of polar lipids and isoprenoid quinones was obtained from the cultures grown in LB broth (M1245, Himedia) prepared in 50 % seawater for 2 days at 30 °C. For the analysis of whole cell fatty acids, strain SSW136^T and *Oceanicola litoreus* M-M22^T were harvested from MA plates after cultivation for 5 days at 30 °C. Fatty acids were saponified, methylated and extracted using the standard procedure of Sherlock microbial identification system (MIDI 6.2B). The fatty acids were analysed by Gas Chromatograph (Hewlett Packard 6890) and identified using TSBA 6.0 database (Sasser 1990). Polar lipids were extracted and examined according to the procedures described by Collins et al. (1980). Briefly, polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second direction was developed in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were detected using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), and α-naphthol reagent (sugars). Isoprenoid quinones were

determined as depicted by Minnikin et al. (1984). The DNA G+C content of strain SSW136^T was determined by reverse-phase HPLC of nucleosides according to Mesbah et al. (1989).

6A.3. Results and Discussion

Morphological observations of 2 days old colonies grown in SWNA media were observed to be punctiform, white, convex and circular in shape. Strain SSW136^T was found to be a Gram negative, non-motile, rod shaped (Fig. 6A.1) and non-spore forming bacterium. The temperature range for growth was determined to be 10–37 °C, with optimal growth at 30 °C. The pH range for growth was determined to be 6–8, with optimal pH 6. Growth of strain SSW136^T was observed at NaCl concentrations between 2 and 11 %, with an optimum of 8 %. Strain SSW136^T was found to be susceptible to all the tested antibiotics. Strain SSW136^T was distinguishable from the closely related taxa by differences in several phenotypic characteristics represented in Table 6A.1.

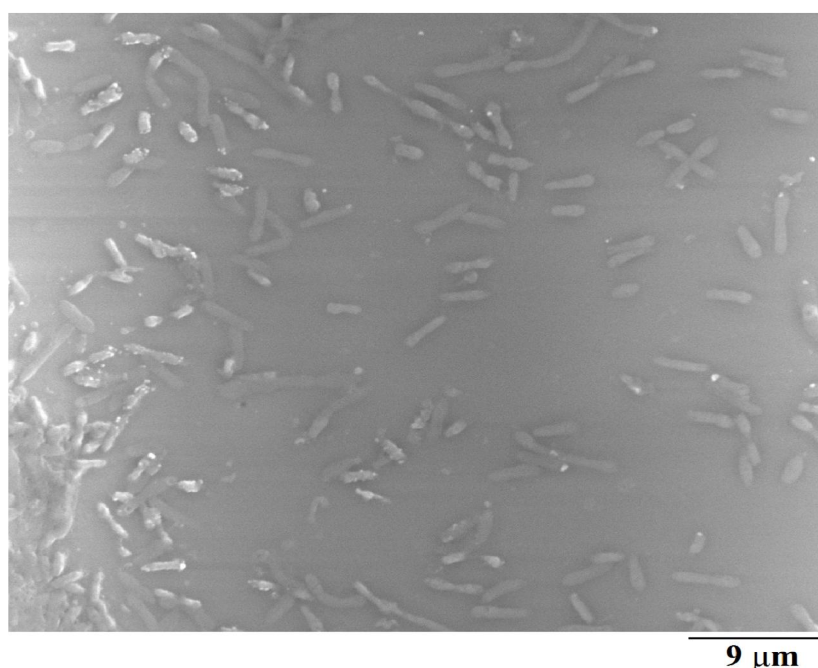


Fig. 6A.1. Scanning electron microscopic image of the strain SSW136^T

Table 6A.1. Characteristics that differentiate strain SSW136^T and closest type strains in the family *Rhodobacteraceae*

Characteristic	1	2	3	4	5
Colony colour[†]	Wh	GY	C	GY	GY
Cell size (>10 µm)	+	-	-	-	-
Motility	-	-	+	-	-
Growth at 4 °C	-	+	-	-	-
Growth at 40 °C	-	+	-	+	+
Growth at 10 % NaCl	+	-	-	-	-
Nitrate reduction	+	-	+	+	-
Hydrolysis of:					
Casein	-	-	+	+	-
Gelatin	+	+	-	-	-
Tween 20	-	+	-	+	-
Tween 80	-	+	-	+	-
Aesculin	+	+	-	+	+
Urea	-	+	-	ND	-
API ZYM results*					
Lucine arylamidase	+	+	-	+	+
Acid phosphatase	+	-	-	+	+
Naphthol-AS-BI-phosphohydrolase	-	-	-	+	W
α-galactosidase	+	-	-	-	-
β-galactosidase	W	-	-	-	+
α-glucosidase	W	-	-	-	-
N-Acetyl-β-glucosaminidase	-	-	-	-	+
Major polar lipids	PC, PG, PE, PL, AL	PC, PG, PE, AL, L	ND	PC, PG, PE, AL	PG, DPG, PE, PL, GL
DNA G+C content (mol%)	63.5	67.6	58.6	59.9	67.0

Strains: 1, SSW136^T; 2, *Oceanicola litoreus* M-M22^T; 3, *Roseovarius aestuarii* SMK-122^T; 4, *Marivita geojedonensis* DPG-138^T; 5, *Pseudoruegeria aquimaris* SW-255^T; 6, *Jannaschia cystaugens* CFPB-A9^T. Data for column 2 (except *asterisk) were taken from Park *et al*. 2013; Data for columns 3, 4, 5 were taken from Yoon *et al*. (2007, 2008 and 2013). Data for column 1 and asterisk (*) shown in column 2 were obtained from this study. +, positive; -, negative; W, weak; ND, No data available; †Wh, white; C, cream; GY, greyish yellow. All five taxa are positive for catalase, oxidase, alkaline phosphatase, esterase (C4) and esterase lipase (C8). All are negative for Gram-staining, starch hydrolysis, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase. Polar lipids: AL, unidentified aminolipid; PL, unidentified phospholipid; GL, unidentified glycolipid; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; L, unidentified lipid.

The major isoprenoid quinone was determined to be Q-10. Polar lipids of the strain SSW136^T consisted of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, two unidentified aminolipids and three unidentified phospholipids (Fig. 6A.2). Cellular fatty acid analysis revealed that $C_{18:1}$ ω7c (46.5 %), Cyclo-$C_{19:0}$ *ω8c* (16.0 %) and $C_{16:0}$ (12.8 %) are the major components (Table 6A.2) observed in 5 days old culture. Whereas, in 2 days old culture $C_{18:1}$ ω7c (62.6 %) and $C_{16:0}$ (20.0 %) are dominant (Table 6A.3). The proportion of Cyclo-$C_{19:0}$ *ω8c* was found to be lower (2.9 %) in 2 days and tried to be higher with longer incubation of 5 days (16.0 %). At the same time, proportion of major fatty acid $C_{18:1}$ ω7c was reduced nearly 16 % as the cultures got older. It may be estimated that there is difference between the growth phases of strain SSW136^T and *Oceanicola litoreus* M-M22^T. Comparative analysis with *Oceanicola litoreus* M-M22^T revealed that Methyl-$C_{19:0}$ *ω7c*, $C_{12:0}$ 3-OH and $C_{16:0}$ 2-OH were detected only in strain SSW136^T. Further, Strain SSW136^T could be distinguished from its closest relative *Oceanicola litoreus* M-M22^T by the differences in proportions of

fatty acids (Table 6A.2). The chemotaxonomic properties of strain SSW136^T i.e., ubiquinone Q-10, the presence of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and the large proportion of unsaturated fatty acid $C_{18:1}$ ω7c are similar to those described for phylogenetically related genera of the order *Rhodobacterales*, class α-*Proteobacteria* (Romanenko et al. 2011). However, strain SSW136^T differed from their closest relatives by some unidentified phospholipids and aminolipids, and the percentage variations of fatty acids (Table 6A.2). The DNA G+C content of the strain SSW136^T was determined to be 63.5 mol% which matched the values reported for related type strains (58.6 – 67.6 mol%).

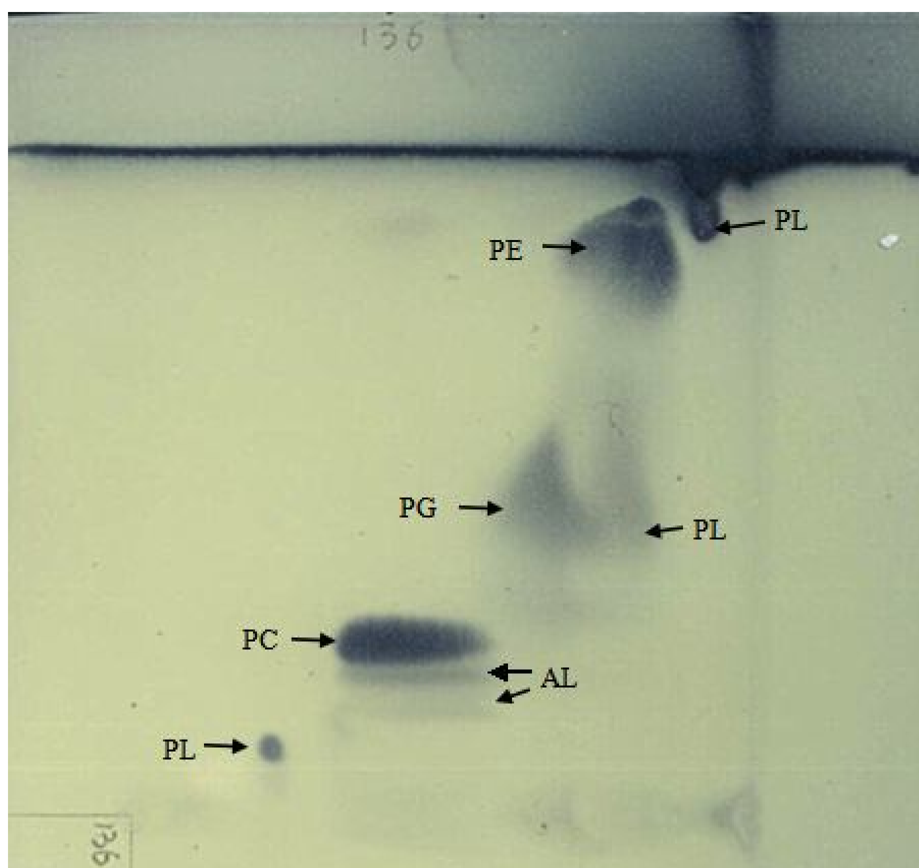


Fig. 6A.2. Two-dimensional TLC of polar lipids of the strain SSW136^T

PL, unidentified phospholipid; AL, unidentified aminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine.

Table 6A.2. Cellular fatty acid compositions of strain SSW136^T and closest type strain *Oceanicola litoreus* M-M22^T.

Fatty acids	1	2
$C_{16:0}$	12.8	7.1
$C_{17:0}$	-	1.7
$C_{18:0}$	2.1	11.5
$C_{10:0}$ 3-OH	3.3	4.3
$C_{12:0}$ 3-OH	3.5	-
$C_{16:0}$ 2-OH	3.7	-
$C_{18:1}\omega 7c$	46.5	65.4
Methyl-$C_{18:1}\omega 7c$	8.4	-
Cyclo-$C_{19:0}$ $\omega 8c$	16.0	5.0
Summed feature 3	-	1.1

Strains: 1, SSW136^T; 2, *Oceanicola litoreus* M-M22^T. Data were obtained from this study. Fatty acids that represented ≥1.0 % are given. Summed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH.

Table 6A.3. Cellular fatty acid compositions of strain SSW136^T with different time interval

Fatty acids	A	B
$C_{14:0}$	1.0	-
$C_{16:0}$	20.0	12.8
$C_{18:0}$	4.0	2.1
$C_{10:0}$ 3-OH	2.3	3.3
$C_{12:0}$ 3-OH	2.0	3.5
$C_{16:0}$ 2-OH	-	3.7
$C_{18:1}\omega 7c$	62.5	46.5
Methyl-$C_{18:1}\omega 7c$	1.7	8.4
Cyclo-$C_{19:0}$ $\omega 8c$	2.9	16.0

Fatty acids extracted from two days old culture (A) and five days old culture (B) of strain SSW136^{T}. Fatty acids that constituted <1.0 % were omitted.

The nearly complete 16S rRNA gene sequence of the strain SSW136^{T} (1,411 nt) has highest similarity values with the type strains of the family *Rhodobacteraceae*. EzTaxon-e (Kim et al. 2012a) showed closest similarities to *Oceanicola litoreus* M-M22^{T} (96.3 %) *Roseovarius aestuarii* SMK-122^{T} (95.9 %), *Marivita geojedonensis* DPG-138^{T} (95.7 %), *Pseudoruegeria aquimaris* SW-255^{T} (95.5 %), *Marivita cryptomonadis* CL-SK44^{T} (95.4 %), *Tropicimonas sediminicola* M97^{T} (95.4 %), *Roseibacterium elongatum* DSM 19469^{T} (95.4 %), *Celeribacter neptunius* H 14^{T} (95.3 %), *Jannaschia cystaugens* CFPB-A9^{T} (95.2 %), *Roseovarius tolerans* EL-172^{T} (95.2 %), *Roseisalinus antarcticus* EL-88^{T} (95.2 %) *Roseovarius nubinhibens* ISM^{T} (95.2 %), *Jannaschia donghaensis* DSW-17^{T} (95.1 %), *Roseovarius lutimaris* 112^{T} (95.1 %) and *Thioclava pacifica* DSM 10166^{T} (95.0 %). The remaining type strains of the *Rhodobacteraceae* members had similarity of <95 %.

Various phylogenetic tree algorithms (neighbour-joining and maximum-parsimony) based on 16S rRNA gene sequences (Fig. 6A.3 and Fig. 6A.4) revealed a close phylogenetic relationship between strain SSW136^{T} and members of the family *Rhodobacteraceae* by forming a separate branch within the type species of closely related genera. In addition, Strain SSW136^{T} exhibited DNA-DNA relatedness value of 14.3±4.5 % to *Oceanicola litoreus* M-M22^{T}. This information confirms that the strain SSW136^{T} belongs to a novel genus within the *Roseobacter* lineage of the α-*Proteobacteria*.

6A.4. Description of *Nioella* gen. nov.

Nioella (N.L. fem. dim. n. *Nioella*, arbitrary name after NIO, the National Institute of Oceanography, where the taxonomic study of this taxon was conducted).

Cells are Gram-negative, aerobic, non-spore forming, rod shaped bacteria that are positive for catalase and oxidase. Sodium ions are necessary for growth. Predominant quinone is Q-10. The polar lipids comprised of phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl choline, two unidentified aminolipids and three unidentified phospholipids. Major fatty acids are $C_{18:1}$ ω7c, Cyclo-$C_{19:0}$ *ω8c* and $C_{16:0}$. The DNA G+C content is 63.5 mol%. Based on 16S rRNA gene sequence, the genus represents a separate branch within the class α-*Proteobacteria,* closely related to the genera, *Oceanicola*, *Roseovarius*, *Marivita* and *Pseudoruegeria*. The type species is *Nioella nitratireducens*.

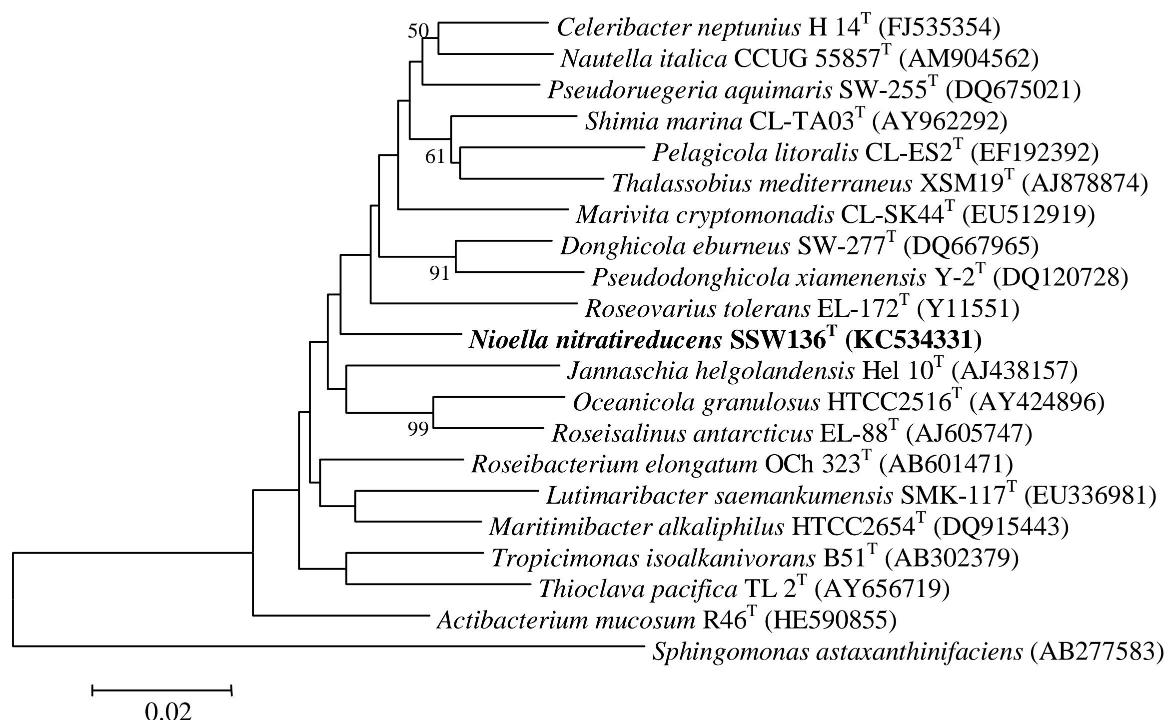


Fig. 6A.3. Neighbour-joining phylogenetic tree showing the relationship of strain SSW136^T and phylogenetically related species of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. Numbers at the nodes indicate percentage bootstrap values above 50 (1000 replicates). Bar, 0.02 substitutions per nucleotide position. *Sphingomonas astaxanthinifaciens* TDMA-17^T (AB277583) was used as an out group.

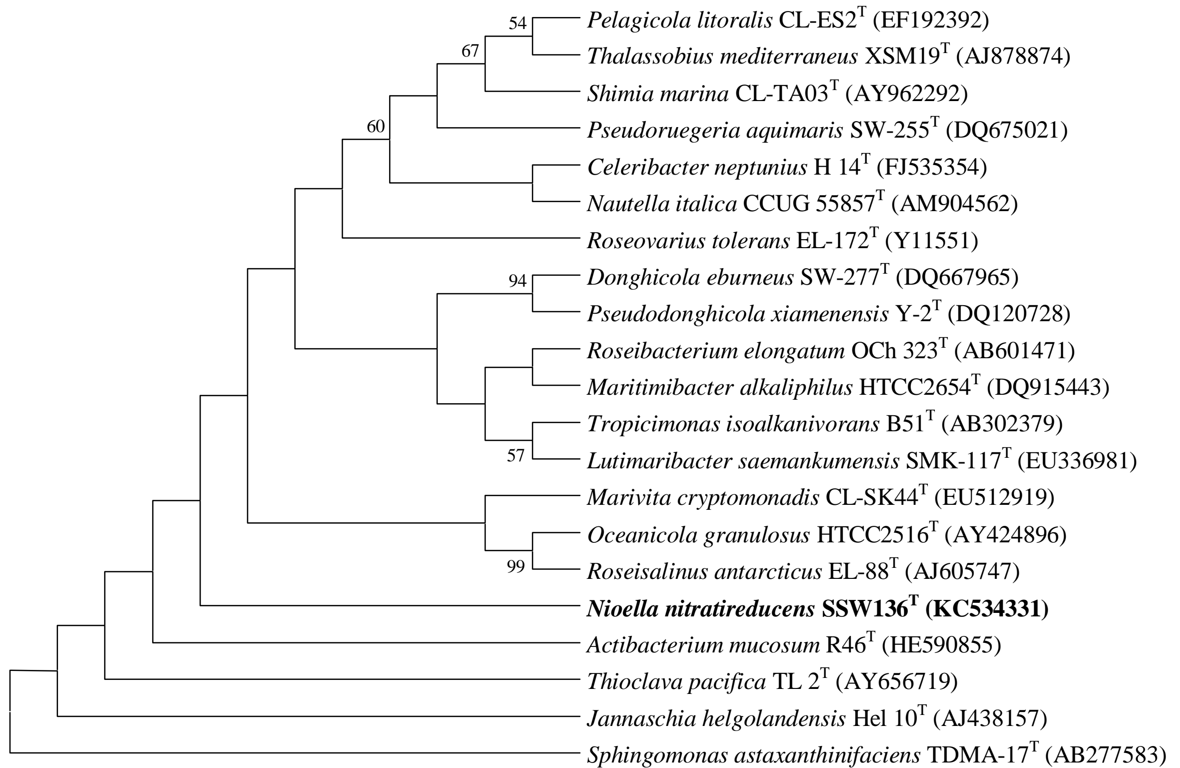


Fig. 6A.4. 16S rRNA gene sequence based Maximum-parsimony phylogenetic tree showing the relationship of strains SSW136^T and its closest neighbours of the class α-*Proteobacteria* from the GenBank database.

6A.5. Description of *Nioella nitratireducens* sp. nov.

Nitratireducens (N.L. n. *nitratum*, nitrate; L. v. *reducens*, bringing back to a state or condition; N.L. part. adj. *nitratireducens*, reducing nitrate).

Cells are aerobic, Gram-staining negative, non-motile and small rods- long rod shaped (0.6–0.9 × 2.4–13.5 μm). Colonies on SWNA are punctiform, white, convex, circular and opaque after incubation at 30 °C for 48 h. Optimal growth temperature is 30 °C; growth occurs between 10 and 37 °C but not at 40 °C. Optimal pH for growth is 6.0;

growth occurs at pH 6.0–8.0. Optimum NaCl concentration for growth is 8 % (w/v); growth occurs in the presence of 2–11 % of NaCl (w/v). Mg^{2+} ions are not required for growth. Catalase and oxidase are positive. Nitrate is reduced to nitrite. Gelatin and aesculin are hydrolyzed but Tweens 20, and 80, casein, starch, agar, alginate, xylan, CMC, DNA, urea and xanthine are not. Acid is produced from erythritol, L-arabinose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose (weak), D-mannitol, D-sorbitol, D-cellobiose, D-maltose, D-lactose (weak), D-turanose, D-fucose and D-arabitol. In assays with the API ZYM system, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase (weak) and α-glucosidase (weak) activities are present, but lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The main respiratory quinone is Q-10. The major fatty acids (>10 %) are $C_{18:1}$ ω7c (46.5 %), Cyclo-$C_{19:0}$ *ω*8*c* (16.0 %) and $C_{16:0}$ (12.8 %). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, two unidentified aminolipids and three unidentified phospholipids. The DNA G+C content of the type strain is 63.5 mol%.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain $SSW136^T$ is KC534331. The type strain, $SSW136^T$ (= KCTC 32417^T = NCIM 5499^T), was isolated from coastal surface seawater at the Espalamaca, Azores, Portugal.

Chapter 6B

Roseovarius azorensis sp. nov., isolated from seawater at Espalamaca, Azores

6B.1. Introduction

The genus *Roseovarius*, a member of the family *Rhodobacteraceae* within the class α-*Proteobacteria*, was first created by Labrenz et al. (1999) with a type strain, *Roseovarius tolerans* EL-172^T. The type species was characterized as Gram negative, rod-shaped, bacteriochlorophyll *a* (BChl *a*) positive, Q-10 as major quinone, $C_{18:1}$ as major fatty acids and to require Na^+ for growth. At the time of writing, the genus *Roseovarius* comprises 14 species with validly published names: *Roseovarius tolerans* (Labrenz et al. 1999), *Roseovarius nubinhibens* (González et al. 2003), *Roseovarius crassostreae* (Boettcher et al. 2005), *Roseovarius mucosus* (Biebl et al. 2005), *Roseovarius aestuarii* (Yoon et al. 2008), *Roseovarius halotolerans* (Oh et al. 2009), *Roseovarius pacificus* (Wang et al. 2009), *Roseovarius nanhaiticus* (Wang et al. 2010a), *Roseovarius indicus* (Lai et al. 2011a), *Roseovarius marinus* (Jung et al. 2011), *Roseovarius halocynthiae* (Kim et al. 2012b), *Roseovarius litoreus* (Jung et al. 2012), with recent proposal of *R. sediminilitoris* (Park and Yoon 2013) and *R. lutimaris* (Choi et al. 2013). In addition the species *Roseovarius marisflavi* has recently been proposed (Li et al. 2013b). The members of the genus have been isolated from various aquatic environments, including seawater, sediment and lake. The main aim of the present study was to determine the precise taxonomic position of strain SSW084^T, which was isolated from Espalamaca, North Atlantic Ocean. This study uses a polyphasic approach that consists of determination of phenotypic and chemotaxonomic properties along with a detailed phylogenetic investigation based on 16S rRNA gene sequences. As a result of these studies, strain SSW084^T is considered to represent a novel species of the genus *Roseovarius*, for which *Roseovarius azorensis* sp. nov is proposed.

6B.2. Materials and Methods

6B.2.1. Isolation and maintenance of the bacterial strain

Surface seawater was collected from the Espalamaca region in the Azores, Portugal (38°33'N; 28°39'W) during September 2010. Strain SSW084^T was isolated from a colony grown at 30 °C for 2 days on seawater nutrient agar (SWNA= nutrient agar prepared in 50 % seawater). Strain SSW084^T was purified by repeated streaking on SWNA. Strain SSW084^T was maintained on SWNA at 4 °C for short-term maintenance and as 15 % glycerol suspensions at -80 °C for long-term preservation.

6B.2.2. Growth studies on various media

The growth of the strain SSW084^T was checked in various media i.e., tryptic soy agar (TSA, Himedia), TSA prepared with 3 % NaCl, TSA prepared in 50 % seawater, nutrient agar prepared in artificial seawater (ASWNA) and Zobell marine agar (MA, Himedia).

6B.2.3. Morphological, physiological and biochemical characterization

Colony morphology (colour, shape, size, opacity, elevation) was observed using a colony counter. Gram staining (K001, Himedia) and motility by hanging drop (Collee et al. 2008) of strain SSW084^T was examined using light microscopy (Olympus BX-60) with 1000× magnification. The cell morphology was examined by scanning electron microscopy (JEOL JSM-5800LV). Salinity tolerance of strain SSW084^T was investigated in nutrient agar medium prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increment) with and without adding Mg^{2+} and Ca^{2+} (4.53 g L^{-1} $MgCl_2$; 5.94 g L^{-1} $MgSO_4$; 1.3 g L^{-1} $CaCl_2$). In addition to this, strain SSW084^T was also tested in nutrient agar prepared with various seawater concentrations

(0, 25, 50, 75 and 100 %). The pH range for growth was determined by adjusting the pH of the SWNB medium (nutrient broth prepared in 50 % seawater) using various buffers to pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer). Growth at various temperatures 4, 10, 15, 20, 30, 37, 40 and 45 °C was also determined by using SWNB medium. In above experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation.

For analysis of BChl *a*, strain SSW084^T was cultivated aerobically in the dark at 30 °C in SWNB medium. The cells were harvested by centrifugation at a speed of 10,000 rpm for 10 min. The cell pellet was used for extraction with methanol and the methanol solution was filtered in the dark. The filtered solution was examined for light absorbance spectrum from 400–900 nm with a spectrophotometer (Cary 300) to detect BChl *a* in the region of 766-772 nm (Shiba et al. 1979; Yoon et al. 2004).

Catalase activity was tested using the 3 % (v/v) H_2O_2 method (Smibert and Krieg 1994) and oxidase activity was determined using oxidase discs (DD018, Himedia, India). Hydrolysis of casein (1 %), gelatin (1 %), Tween 20 and 80 (1 %), tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert and Krieg 1994) were tested on SWNA medium. Hydrolysis of urea and DNA was determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Xylan, starch and carboxymethyl cellulose (CMC) hydrolysis were determined using the methods provided by Khandeparker et al. (2011). Other enzyme activities were determined using API ZYM strips (BioMérieux) and acid production from carbohydrates was tested with API-CH-50 (BioMérieux) tests using inoculation medium API CHB/E (BioMérieux) amended with marine cations supplement (MCS, Farmer and Hickman-Brenner 2006) according to the manufacturers' instructions. Antibiotic susceptibility tests were performed using

antibiotic discs (Himedia) containing the following concentrations (µg per disc unless indicated): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyl (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30), ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin-G (2 IU), polymyxin-B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5).

6B.2.4. Chemotaxonomic analysis

Cell biomass of strain SSW084^T for the analysis of isoprenoid quinones and polar lipids was obtained from cultures grown in LB broth (M1245, Himedia) prepared in 50 % seawater for 2 days at 30 °C. Analysis of whole cell fatty acids of the isolate was performed according to the Microbial Identification System TSBA 6.0 (Sasser 1990) after cultivation on SWNA for 3 days at 30 °C. Polar lipids were extracted and examined according to the procedures described by Collins et al. (1980). Briefly, Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were detected using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), and α-naphthol reagent (sugars). Isoprenoid quinones were determined as described by Minnikin et al. (1984). The genomic DNA for G+C content analysis was extracted using phenol method provided by Mesbah et al. (1989). The DNA G+C content of strain SSW084^T was determined by reverse-phase HPLC of nucleosides according to Mesbah et al. (1989).

6B.2.5. Phylogenetic analyses

Genomic DNA of strain SSW084^T was isolated using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was amplified using the bacterial consensus primers 27F and 1492R (Lane 1991). The PCR amplified 16S rRNA gene was purified with a PCR cleanup kit (Sigma) according to the manufacturer's instructions. DNA sequencing was performed using an automated 3130xl Genetic analyzer (Applied Biosystems). The sequence was aligned using Clustal W sequence alignment program (Thompson et al. 1994). The 16S rRNA gene sequences of the neighbouring taxa were obtained from the GenBank database. Phylogenetic trees were established with neighbour-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971) algorithms using MEGA 5.0 software (Tamura et al. 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985).

6B.3. Results and Discussion

Cells of strain SSW084^T appeared as rod shaped, Gram-negative, approximately 0.6–0.8 µm wide and 1.3–2.0 µm long (Fig. 6B.1); Cells were found to be motile and non-spore forming. The colonies of the strain SSW084^T were observed to be punctiform, white, circular, convex, smooth and translucent on SWNA media after 48 h. Strain SSW084^T did not grow on medium with NaCl as a sole salt and was found to require 25 – 100 % seawater or 0.5 – 7.0 % NaCl with Mg^{2+} and Ca^{2+}. Strain SSW084^T did not grow on marine agar and tryptic soy agar even when seawater supplemented. When tested on SWNB, growth of the strain SSW084^T was observed at temperatures between 15 and 40 °C with optimum growth at 30 °C, but no growth was observed at 10 and 45 °C. Growth of strain SSW084^T was found to occur at a pH range of 7.0–9.0 with an optimum pH of 7.0. Differences between morphological and physiological characteristics of strain

SSW084^T compared with the closest related type strains of the genus *Roseovarius* are given in Table 6B.1. Usually *Roseovarius* species are either pigmented or cream in colour and some of them are able to produce the pigment BChl *a*, whereas strain SSW084^T is non-pigmented and could not produce BChl *a*. In addition strain SSW084^T was found to be susceptible to the following antibiotics: amikacin, amoxicillin, ampicillin, bacitracin, cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, kanamycin, lincomycin, neomycin, norfloxacin, novobiocin, penicillin-G, polymyxin-B, rifampicin, streptomycin, tetracycline, tobramycin and vancomycin.

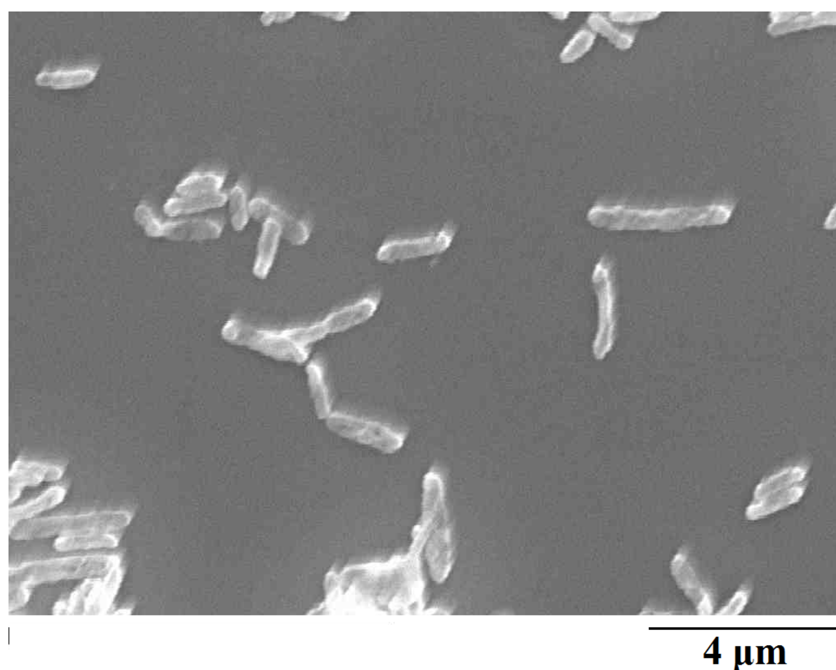


Fig. 6B.1. Scanning electron micrograph of strain SSW084^T after growth for 3 days at 30 °C on SWNA

Table 6B.1. Characteristic that differentiate strain SSW084^T and the closely related type strains of the genus *Roseovarius*

Characteristic	SSW084^T	*R. tolerans*	*R. mucosus*	*R. lutimaris*	*R. aestuarii*
Pigmentation	Non-pigmented	Red	Faintly pink	Beige	Cream
Cell morphology	Rod	Rod	Rod	Ovoid/Rod	Ovoid/Rod
Cell size	0.6–0.8×1.3–2.0	0.7–1.0×1.1–2.2	0.5–0.7×1.3–3.0	0.4–0.8×2.0–3.0	0.3–0.6×0.8–3.5
Motility	+	+	-	+	+
Catalase	-	+	+	+	+
Oxidase	+	W	+	+	+
Growth ranges					
pH	7.0–9.0	5.9–9.0	6.0–8.8	5.0–9.0	7.0–8.0
Temperature (°C)	15–40	8–33.5	20–40	15–40	10–37
NaCl (%)	0.5–7.0	1.0–10.0	1.0–7.0	1.0–6.0	0.5–7.0
BChl *a*	-	+	+	-	-
Nitrate reduction	+	-	-	+	+
Gelatin liquefaction	-	-	+	-	-
Enzyme activities*					
Alkaline phosphatase	-	+	+	+	W
Valine arylamidase	-	+	+	W	-
Cystine arylamidase	-	+	W	-	-
Trypsin	-	W	-	-	-

Acid phosphatase	-	+	+	-	-
Acid production from*					
D-Arabinose	-	+	+	+	ND
D-Galactose	-	+	+	+	-
D-Glucose	-	-	+	W	-
D-Fructose	-	+	+	+	-
D-Mannitol	-	-	+	-	-
D-Sorbitol	-	-	+	W	-
DNA G+C content (mol%)	61.9	62.2–63.8	60.9–62.9	58.2	58.6
Isolation source	Sea water	Lake water	Dinoflagellate	Tidal flat	Tidal flat

Data for *R. tolerans*, *R. mucosus*, *R. lutimaris* and *R. aestuarii* are taken from Labrenz et al. (1999), Biebl et al. (2005), Choi et al. (2013) and Yoon et al. (2008) respectively. All strains are positive for esterase (C4) and esterase lipase (C8). All strains are negative for citrate utilization, indole production, hydrolysis of starch, Tween 20 & 80, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. +, positive; -, negative; W, weak; ND, not determined; *Data for *R. tolerans*, *R. mucosus*, *R. lutimaris* taken from Choi et al. (2013).

The 16S rRNA gene sequence obtained from the strain SSW084^T was found to be closely related to those of *R. tolerans* EL-172^T, *R. mucosus* DFL-24^T and *R. lutimaris* with sequence similarities of 95.72, 95.39 and 95.30 % respectively, which are below the threshold generally considered necessary of species delineation (Stackebrandt and Goebel 1994). Other related species of the genus *Roseovarius* showed <95 % similarity in EzTaxon-e (Kim et al. 2012a). The nearly full sequence (1,379 nucleotides) of the 16S rRNA gene of strain SSW084^T has been deposited in GenBank under accession number KC534172. Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain SSW084^T formed a phylogenetic lineage with *R. tolerans* EL-172^T and *R. mucosus* DFL-24^T (bootstrap value 79; Fig. 6B.2). The maximum-parsimony algorithm also confirmed the relationship of the strain SSW084^T with *R. tolerans* EL-172^T and *R. mucosus* DFL-24^T (Fig. 6B.3).

Strain SSW084^{T}was found to contain Q-10 as the major respiratory quinone, which is consistent with other *Roseovarius* species (Garrity and Holt 2001). The polar lipid profile of strain SSW084^{T}was found to consist of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unidentified lipid and one unidentified aminolipid (Fig. 6B.4). The polar lipids are thus very similar to those of *R. mucosus* and *R. tolerans* (Labrenz et al. 1999; Biebl et al. 2005). The major fatty acids found in SSW084^T were $C_{18:1}$ ω7c (52.5 %) and $C_{16:0}$ (13.8 %), which are similar to the profiles of the type strains of *R. mucosus* and *R. tolerans* (Table 6B.2). However, $C_{18:2}$ reported as second major fatty acid in *R. tolerans* which was not detected in the strain SSW084^T. The genomic G+C content was found to be 61.9 mol% which is consistent with species of the genus *Roseovarius*.

Table 6B.2. Fatty acid composition of strain SSW084^T and the closest related type strains *R. tolerans* and *R. mucosus.*

Fatty acids	SSW084^T	*R. tolerans*	*R. mucosus*
$C_{12:0}$	4.5	-	3.1
$C_{16:0}$	13.8	6.2	13.1
$C_{18:0}$	4.1	0.8	1.8
$C_{18:2}$	-	10.6	-
$C_{12:0}$ 2-OH	-	2.4	2.5
$C_{12:0}$ 3-OH	3.8	-	-
$C_{12:1}$ 3-OH	-	3.6	1.7
iso-$C_{11:0}$ 3-OH	8.3	-	-
iso-$C_{18:1}$ H	1.3	-	-
$C_{18:1}\omega 7c$	52.5	70.2	69.4
Methyl-$C_{18:0}$	-	-	2.1
Methyl-$C_{18:1}\omega 7c$	1.7	-	2.1
$C_{18:1}\omega 9c$	2.4	-	-
Cyclo-$C_{19:0}\omega 8c$	2.1	-	-
Cyclo-$C_{19:1}$	-	-	3.7
$C_{16:1}\omega 6c$/ $C_{16:1}\omega 7c$	1.9	-	-

Data for *R. tolerans* and *R. mucosus* are taken from Labrenz et al. (1999) and Biebl et al. (2005) respectively.

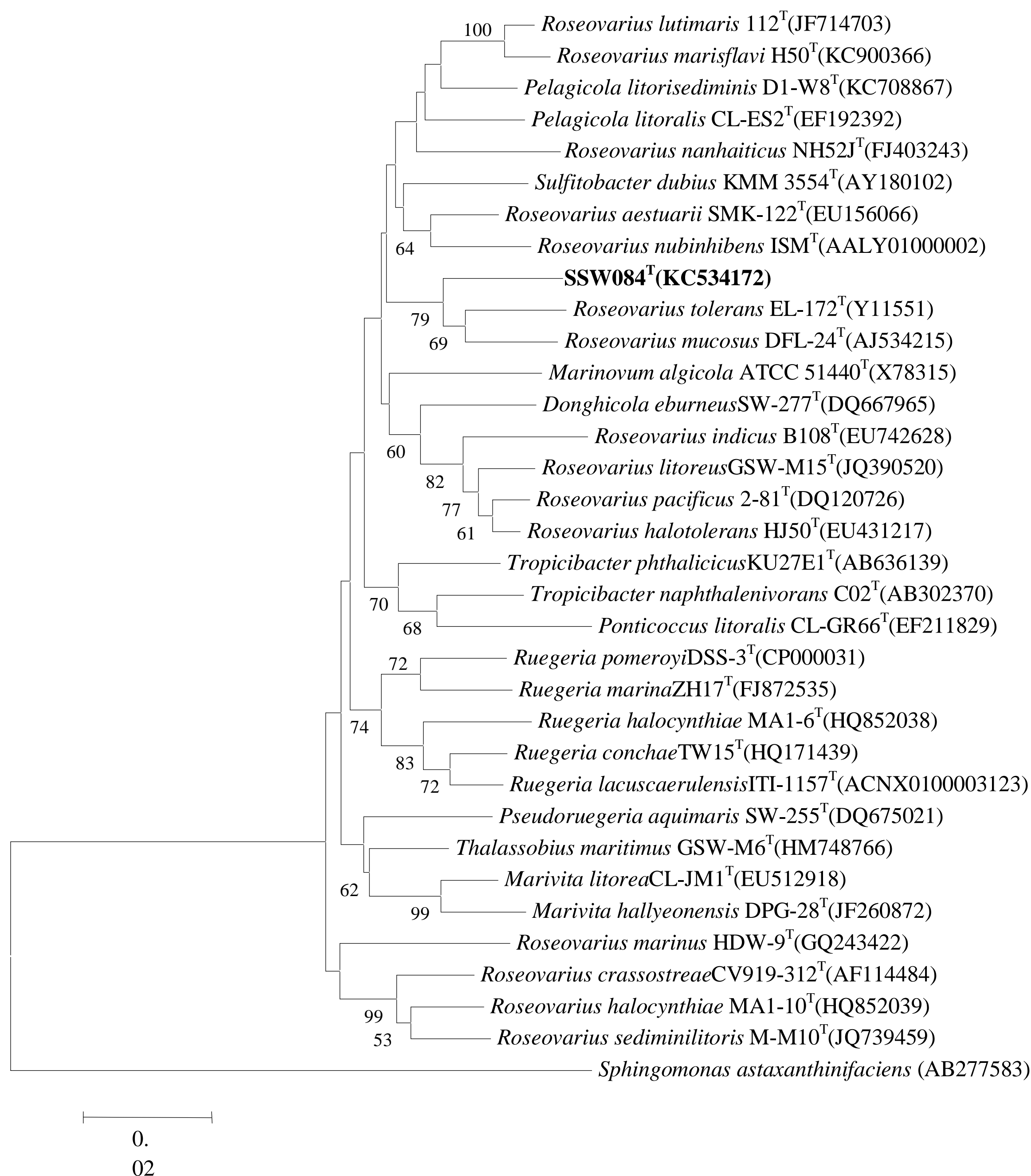


Fig. 6B.2. Neighbour-joining tree showing the phylogenetic relationship of strain SSW084^T and phylogenetically related species of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. Bootstrap percentages (1,000 replications) are shown at branching points and the values >50 % are shown. *Sphingomonas astaxanthinifaciens* TDMA-17^T (AB277583) was used as an out group. Scale bar 0.02 substitutions per nucleotide position.

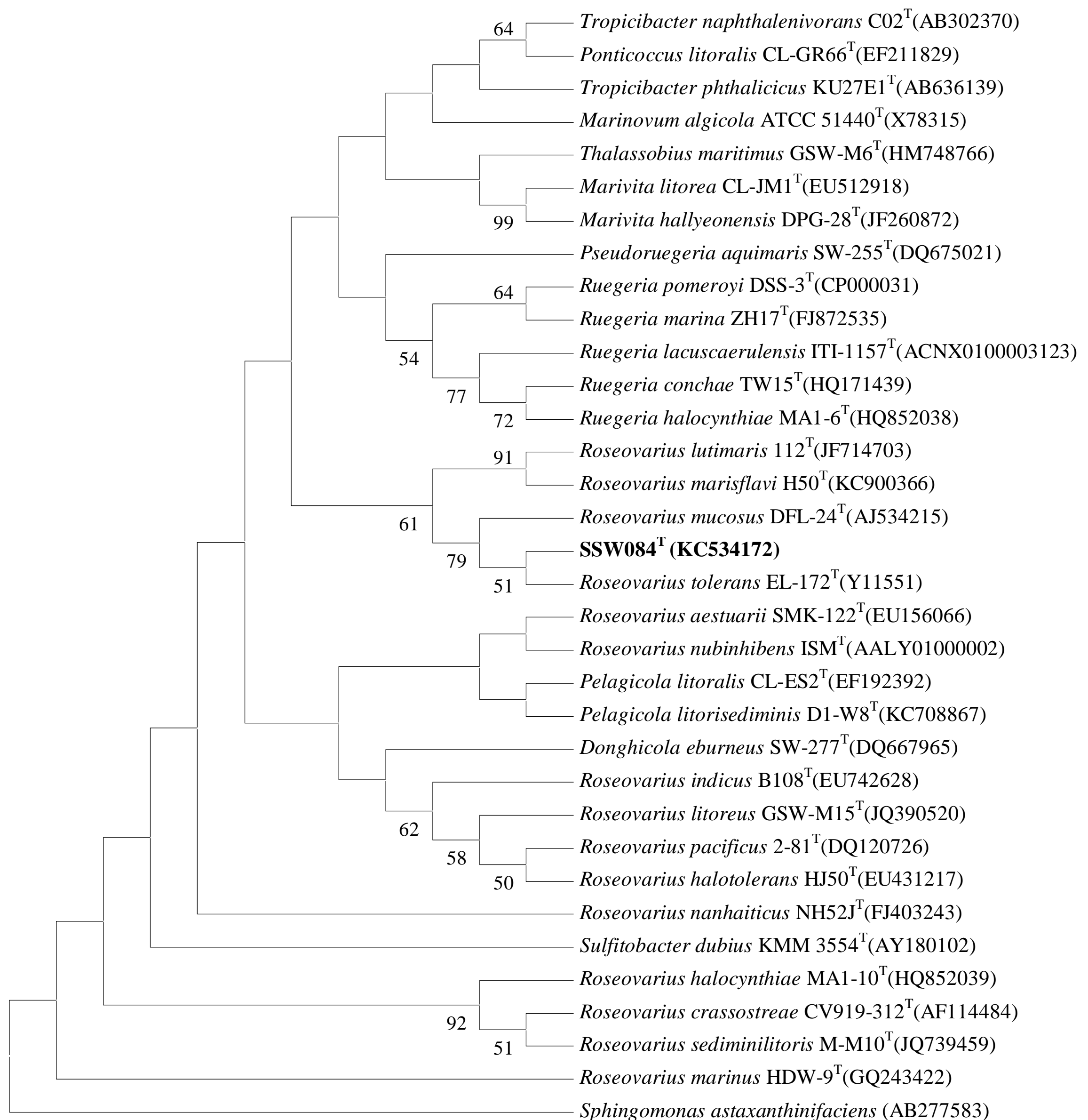


Fig. 6B.3. Maximum-parsimony tree showing the phylogenetic relationship of strain SSW084^T and phylogenetically related species of the family *Rhodobacteraceae* based on 16S rRNA gene sequences

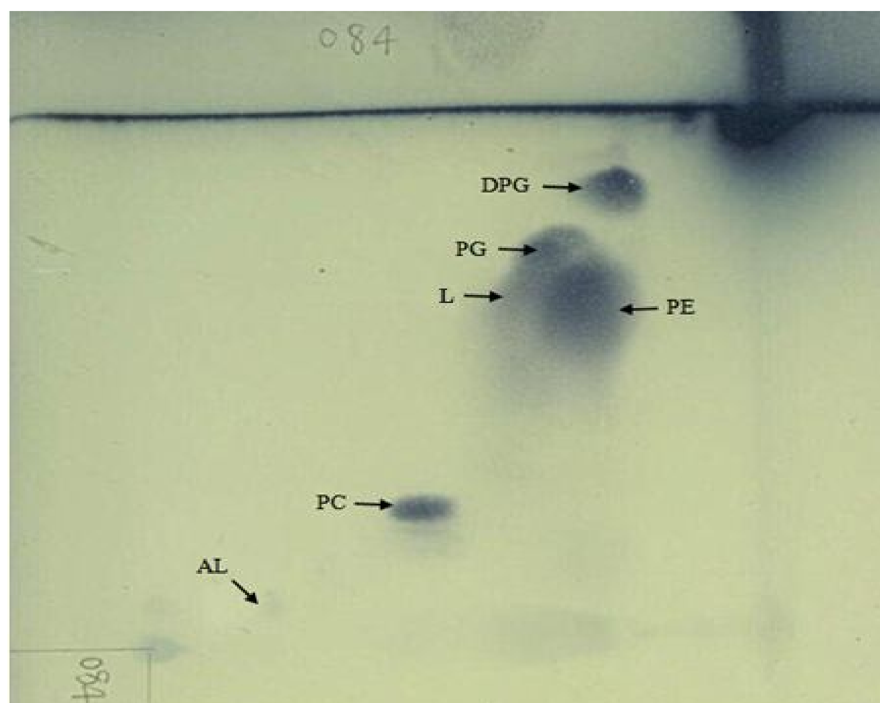


Fig. 6B.4. Two-dimensional TLC of total polar lipids of the strain SSW084^{T}. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; AL, unidentified aminolipid; DPG, diphosphatidylglycerol; L, unidentified lipid

In summary, from the phenotypic, genotypic and chemotaxonomic data presented above it can be concluded that the strain SSW084^{T} is a member of the genus *Roseovarius* but can be distinguished from previously described species of the genus (Table 6B.1). Strain SSW084^{T} therefore represents a novel *Roseovarius* species, for which we proposed the name *Roseovarius azorensis* sp. nov.

6B.4. Description of *Roseovarius azorensis* sp. nov

Roseovarius azorensis (*azo.ren'sis,* N.L. masc. adj. azorensis, after the portug. Islas dos Azores, pertaining to the Azorean Islands, the source of the sample from which the type strain was isolated).

Gram-negative, rod shaped (0.6–0.8 × 1.3–2.0 μm), motile by gliding and non-spore forming. The colonies are punctiform, non-pigmented, circular, convex, smooth and translucent on SWNA medium. Requires 25 – 100 % seawater for its growth or can grow with salinity range of 0.5 – 7.0 % NaCl in the presence of Mg^{2+} and Ca^{2+}; does not grow with NaCl alone. Growth is observed at temperature ranges from 15–40 °C and at pH 7.0–9.0. The optimum temperature and pH for growth are 30 °C and 7.0 respectively. BChl *a* is not produced. Oxidase positive but catalase negative. Nitrate reduction is positive. Acid production is observed from the substrate potassium 5-keto gluconate but not from other carbohydrates in the API-CH-50 gallery. Aesculin is hydrolyzed but starch, DNA, gelatin, Tween 20 and 80, casein, tributyrin, xanthine, alginate, urea, xylan and CMC are not hydrolysed. Positive (in API ZYM strips) for esterase C4, esterase lipase C8, lucine arylamidase and naphthol-AS-BI-phosphohydrolase but negative for alkaline phosphatase, trypsin, α-glucosidase, β-glucosidase, acid phosphatase, lipase C14, valine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, α-chymotrypsin. The polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unidentified lipid and one unidentified aminolipid. The predominant ubiquinone is Q-10. The major fatty acids are $C_{18:1}$ ω7c and $C_{16:0}$. The G+C content of the type strain is 61.9 mol%.

The type strain, $SSW084^T$ (= KCTC 32421^T = MTCC 11812^T), was isolated from surface seawater at Espalamaca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain $SSW084^T$ is KC534172.

Chapter 6C

Vitellibacter nionensis sp. nov., isolated from a shallow water hydrothermal vent in Espalamaca, Azores

6C.1. Introduction

The genus *Vitellibacter*, a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, was first established by Nedashkovskaya et al. (2003). At the time of writing the genus *Vitellibacter* comprised of only three validly described species, *Vitellibacter vladivostokensis* (Nedashkovskaya et al. 2003), *Vitellibacter aestuarii* (Kim et al. 2010) and *Vitellibacter soesokkakensis* (Park et al. 2014). During our investigation on culturable bacterial diversity from shallow water hydrothermal vent of Espalamaca (Azores, Portugal), we have revealed some novel bacterial taxa belong to the phylum *Bacteroidetes*. Strain VBW088^T was isolated and characterized in this study. The main aim of the present investigation was to determine the precise taxonomic place for strain VBW088^T using a polyphasic approach.

6C.2. Materials and methods

6C.2.1. Sampling and strain isolation

A shallow water hydrothermal vent in Espalamaca (38°33'N; 28°39'W) which is located in the Faial Island, Azores, North Atlantic Ocean, was identified in 2010. Gaseous discharges from the vents are mainly composed of CO_2, low concentration of methane, temperature 35 °C and pH 5.7 (Colaço, personal communication). Bottom water sample from the venting area was collected in September 2010 by scuba diving under Indo-Portugal bilateral program. For isolation of heterotrophic bacteria, serial dilutions of the samples were spread plated on seawater nutrient agar (SWNA: peptone 5.0 g L^{-1}, beef extract 1.5 g L^{-1}, yeast extract 1.5 g L^{-1}, 1.5 % agar prepared in 50 % seawater). The plates were incubated for 2 days at 30 ± 1 °C. Morphologically different colonies were picked and repeatedly quadrant streaked on the same medium to get pure cultures.

Subsequently, the growth of strain VBW088^T was checked on tryptic soy agar (TSA) prepared in 50 % seawater, marine agar 2216 (MA: M384, Himedia) and nutrient agar prepared with artificial seawater (ASWNA). Strain VBW088^T was maintained at 4 °C on SWNA and as 15 % glycerol suspensions at -80 °C for long-term storage.

6C.2.2. Phylogenetic analysis

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. For 16S rRNA genes amplification, eubacterial primers 27F and 1492R were used (Lane 1991). The PCR amplified 16S rRNA gene product was purified with PCR cleanup kit (Sigma) according to the manufacturer's instructions. The purified 16S rRNA gene was sequenced on automated DNA sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) with the bacterial primers 27F, 518F, 1053F and 1492R. The acquired near-full-length sequences were subjected to a BLASTn on the National Center for Biotechnology Information (NCBI) and EzTaxon-e (Kim et al. 2012a) server to identify the sequences with the highest similarity. Multiple and pairwise sequence alignment were performed using Clustal W sequence alignments program (Thompson et al. 1994). Neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) methods were used to reconstruct phylogenetic trees using MEGA5 software program (Tamura et al. 2011). The topology of the phylogenetic trees was evaluated by bootstrap analysis method of Felsenstein (1985) with 1,000 replications. The genomic DNA isolation and determination of G+C content by reverse-phase HPLC of nucleosides were done according to the protocol described by Mesbah et al. (1989). DNA-DNA hybridization was performed fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes in microdilution wells. Hybridization was performed with five

replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA-DNA relatedness values.

6C.2.3. Morphological, physiological and biochemical characterization

Gram staining was carried out by using the standard Gram reaction (K001, Himedia) and by using the KOH method (Cerny 1978). Colony morphology was observed using a colony counter and motility by hanging drop method. Cell morphology was examined using light microscopy (Nikon TS100) and scanning electron microscopy (Hitachi TM3000). Growth under anaerobic condition was determined on MA after incubation for 10 days in an anaerobic system with an AnaeroPack (Himedia, Mumbai). For growth at different pH, the nutrient broth was buffered with citrate (pH 4-5) phosphate (pH 6-8) and carbonate (pH 9-10) buffer systems. Growth was tested on nutrient broth at 4, 10, 20, 25, 30, 37, 40, 45 and 50 °C. In above experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days of incubation. Growth in the presence of 0–15 % NaCl (w/v, at 1.0 % intervals) was investigated in nutrient agar prepared in distilled water. Requirement of Mg^{2+} was investigated on nutrient agar prepared in distilled water with 4.53 g L^{-1} $MgCl_2.6H_2O$ without NaCl. Acid production from carbohydrates was determined using API 50CH kit (bioMérieux) according to the manufacturer's instructions using inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer & Hickman-Brenner 2006). The methyl red and Voges-Proskauer tests, production of indole and H_2S, sodium malonate utilisation and nitrate reduction were assessed using Hi25^{TM} Enterobacteriaceae identification kit (Himedia).

Catalase activity was tested using 3 % (v/v) H_2O_2 drop method (Smibert and Krieg 1994) and oxidase activity was determined using the oxidase discs (DD018, Himedia). Carboxymethyl cellulose (CMC), xylan and starch hydrolysis were determined as per the methods of Khandeparker et al. (2011). Hydrolysis of urea and DNA was determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Hydrolysis of casein (1 %), gelatin (1 %), agar (2 %) Tweens 20 and 80 (1 %), tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert and Krieg 1994) were tested on SWNA medium. Other enzyme activities were assayed using API ZYM strips (bioMérieux) according to the manufacturer's protocol. Antibiotic susceptibility tests were performed in SWNA medium using antibiotic discs (Himedia) containing the following concentrations (µg/disc): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyl (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30), ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (2 IU), polymyxin B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5). The results were observed after 2 days incubation at 30 °C.

6C.2.4. Chemotaxonomic analysis

Strain VBW088^T and *V. soesokkakensis* RSSK-12^T were cultivated on MA at 30 °C for 3 days and harvested for Fatty acid methyl ester (FAME). Fatty acids were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.2B). The fatty acids were analysed by GC (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sasser 1990). Cell biomass of strain VBW088^T for the analysis of isoprenoid quinones and polar lipids was obtained from cultures grown for 2 days at 30

°C in seawater LB broth (M1245, Himedia). Isoprenoid quinones were analyzed by using high performance liquid chromatography (HPLC) and polar lipids were extracted according to the method of Minnikin et al. (1984). Briefly, Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were identified using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), and α-naphthol reagent (sugars). In addition, three days old cells grown on SWNA medium were suspended in methanol and pigments were extracted. UV-Vis scan was recorded from 200 to 900 nm in methanol using UV-Vis spectrophotometer (Cary 300). The KOH test (Reichenbach 1992) was carried out to assess the flexirubin type pigments following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet et al. 2002). The results are presented in a standardized format as described by Kämpfer et al. (2003).

6C.3. Results and discussions

The colonies of strain VBW088^T were small, yellow and circular on MA and SWNA. The cells were Gram-negative and non-spore forming short-rods that are 0.3–0.4 µm wide and 0.8–1.0 µm long (Fig. 6C.1). Growth occurs between 10 and 37 °C (optimum 30 °C) at pH 6–8 (optimum pH 6.0) with NaCl concentration of 1–5 % NaCl (optimum 1–2 %). The results obtained from this study concluded that strain VBW088^T showed characteristics that were consistent with those of the genus *Vitellibacter*, for instance being Gram-negative, rod-shaped, non-motile, yellow-orange pigmented and oxidase

positive (Nedashkovskaya et al. 2003; Kim et al. 2010). Differences noticed on morphological and physiological characteristics of strain VBW088^T with the closest type strains of *Vitellibacter* are given in Table 6C.1.

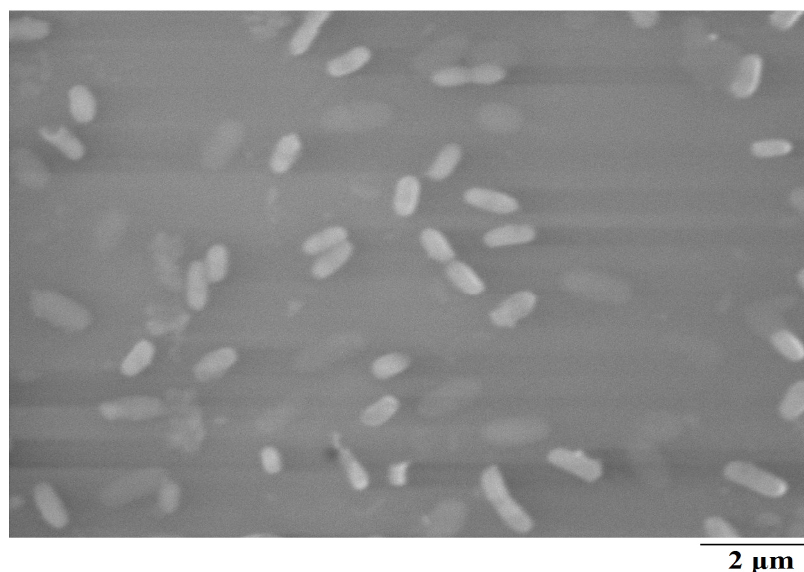


Fig. 6C.1. Scanning electron microscopic picture of strain VBW088^T

Nearly full length 16S rRNA gene sequence of strain VBW088^T (GenBank accession number KC534174) was compared with the sequences from the GenBank database (http://www.ncbi.nlm.nih.gov). 16S rRNA gene sequence similarity values between strain VBW088^T and other validly named species of the genus *Vitellibacter* in EzTaxon-e database are *V. soesokkakensis* RSSK-12^T (97.3 %), *V. vladivostokensis* KMM 3516^T (96.2 %) and *V. aestuarii* JC2436^T (95.1 %). On the other hand, the species of the genus *Aequorivita* also very close to the genus *Vitellibacter* and had similarity values between 94.6 – 96.5 %. However, both neighbour-joining and maximum-parsimony phylogenetic trees confirmed that strain VBW088^T clustered within the clade comprising *Vitellibacter* species (Fig. 6C.2 and Fig. 6C.3). In addition, DNA-DNA hybridization between strain VBW088^T and *V. soesokkakensis* RSSK-12^T showed a low DNA-DNA relatedness (12.7±3.5 %).

Table 6C.1. Differential characteristic between strain VBW088^{T} and type strains of *Vitellibacter* species

Characteristic	1	2	3	4
Pigmentation	Y	Y	Y-O	Y-O
Cell size (≥5 μm)	-	+	+	+
Gliding motility	-	+	-	-
Growth at 40 °C	-	-	+	+
Growth at 4 °C	-	-	+	-
Catalase	+	+	+	-
NaCl range for growth (%)*	1-5	0-12	1-6	2-6
Hydrolysis of:†				
DNA	-	ND	+	+
Tween 80*	+	+	-	-
Acid production from†				
Potassium 5-keto gluconate	+	ND	-	-
D-galactose*	-	+	-	-
API ZYM*				
Lipase (C14)	-	-	-	W
Cystine arylamidase	W	+	-	+
Trypsin	-	+	+	-
α-chymotrupsin	-	-	+	-
DNA G+C content (mol%)	36.7	38.9	41.3	48.7

Strains: 1, VBW088^{T} (present study); 2, *V. soesokkakensis* RSSK-12^{T} (Park et al. 2014); 3, *V. vladivostokensis* KMM 3516^{T} (Nedashkovskaya et al. 2003); 4, *V. aestuarii* JC2436^{T} (Kim et al. 2010). *Data for column 1, 2 and 3 from this study; †Data for column 1 and 3 from present study. Y, yellow; O, orange; Y-O, yellow to orange; +, positive reaction; -, negative reaction; W, weakly positive; ND, not determined. All strains were positive for oxidase; hydrolysis of casein, gelatin and Tween 20; alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains were negative for anaerobic growth; Gram-staining; nitrate reduction; hydrolysis of starch; α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase.

Strain VBW088^T was sensitive to the following antibiotics: amoxicillin, ampicillin, cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, lincomycin, norfloxacin, novobiocin, penicillin G, rifampicin, streptomycin, tetracycline and vancomycin; resistant to the following antibiotics: amikacin, bacitracin, kanamycin, neomycin, polymyxin B and tobramycin.

The whole cell fatty acid profile of strain VBW088^T could not provide a precise identification in MIDI database, which concluded that this strain belonged to a novel species. The predominant fatty acids of strain VBW088^T are iso-$C_{15:0}$ (33.5 %) and iso-$C_{17:0}$ 3-OH (32.0 %). The fatty acids profile of strain VBW088^T and the type strains of *Vitellibacter* are almost similar although differences in the percentage (Table 6C.2). Even though strain VBW088^T and *V. soesokkakensis* RSSK-12^T were only analysed from this study, fatty acids profile of other two type strains *V. vladivostokensis* KMM 3516^T and *V. aestuarii* JC2436^T were also compared since same media and growth conditions had been used in Park et al. (2014) for the fatty acid analysis. The polar lipid profile consisted of phosphatidylethanolamine as major component followed by three unidentified phospholipids and one unidentified aminolipid (Fig. 6C.4). The major isoprenoid quinone was menaquinone (MK-6) that is consistent with other *Vitellibacter* species. The G+C content of strain VBW088^T was 36.7 mol% which is very close to *V. soesokkakensis* RSSK-12^T but however, other two type strains were reported to have more than 40 mol% (Nedashkovskaya et al. 2003; Kim et al. 2010).

Table 6C.2. Fatty acid profile of the strain VBW088^T and type strains of *Vitellibacter* species

Fatty acid	1	2	3	4
$C_{16:0}$	1.2	1.4	2.9	1.4
$C_{17:0}$ 2-OH	1.4	1.4	1.0	-
$C_{17:0}$ 3-OH	2.1	1.3	-	-
iso-$C_{15:0}$	33.5	34.6	40.1	36.7
anteiso-$C_{15:0}$	2.6	3.2	4.5	5.4
iso-$C_{15:0}$ 3-OH	4.0	4.4	3.3	3.9
iso-$C_{16:0}$	2.4	2.4	1.7	1.7
iso-$C_{16:0}$ 3-OH	2.4	2.3	-	1.4
iso-$C_{17:0}$ 3-OH	32.0	31.0	25.3	20.8
iso-$C_{15:1}$ G	2.6	2.2	1.6	4.7
iso-$C_{17:1}\omega 9c$	8.3	7.1	6.8	6.7
Summed feature 3	4.2	5.6	5.6	10.9

Strains: 1, VBW088^T (present study); 2, *V. soesokkakensis* RSSK-12^T (present study); 3, *V. vladivostokensis* KMM 3516^T and 4, *V. aestuarii* JC2436^T (Park et al. 2014). Media and growth conditions are similar for all the type strains. Summed feature 3 contained $C_{16:1}\omega 6c$/ $C_{16:1}\omega 7c$; Fatty acids amounting $\geq$1 % of the totals fatty acids are shown.

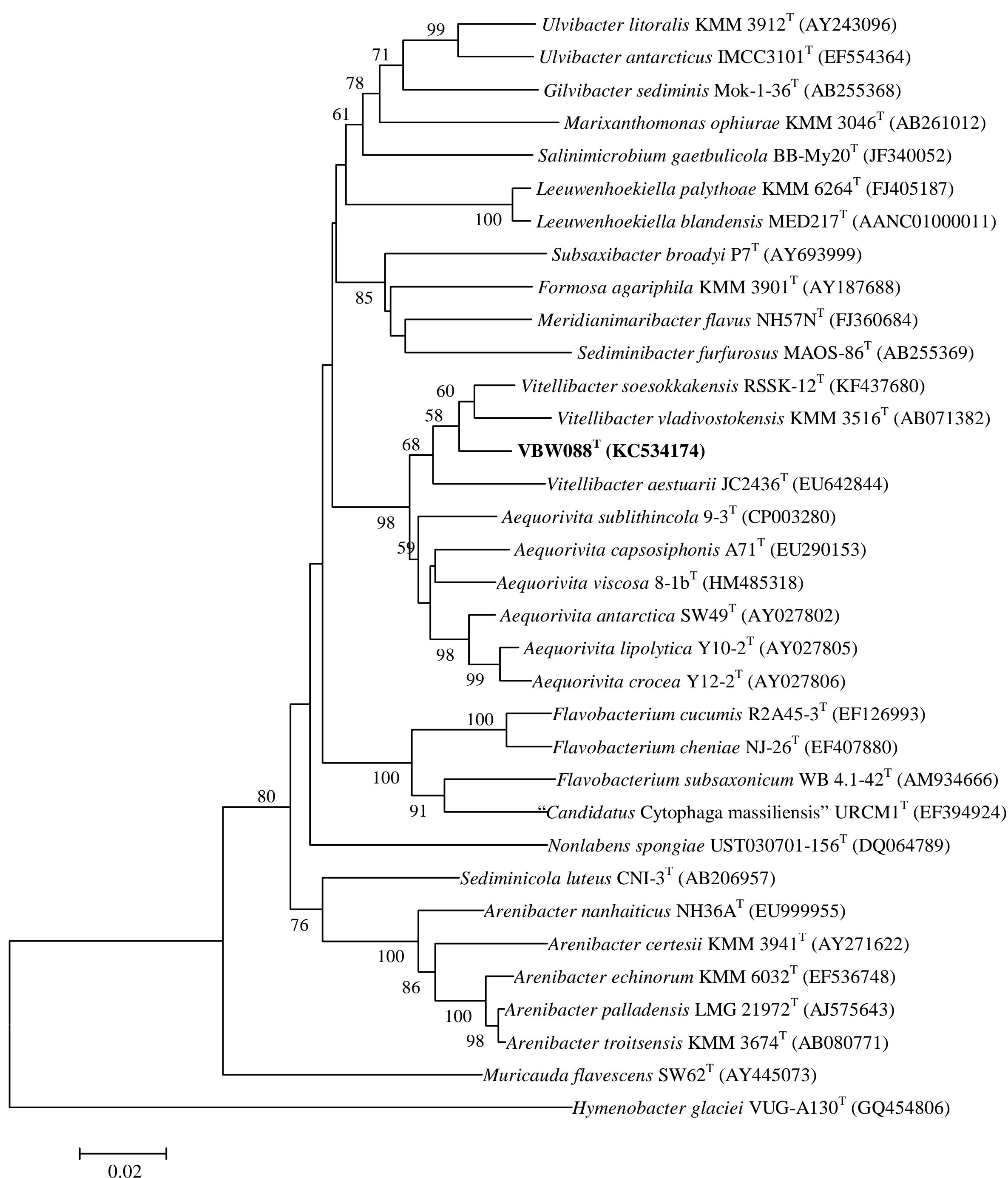


Fig. 6C.2. Neighbour-joining tree showing the phylogenetic relationship of strain VBW088^T and phylogenetically related species based on 16S rRNA gene sequences. Numbers at the nodes are bootstrap values >50 %. *Hymenobacter glaciei* VUG-A130^T (GQ454806) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

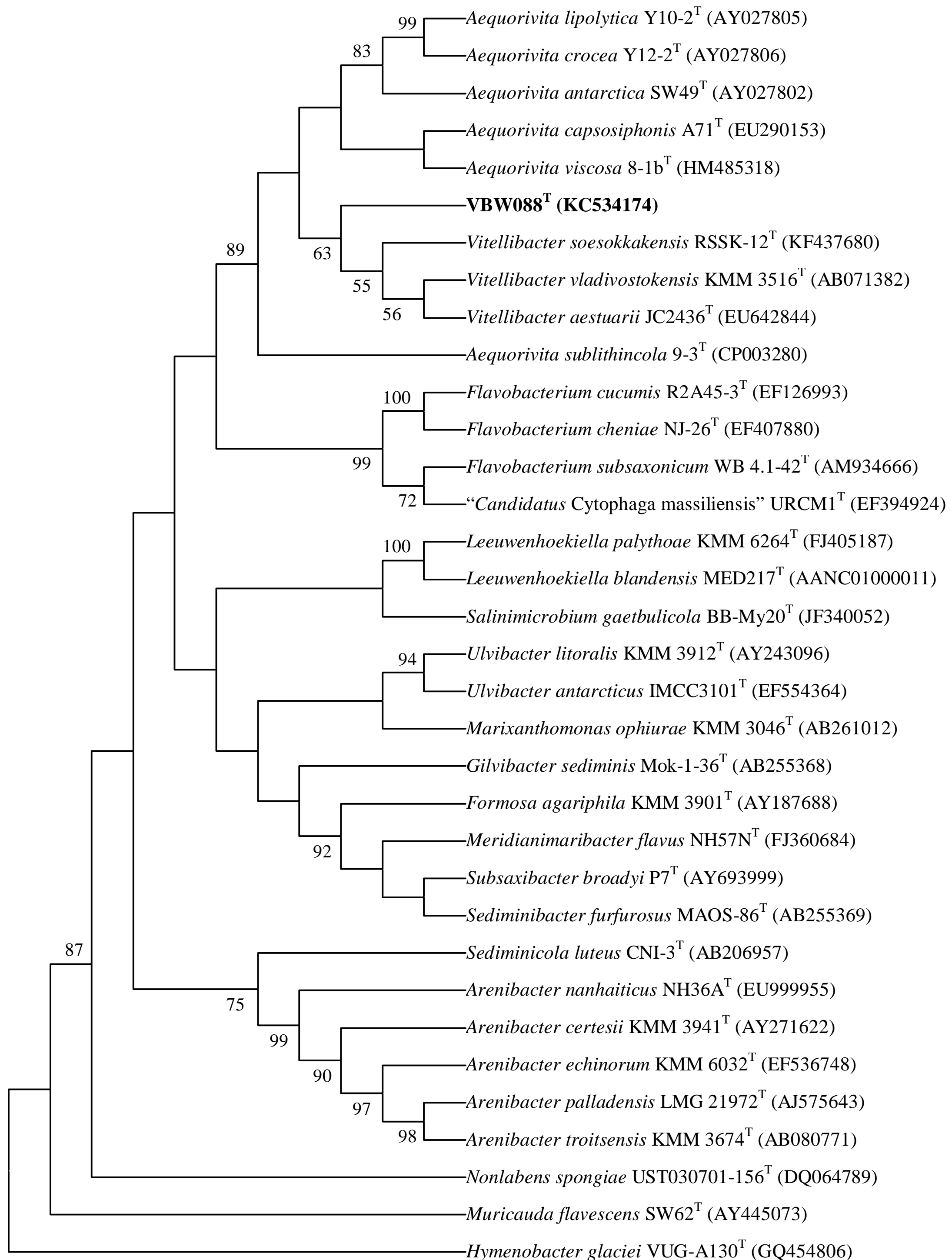


Fig. 6C.3. Maximum-parsimony tree based on 16S rRNA gene showing the phylogenetic position of the strain VBW088^T and related representative taxa.

The methanol extract of the yellow pigmented strain VBW088^{T} has absorption peak at 450 nm (Fig. 6C.5). Some of the yellow pigmented *Flavobacterium* and *Zeaxanthinibacter* are known to produce carotenoids with absorption maxima of around 450 nm (Asker et al. 2007). When the extracts were tested for the presence of flexirubin pigments by exposing them to KOH, resulted no change of colour which indicated that the pigment is not belong to flexirubin (Reichenbach et al. 1980). Interestingly, two of the *Vitellibacter* species were able to produce flexirubin pigments (Nedashkovskaya et al. 2003; Kim et al. 2010).

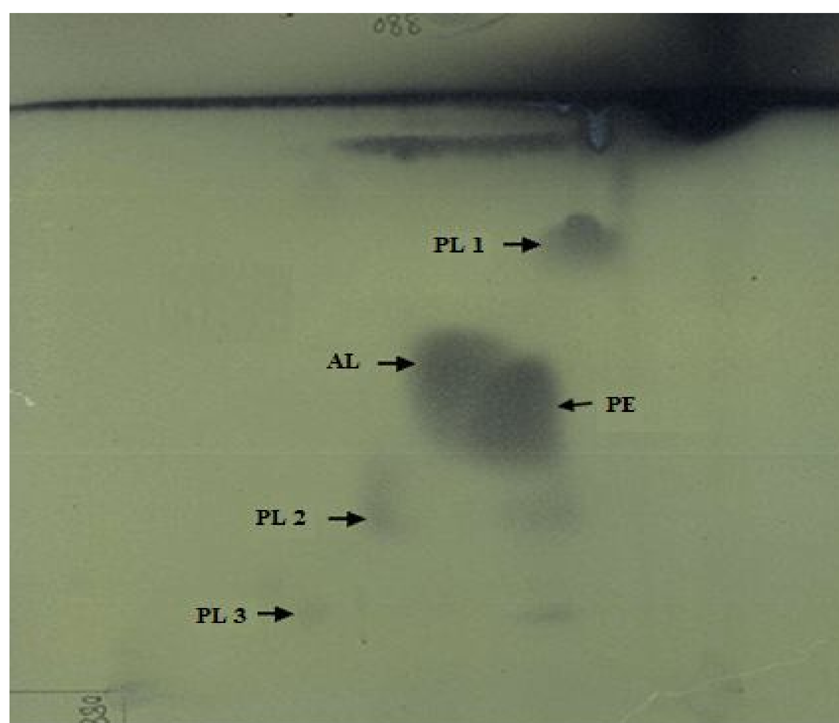


Fig. 6C.4. Two-dimensional TLC of polar lipids of the strain VBW088^{T}. PL 1-3, unidentified phospholipids; PE, phosphatidyl ethanolamine; AL, unidentified aminolipid

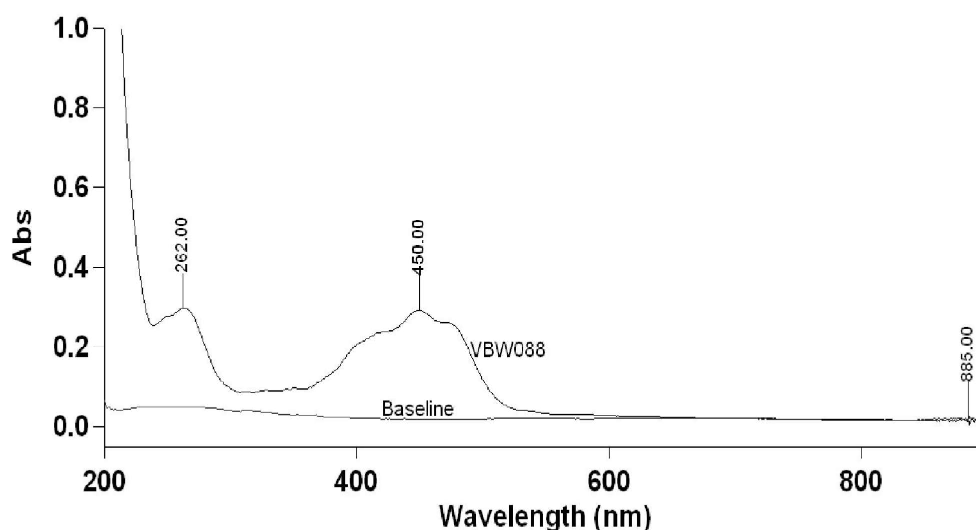


Fig. 6C.5. UV-VIS absorption spectrum of a methanolic extract of the pigment from strain VBW088^{T}

Based on low level of DNA-DNA relatedness (12.7±3.5 %), chemotaxonomic data, phenotypic, and phylogenetic inference presented above, it can be concluded that strain VBW088^{T} represents a novel species of the genus *Vitellibacter*, for which the name *Vitellibacter nionensis* sp. nov. is proposed.

6C.4. Description of *Vitellibacter nionensis* sp. nov.

Vitellibacter nionensis (ni.o.nen'sis. N.L. masc. adj. *nionensis*, arbitrary name from the acronym of National Institute of Oceanography, NIO, where taxonomic studies were performed).

Cells are aerobic, Gram-negative, non-spore forming, non-motile and short-rods (0.3–0.4 × 0.8–1.0 µm). Colonies on MA are circular, raised, smooth, yellow in colour after incubation for 48 h at 30 °C. Optimal growth occurs at 30 °C; growth occurs between 10 and 37 °C. Optimal pH for growth is 6.0; growth occurs at pH 6.0 to 8.0. Optimal growth

occurs in the presence of 1 – 2 % (w/v) NaCl; growth occurs in the presence of 5.0 % (w/v) NaCl, but not in the absence of NaCl or above 5.0 % (w/v) NaCl. Mg^{2+} not required for growth. Catalase and oxidase are positive. Anaerobic growth does not occur on MA. Carotenoid pigment is produced but not flexirubin. Nitrate is not reduced to nitrite. Citrate utilization, H_2S and indole production, and the ONPG, methyl red and Voges Proskauer tests are negative. Gelatin, casein, Tween 20 and Tween 80 are hydrolysed, but starch, DNA, CMC, xylan, urea, tributyrin, xanthine and alginate are not hydrolysed. Sodium malonate is utilised as a sole carbon source. Detectable levels of acid are not produced from carbohydrates except potassium 5-keto gluconate. In assays with the API ZYM strips, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase, valine arylamidase, cystine arylamidase (weak), acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but lipase (C 14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The predominant menaquinone is MK-6. The major fatty acids are iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The major polar lipids are phosphatidyl ethanolamine, one unidentified aminolipid and three unidentified phospholipids. The DNA G+C content of the type strain is 36.7 mol%.

The type strain is VBW088^T (= KCTC 32420^T = MCC 2354^T), isolated from shallow water hydrothermal vent of Espalamaca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VBW088^T is KC534174.

Chapter 6D

Citreicella manganoxidans sp. nov., a manganese oxidizing bacteria isolated from a shallow water hydrothermal vent in Espalamaca (Azores)

6D.1. Introduction

The genus *Citreicella*, a member of the phylum α-*Proteobacteria*, was proposed by Sorokin et al. (2005). At the time of writing, the genus *Citreicella* includes only three validly published species, *Citreicella thiooxidans* (Sorokin et al. 2005), *Citreicella aestuarii* (Park et al. 2011) and *Citreicella marina* (Lai et al. 2011b). During our investigation on culturable bacterial diversity from a shallow water hydrothermal vent in Espalamaca (Azores, Portugal), several manganese resistant bacteria were isolated (Chapter 4). In this study, we characterized one of these isolates, designated strain VSW210^T which was isolated from surface sea water. The aim of the present investigation was to determine the precise taxonomic position of strain VSW210^T using a polyphasic approach.

6D.2. Materials and methods

6D.2.1. Study area and strain isolation

Surface seawater was collected from shallow hydrothermal vent in Espalamaca region (38°33'N; 28°39'W), Azores, Portugal through an Indo-Portugal bilateral program during September 2010. For isolation of this bacteria, serially diluted water samples were spread plated on diluted seawater nutrient agar (dSWNA: peptone 1.25 g L^{-1}, beef extract 0.75 g L^{-1}, 1.8 % agar prepared in 50 % seawater) amended with 1 mM $MnCl_2$. The plates were incubated for up to 3 days at 30 ± 2 °C and morphologically different colonies were isolated. Growth of strain VSW210^T on marine agar 2216 (MA: M384, Himedia) was confirmed and maintained at 4 °C. Strain VSW210^T was also stored at -80 °C in dSWNA with 15 % glycerol. Type strain *Citrecella marina* CK-I3-6^T was

purchased from LMG culture collection (LMG 25230) and parallel study was conducted with our strain VSW210^T.

6D.2.2. Phylogenetic analysis

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions and the 16S rRNA gene was PCR amplified with eubacterial primers 27F/1492R (Lane 1991). The PCR amplified 16S rRNA gene was purified with PCR cleanup kit (Sigma) and sequenced on 3130xl Genetic Analyzer (Applied Biosystems). Nearly full length 16S rRNA gene sequence (1373 nt) was subjected to a BLASTn on the National Center for Biotechnology Information (NCBI) to determine an approximate phylogenetic affiliation and EzTaxon-e (Kim et al. 2012a) to identify similarity values between the isolate and related taxa. Sequences were aligned using Clustal W (Thompson et al. 1994) and phylogenetic trees were constructed using neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein, 1981) methods with MEGA version 5.2 (Tamura et al. 2011). The topology of the phylogenetic trees was estimated by bootstrap analysis with 1,000 replications (Felsenstein 1985).

For DNA-DNA hybridization experiments, genomic DNA was extracted and purified according to the method of Marmur (1961). The analysis was done by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with 96 well thermal cycling block in 96 well plate (Loveland-Curtze et al. 2011). The reassociation of DNA was carried out at optimum reassociation temperature 78.0 °C [Tor = 0.51 × (% G+C) + 47.0] according to De Ley et al. (1970) and Gillis et al. (1970). Resulted DNA-DNA relatedness values are the average of triplicates. DNA G+C content was

determined by reverse-phase HPLC of nucleosides following the protocol described by Mesbah et al. (1989).

6D.2.3. Morphological and biochemical characteristics

Gram staining (K001, Himedia) and motility (Collee et al. 2008) of strain VSW210^T was examined using light microscopy (Olympus BX-60) with 1000× magnification. The cell morphology was examined using a scanning electron microscope (JEOL JSM-5800LV). Growth under anaerobic condition was determined on MA after incubation for 10 days in an anaerobic system with an AnaeroPack (Himedia, Mumbai). The pH range for growth was determined by adjusting the pH of the marine broth 2216 (MB: M385, Himedia) using various buffers to pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer) and cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 3 days of incubation. Temperature dependent growth was tested on MA at 4, 10, 20, 25, 30, 37, 40, 45 and 50 °C. Salinity tolerance of strain VSW210^T was investigated in nutrient broth (NB: M088, Himedia) prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increment) with and without adding Mg^{2+} and Ca^{2+} (4.53 g L^{-1} $MgCl_2$; 5.94 g L^{-1} $MgSO_4$; 1.3 g L^{-1} $CaCl_2$).

Catalase activity was tested using 3 % (v/v) H_2O_2 drop method (Smibert and Krieg 1994) and oxidase activity was determined using the oxidase discs (DD018, Himedia). Hydrolytic enzyme tests were determined as described previously (section 6C.2.3.) on MA and other enzymes were tested using API ZYM (bioMérieux) according to the manufacturer's protocol. Acid production from carbohydrates was determined using API 50CH kit (bioMérieux) following the manufacturer's instructions using inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer and Hickman-Brenner 2006). Antibiotic susceptibility tests were performed in seawater

nutrient agar (SWNA: peptone 5.0 g L^{-1}, beef extract 1.5 g L^{-1}, yeast extract 1.5 g L^{-1}, 1.5 % agar prepared in 50 % seawater) using antibiotic discs (Himedia) containing the following concentrations (µg/disc): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyl (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30), ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (2 IU), polymyxin B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5). The results were observed after 3 days incubation at 30 °C.

6D.2.4. Chemotaxonomic analysis

Strain VSW210^T was cultivated on MA at 30 °C for 2 days and harvested for Fatty acid methyl ester (FAME). Fatty acids were saponified, methylated and extracted using the standard protocol of Microbial Identification System TSBA 6.0 (Sasser 1990). Cell biomass of strain VSW210^T for the analysis polar lipids was obtained from cultures grown for 2 days at 30 °C in MB. Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck) as described previously (section 6C.2.4).

6D.3. Results and discussion

Cells of strain VSW210^T appeared as Gram-staining-negative, rod shaped (Fig. 6D.1), non-motile and non-spore forming. The colonies of the strain VSW210^T were observed to be small, non-pigmented, circular, convex and smooth on MA and dSWNA media after 48 h. Strain VSW210^T did not grow on medium with NaCl as a sole salt and was found to require Mg^{2+}. Growth of the strain VSW210^T was observed at temperatures between 4 and 40 °C with optimum growth at 30 °C and at a pH range of 6.0–9.0 with an

optimum pH of 7.0. Differences between morphological and physiological characteristics of strain VSW210^{T} compared with the closest related type strains of the genus *Citreicella* are given in Table 6D.1. Strain VSW210^{T} was susceptible to all the antibiotics tested. Further, strain VSW210^{T} was detected to oxidize soluble Mn(II) to insoluble Mn(III/IV) oxides (detailed results are given in Chapter 4.3).

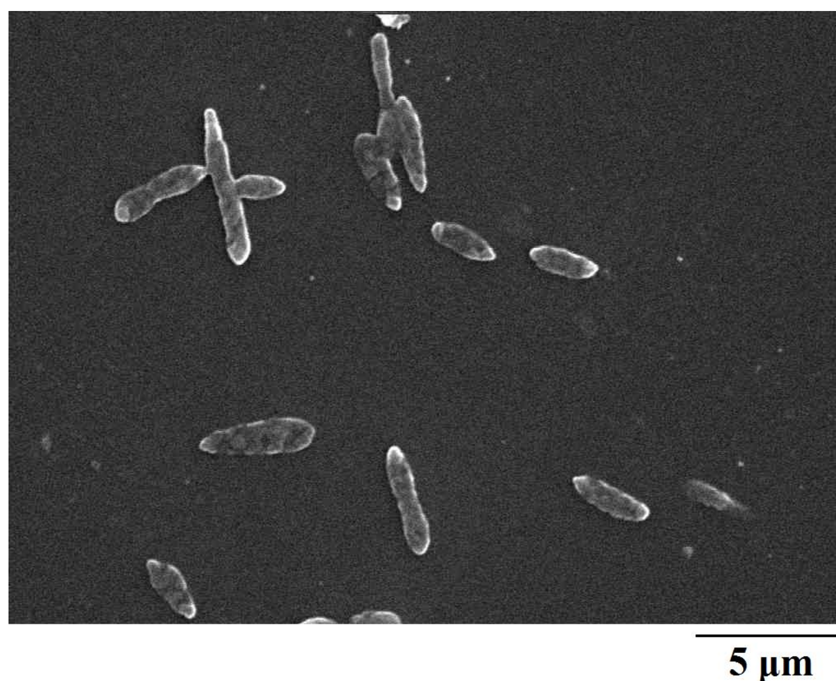


Fig. 6D.1. Scanning electron microscopic picture of strain VSW210^{T} showing rod-shaped cells

The 16S rRNA gene sequence of the strain VSW210^{T} was closely related with *Citreicella marina* CK-I3-6^{T} with sequence similarity of 99.6 %. However, DNA-DNA hybridization between VSW210^{T} and *C. marina* CK-I3-6^{T} showed a low DNA-DNA relatedness (55.8 %). The determined DNA-DNA relatedness was less than 70 %, the recommended threshold value generally considered for species delineation (Stackebrandt and Goebel 1994). 16S rRNA gene sequence similarity values between strain VSW210^{T} and other validly named species of the genus *Citreicella* in EzTaxon-e database were *C.*

thiooxidans CHLG 1^T (96.9 %) and *C. aestuarii* AD8^T (96.5 %). Neighbour-joining and maximum-parsimony phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain VSW210^T formed a separate subclade with *C. marina* CK-I3-6^T (Fig. 6D.2 and Fig. 6D.3).

Table 6D.1. Differential characteristics between strain VSW210^T and closest type strains of species of the genus *Citreicella*

Characteristic	1	2	3
NaCl range for growth (%, w/v)	0-12	0-12	0.5-16*
pH range for growth	6-9	6-9	6.5-8.5*
Temperature range for growth (°C)	4-40	4-37	8-35*
API-CH50			
Glycerol, L-arabinose, D-xylose, D-galactose, L-rhamnose, methyl-bD-xylopyranoside, D-lactose, D-melibiose, D-arabitol, L-fucose	+	+	-
Erythritol, salicin, D-trehalose, D-lyxose, potassium gluconate, potassium 5-keto gluconate	-	-	+
L-xylose	-	+	+
D-adonitol	-	+	-
L-sorbose	+	-	-
D-sorbitol	w	+	+
Gentibiose	-	w	-
API ZYM			
Alkaline phosphatase	w	+	+
Esterase (C4), Esterase lipase (C8)	+	+	w
Lipase (C14), Cystine arylamidase, α-chymotrypsin, β-glucosidase	-	-	w
Valine arylamidase	-	-	+
Naphthol-AS-BI-phosphoamidase	w	+	-
DNA G+C content (mol%)	67.4	67.5†	67.5-69.2*

Strains: 1, VSW210^T (present study); 2, *Citreicella marina* CK-I3-6^T (present study) ([†]Lai et al. 2011b); 3, *Citreicella thiooxidans* CHLG 1^T (Lai et al. 2011b; *Sorokin et al. 2005). All strains were positive for catalase and oxidase; leucine arylamidase, α-glucosidase; acid production from D-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, esculin, D-cellobiose, D-maltose, xylitol and D-fucose. All strains were negative for Gram-staining; enzymes trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase; acid production from dulcitol, inocitol, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, D-melazitose, D-raffinose, amidon, glycogen, D-turanose, D-tagatose, L-arabitol, and potassium 2-ketogluconate. For column 1 and 2, hydrolysis of Tween 20, DNA and agar were positive and casein, gelatin, Tween-80, xanthine, xylan, CMC and tributyrin were negative. +, Positive; -, negative; w, weak reaction

Table 6D.2. Fatty acid profile of strain VSW210^T and closest type strains of species of the genus *Citreicella*

Fatty acids	1	2	3
$C_{12:1}$ 3-OH	2.8	2.3	tr
$C_{12:0}$ 3-OH	-	-	1.7
$C_{10:0}$	1.7	-	-
$C_{16:0}$	9.2	7.8	8.6
$C_{17:0}$	1.1	tr	tr
$C_{18:1}$ ω7*c*	80.1	66.6	74.6
$C_{18:0}$	1.3	2.0	2.6
$C_{18:1}$ ω7*c* 11-methyl	-	1.9	tr
$C_{19:0}$ ω8c cyclo	-	10.0	6.6
Summed feature 3*	3.9	6.3	1.9

Strains: 1, VSW210^T (present study); 2, *Citreicella marina* CK-I3-6^T (Lai et al. 2011b); 3, *Citreicella thiooxidans* CHLG 1^T (Lai et al. 2011b). Only fatty acids amounting to ≥1 % of the total are shown. -, not detected; tr, trace amount (<1 %). *Summed features contain fatty acids that cannot be separated using standard procedures. Summed feature 3 contained $C_{16:1}$ ω7*c*/ $C_{16:1}$ ω6*c*.

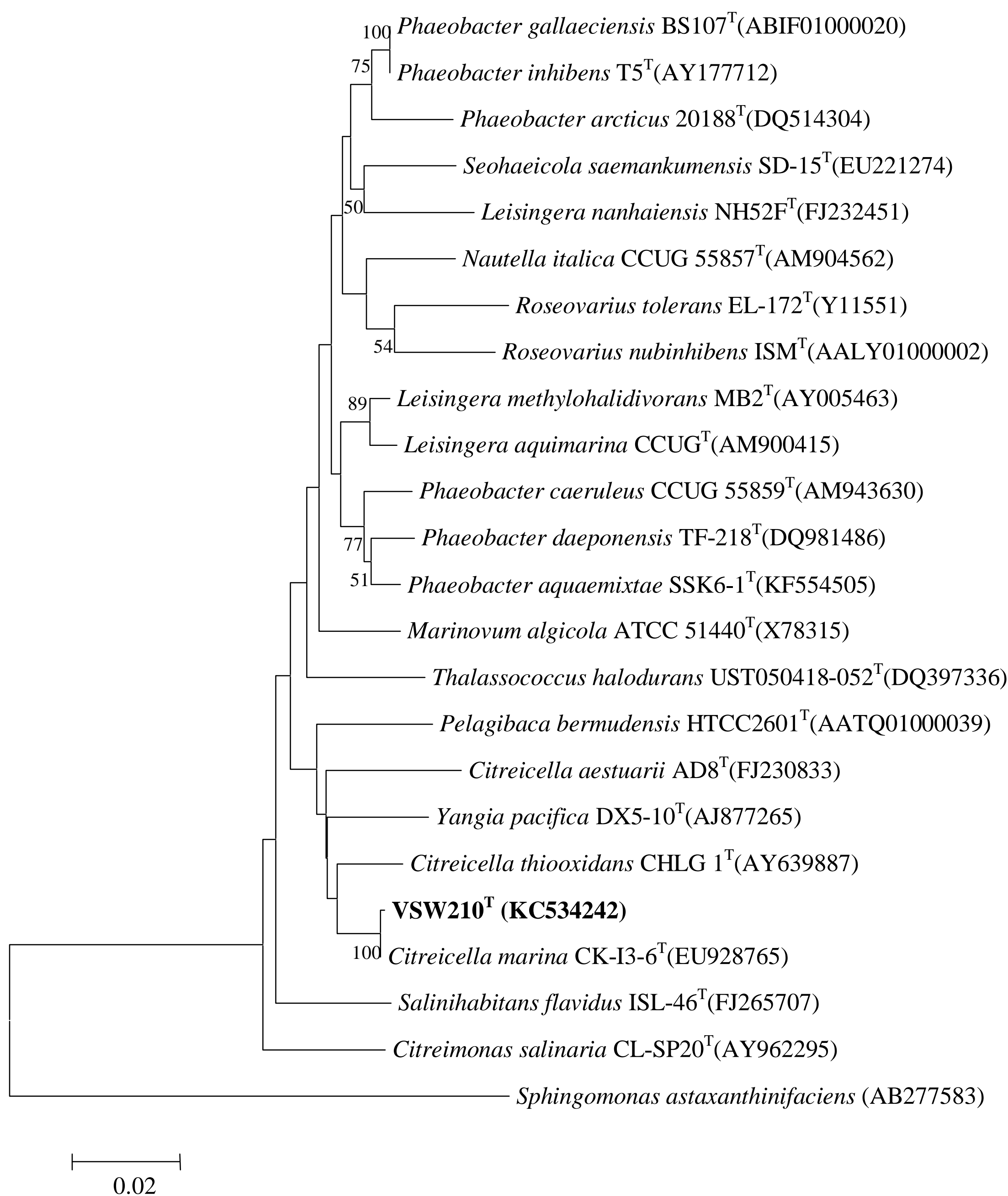


Fig. 6D.2. 16S rRNA gene sequence based neighbour-joining tree showing the phylogenetic relationship of strain VSW210^T. Numbers at the nodes are bootstrap values >50 %. *Sphingomonas astaxanthinifaciens* TDMA-17^T (AB277583) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

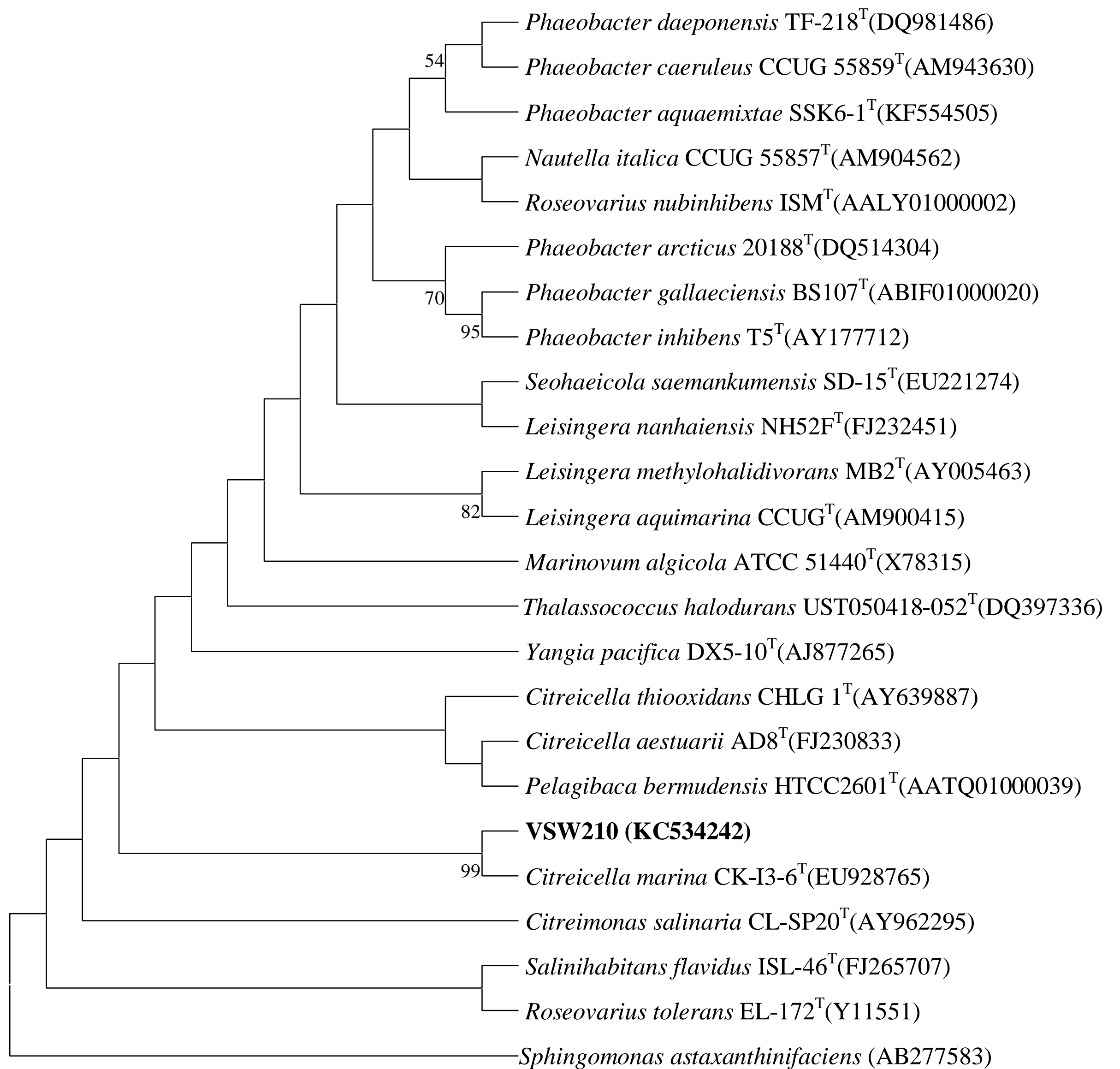


Fig. 6D.3. Maximum-parsimony tree based on 16S rRNA gene sequence showing the phylogenetic position of the strain VSW210^T and related members.

The polar lipid profile of strain VSW210^T was found to consist of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid (Fig. 6D.4). The major fatty acids found in strain VSW210^T were $C_{18:1}$ $\omega 7c$ (80.1 %) and $C_{16:0}$ (9.2 %), which were almost comparable to the profiles of the type strains *Citreicella marina* CK-I3-6^T and *C. thiooxidans* CHLG 1^T (Table 6D.2). However, the fatty acid $C_{19:0}$ ω8c cyclo which was reported (>5 %) in *Citreicella marina* CK-I3-6^T and *C. thiooxidans* CHLG 1^T, was not detected in strain VSW210^T. In addition to that, fatty acid $C_{10:0}$ which was observed from strain VSW210^T, was not observed from the closest type strains. The genomic G+C content was found to be 67.4 mol% which is unfailing with species of the genus *Citreicella*.

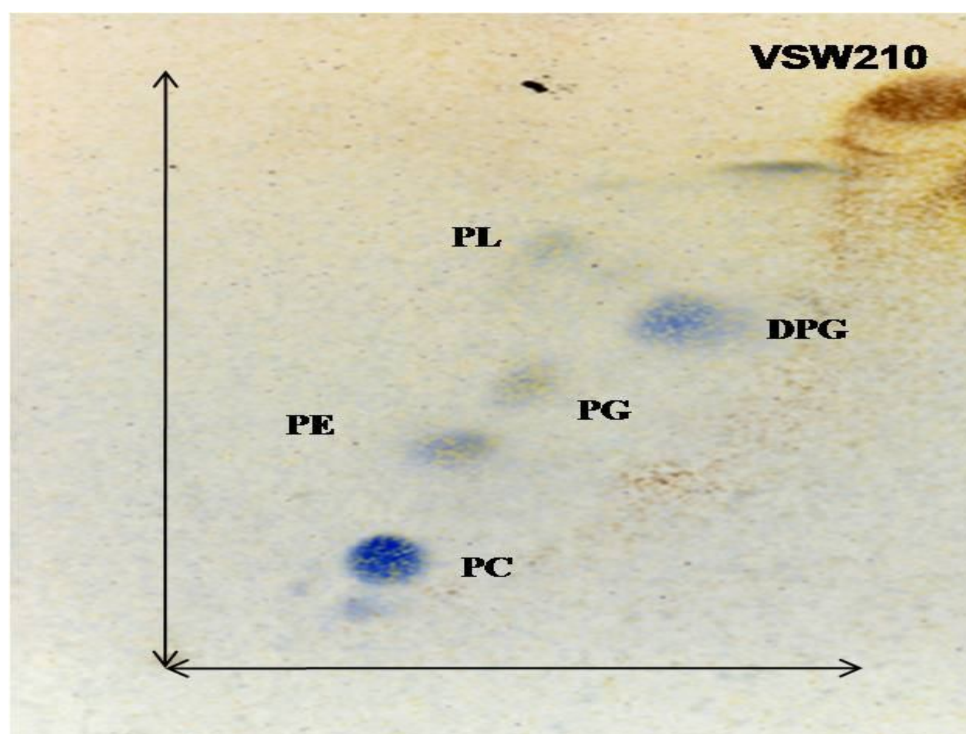


Fig. 6D.4. Two-dimensional TLC of polar lipids of the strain VSW210^T. PL, unidentified phospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol

Based on phylogenetic analysis, phenotypic characteristics, chemotaxonomic data and low level of DNA-DNA relatedness (55.8 %), it can be concluded that strain VSW210^T represents a novel species of the genus *Citreicella*, for which the name *Citreicella manganoxidans* sp. nov. is proposed.

6D.4. Description of *Citreicella manganoxidans* sp. nov.

Citreicella manganoxidans (man.gan.ox'i.dans. N.L. neut. n. *manganum* manganese; M.L. part. adj. *oxidans* oxidizing; N.L. part. adj. *manganoxidans* manganese-oxidizing).

Cells are aerobic, Gram-staining-negative, non-spore-forming and non-motile rods (0.4 – 1.1 × 1.9 – 5.8 μm). Colonies on MA and dSWNA are circular, raised, smooth and non-pigmented after incubation for 48 h at 30 °C. Grows in 0–12 % NaCl (optimum 2–6 %), at pH 6–9 (optimum 7) and at 4–40 °C (optimum 30 °C). Mg^{2+} is required for growth. Positive for catalase and oxidase. Anaerobic growth does not occur on MA. Nitrate is not reduced to nitrite. Tween 20, DNA and agar are hydrolysed, but casein, gelatin, Tween-80, xanthine, xylan, CMC and tributyrin are not hydrolysed. In assays with API ZYM strips, alkaline phosphatase (weak), esterase (C 4), esterase lipase (C 8), leucine arylamidase, acid phosphatase (weak), naphthol-AS-BI-phosphohydrolase (weak) and α-glucosidase activities are present, but lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Acids produced from the substrates, glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, methyl-bD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol (weak), esculin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, xylitol, D-fucose, L-fucose and D-arabitol. Major fatty acid is $C_{18:1}$ ω7c. The major polar lipids are phosphatidylcholine,

phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and one unidentified phospholipid.

The type strain, VSW210^T (= KCTC 32497^T = MCC 2286^T), was isolated from surface seawater at the shallow hydrothermal vent region in Espalamaca, Azores, Portugal. The DNA G+C content of the type strain is 67.4 mol%. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VSW210^T is KC534242.

Vibrio azorensis sp. nov., isolated from shallow water hydrothermal vent sediment (Espalamaca, Azores)

6E.1. Introduction

The genus *Vibrio* has been reported from various marine environments including seawater, sediments and as symbiont on various organisms ranging from plankton to fish (Thompson et al. 2004). Taxonomy of Vibrios has been studied in detail, and has been subjected to repeated rearrangements in last 3 decades due to the developments and use of molecular methods such as DNA-DNA hybridization, multilocus sequence analysis (MLSA) and amplified fragment length polymorphism (AFLP) (Colwell 2006; Thompson and Swings 2006; Beaz-Hidalgo et al. 2010; Dieguez et al. 2011). These techniques have enhanced the understanding of the taxonomic structure and phylogeny of Vibrios (Lasa et al. 2014). At the time of writing, there are 120 validly described species of the genus *Vibrio*, which includes two subspecies. During our investigation on culturable bacterial diversity from a shallow water hydrothermal vent in Espalamaca (Azores, Portugal), several lead (Pb) resistant bacteria were isolated (Chapter 4). In this study, we characterized one of these isolates, designated strain VSD707^T which was isolated from hydrothermal vent sediments. The main aim of the present investigation was to determine the precise taxonomic position of strain VSD707^T using a polyphasic approach.

6E.2. Material and methods

6E.2.1. Strain isolation

Strain VSD707^T was isolated from the sediment sample collected from the shallow water hydrothermal vent in Espalamaca region in the Azores, Portugal (38°33'N; 28°39'W) through an Indo-Portugal bilateral program during August 2012. For isolation, serially diluted sediment samples were spread plated on diluted seawater nutrient agar (dSWNA:

peptone 1.25 g L^{-1}, beef extract 0.75 g L^{-1}, 1.8 % agar prepared in 50 % seawater) amended with 1 mM $Pb(NO_3)_2$. The plates were incubated for 2 days at 30 ± 2 °C and morphologically different colonies were isolated. Strain VSD707^T was isolated and maintained on marine agar 2216 (MA: M384, Himedia) at 4 °C and as 15 % glycerol suspensions at -80 °C for long-term maintenance.

6E.2.2. Morphological, physiological and biochemical characterization

Gram staining (K001, Himedia) and motility (by hanging drop) of strain VSD707^T was examined using light microscopy (Olympus BX-60) with 1000× magnification. The cell morphology was examined by scanning electron microscopy (JEOL JSM-5800LV). Salinity tolerance of strain VSD707^T was investigated on nutrient broth (NB: M088, Himedia) with 1.5 % agar prepared in distilled water with NaCl concentrations of 0 – 15 % (w/v; 1 % increment). The pH range for growth was determined by adjusting the pH of the SWNB medium (nutrient broth prepared in 50 % seawater) using various buffers to pH 4–5 (citrate buffer), pH 6–8 (phosphate buffer) and pH 9–10 (carbonate buffer). Growth at various temperatures 4, 10, 15, 20, 30, 37, 40 and 45 °C was also determined by using SWNB medium. In above experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation.

Catalase activity was determined using 3 % (v/v) H_2O_2 drop method (Smibert and Krieg, 1994) and oxidase activity was tested with oxidase discs (DD018, Himedia). Hydrolytic enzyme tests were determined as described previously (section 6C.2.3) on MA and other enzymes were tested using API ZYM (bioMérieux) according to the manufacturer's protocol. Acid production from carbohydrates was determined using API 50CH kit (bioMérieux) following the manufacturer's instructions using inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer & Hickman-Brenner,

2006). Antibiotic susceptibility tests were performed in seawater nutrient agar as described previously (section 6C.2.3).

6E.2.3. Chemotaxonomic analysis

Cell biomass of strain VSD707^T for the analysis of isoprenoid quinones and polar lipids was obtained from cultures grown in NB prepared in 50 % seawater for 2 days at 30 °C. Analysis of whole cell fatty acids of the isolate was performed according to the Microbial Identification System TSBA 6.0 (Sasser 1990) after cultivation on MA for 2 days at 30 °C. Polar lipids were extracted and examined as described previously (Section 6C.2.4). The DNA G+C content of strain VSD707^T was determined by reverse-phase HPLC of nucleosides according to Mesbah et al. (1989).

6E.2.4. Phylogenetic analyses

Genomic DNA of strain VSD707^T was isolated using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. The 16S rRNA gene was amplified using the consensus bacterial primers 27F and 1492R (Lane 1991). PCR amplification of *pyrH* (uridine monophosphate kinase gene), *recA* (recombinase A gene) and *gyrB* (gyrase B gene) were done as described previously by Wang et al. (2010b). The PCR amplified 16S rRNA gene was purified with a PCR cleanup kit (Sigma) following the manufacturer's instructions. DNA sequencing was performed using Genetic analyzer 3130xl (Applied Biosystems). The sequence was aligned using Clustal W sequence alignment program (Thompson et al. 1994). The 16S rRNA gene sequence was subjected to BLASTn in NCBI database and EzTaxon-e (Kim et al. 2012a) to identify similarity values between the isolate and related taxa. Phylogenetic trees were established with neighbour-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971)

algorithms using MEGA 5.0 software (Tamura et al. 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985).

6E.3. Results and discussion

The colonies of strain VSD707^T were small, colourless to pale yellow and circular on MA and SWNA. The cells were Gram-negative, motile and non-spore forming curved rods (Fig. 6E.1).

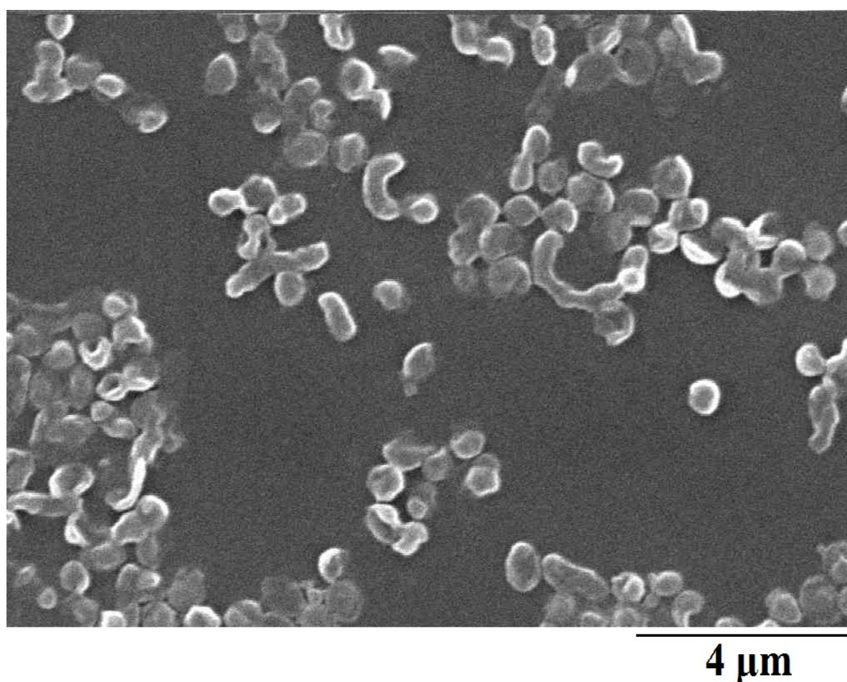


Fig. 6E.1. Scanning electron microscopic picture of strain VSD707^T

Growth occurs between 4 and 32 °C (optimum 25 °C) at pH 5–9 (optimum pH 6.0) with NaCl concentration of 1–10 % (optimum 1–2 %). Mg^{2+} was not required for growth. Strain VSD707^T was sensitive to the following antibiotics: amoxicillin, ampicillin,

cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, norfloxacin, novobiocin, rifampicin, amikacin, streptomycin, tetracycline, kanamycin, neomycin, polymyxin B and tobramycin; resistant to the following antibiotics: vancomycin, bacitracin, penicillin G and lincomycin. Differences noticed on morphological and physiological characteristics of strain VSD707^T with the closest type strain of *Vibrio* are given in Table 6E.1.

Table 6E.1. Characteristics that differentiate strain VSD707^T from its closest neighbour *Vibrio plantisponsor* MSSRF60^T

Characteristics	VSD707^T	MSSRF60^T
Growth temperature (°C)	4-32 (25)	10-40 (28)
Salinity range (%)	1.0-10	0.5-8.0
Indole production	-	+
Casein hydrolysis	-	+
Citrate utilization	-	+
Acid production from		
D-xylose	-	+
D-sorbitol	+	-
Amygdalin	-	+
D-lactose	-	+
D-raffinose	-	+
Glycogen	-	+
D-arabitol	+	-
Potassium gluconate	-	+
β-galactosidase	-	+
DNA G+C content (mol%)	46.9	41.8
Isolation source	Hydrothermal vent sediment	Mangrove roots

Strains: VSD707^T, *Vibrio azorensis* sp. nov. (present study); MSSRF60^T, *Vibrio plantisponsor* (Rameshkumar et al. 2011). Both the taxa are positive for motility, catalase, oxidase, nitrate reduction, hydrolysis of starch, acid production from L-

arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, N-acetyl glucosamine, arbutin, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose and gentibiose. Both the taxa are negative for Gram-staining, H2S production, lysine utilization, Voges Proskauer's test, hydrolysis of gelatin, Tween 80, DNA, alginate, acid production from glycerol, erythritol, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, dulcitol, inocitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, D-melibiose, inulin, D-melazitose, D-turanose, D-tagatose, D-fucose, L-arabitol, potassium 2-keto gluconate and potassium 5-keto gluconate. +, Positive; -, negative

Nearly full length 16S rRNA gene sequence of strain VSD707^T (GenBank accession number KC534342) was compared with the sequences from the GenBank database (http://www.ncbi.nlm.nih.gov). 16S rRNA gene sequence similarity values between strain VSD707^T and closest validly named species of the genus *Vibrio* in EzTaxon-e database are *V. plantisponsor* MSSRF60^T (96.9 %), *V. tapetis* subsp. *brittanicus* HH6087^T (96.8 %), *V. diazotrophicus* ATCC 33466^T (96.8 %), *V. tasmaniensis* LMG 21574^T (96.7 %) and V. atlanticus Vb 11.11^T (96.6 %). Neighbour-joining phylogenetic analysis based on 16S rRNA, *pyrH*, *recA* and *gyrB* revealed that strain VSD707^T formed a separate branch among other *Vibrio* species (Fig. 6E.2 – 6E.5). Every housekeeping gene sequences indicated different *Vibrio* species as the closest relative in BLASTn, with maximum similarity values of 82 %, 84 % and 87 % for *gyrB*, *pyrH* and *recA* genes respectively. Studies conducted by Rameshkumar et al. (2011) concluded that gene sequences of *pyrH*, *recA* and *gyrB* had a higher phylogenetic resolution when compared to *mreB* and *gapA*.

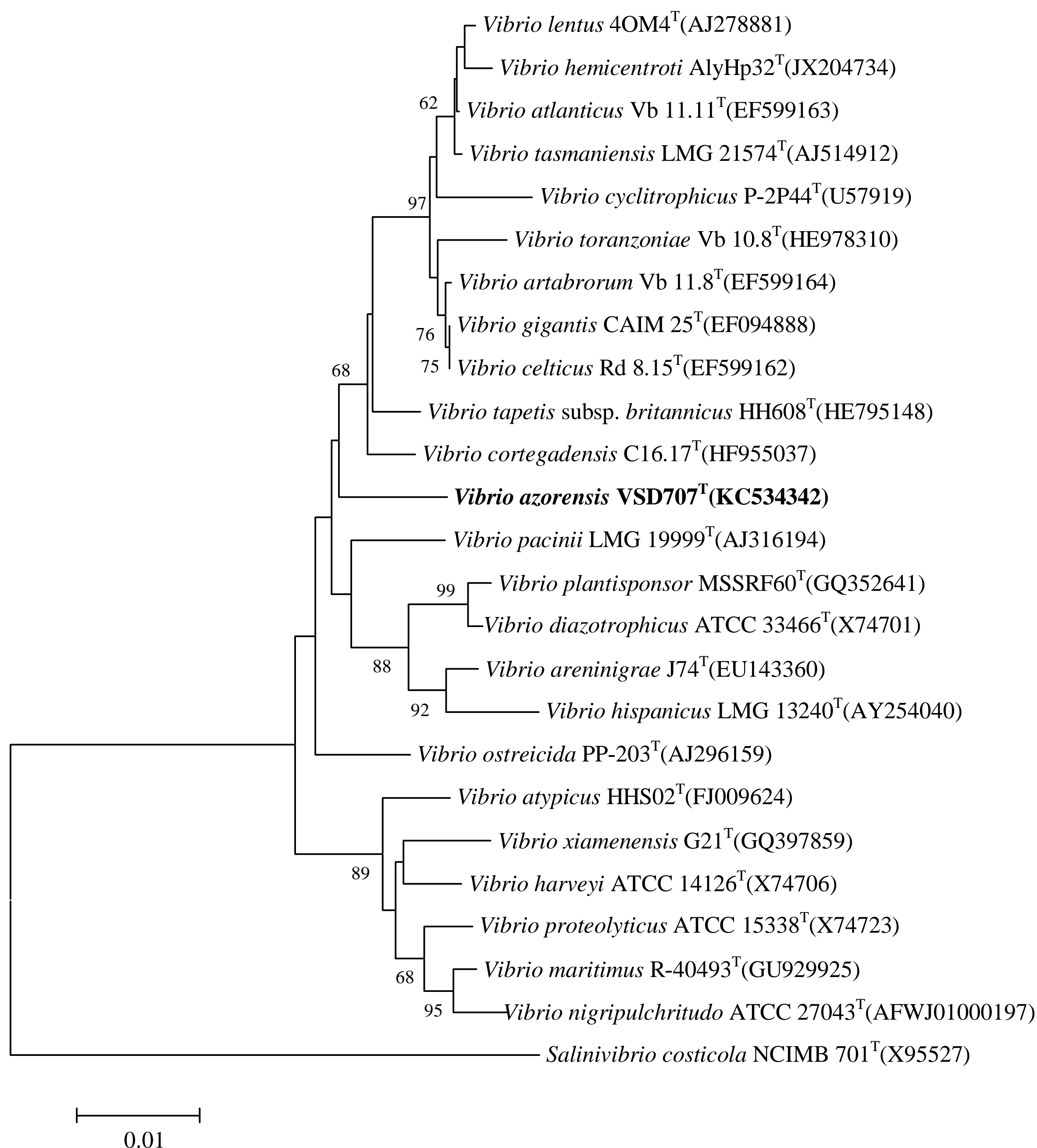


Fig. 6E.2. 16S rRNA gene sequence based neighbour-joining tree showing the phylogenetic relationship of strain VSD707^T among closely related *Vibrio* species. Numbers at the nodes are bootstrap values >50 %. *Salinivibrio costicola* NCIMB 701^T (X95527) was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

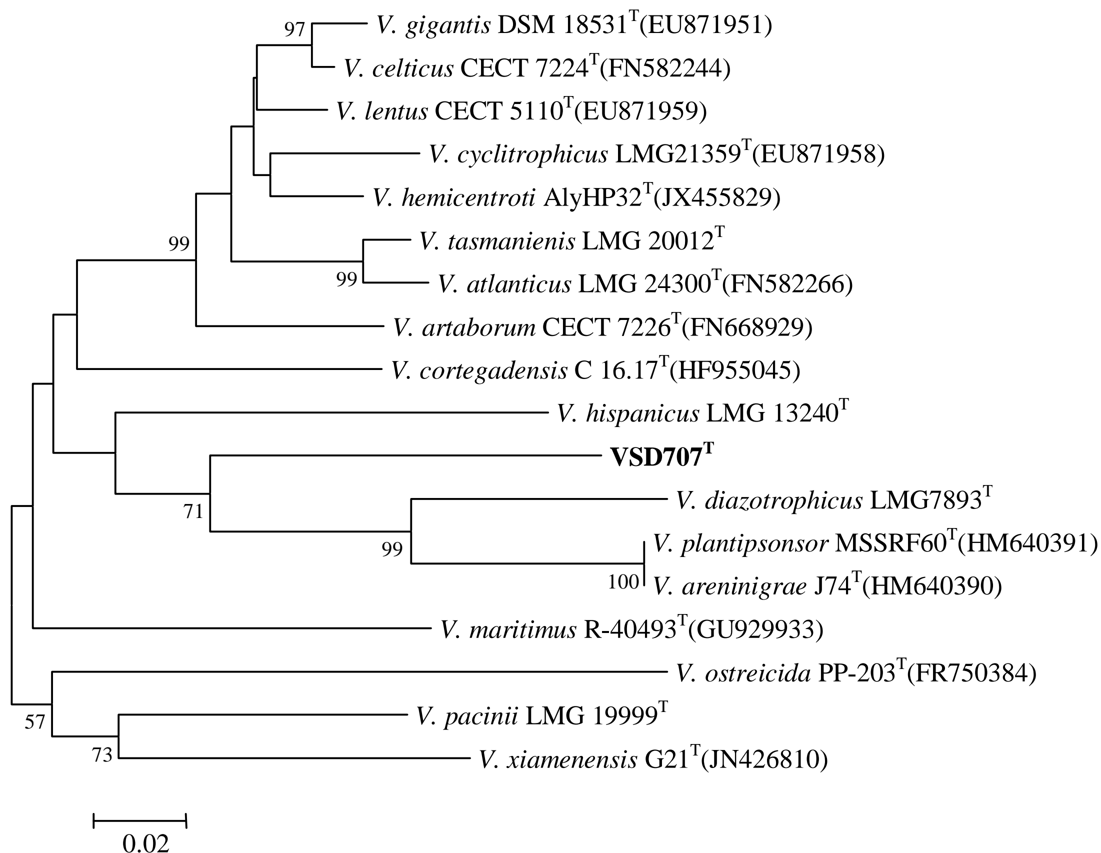


Fig. 6E.3. Phylogenetic tree based on *pyrH* gene sequences constructed using neighbour-joining method, showing the phylogenetic placement of strain VSD707^T and their phylogenetic neighbours of the genus *Vibrio*. Bootstrap percentages (based on 1000 replicates) are indicated at branching points (values >50 % are shown). Gene sequences for *V. hispanicus* LMG 13240^T, *V. tasmanienis* LMG 20012^T, *V. diazotrophicus* LMG7893^T and *V. pacinii* LMG 19999^T were obtained from TaxVibrio database (http://www.taxvibrio.lncc.br/)

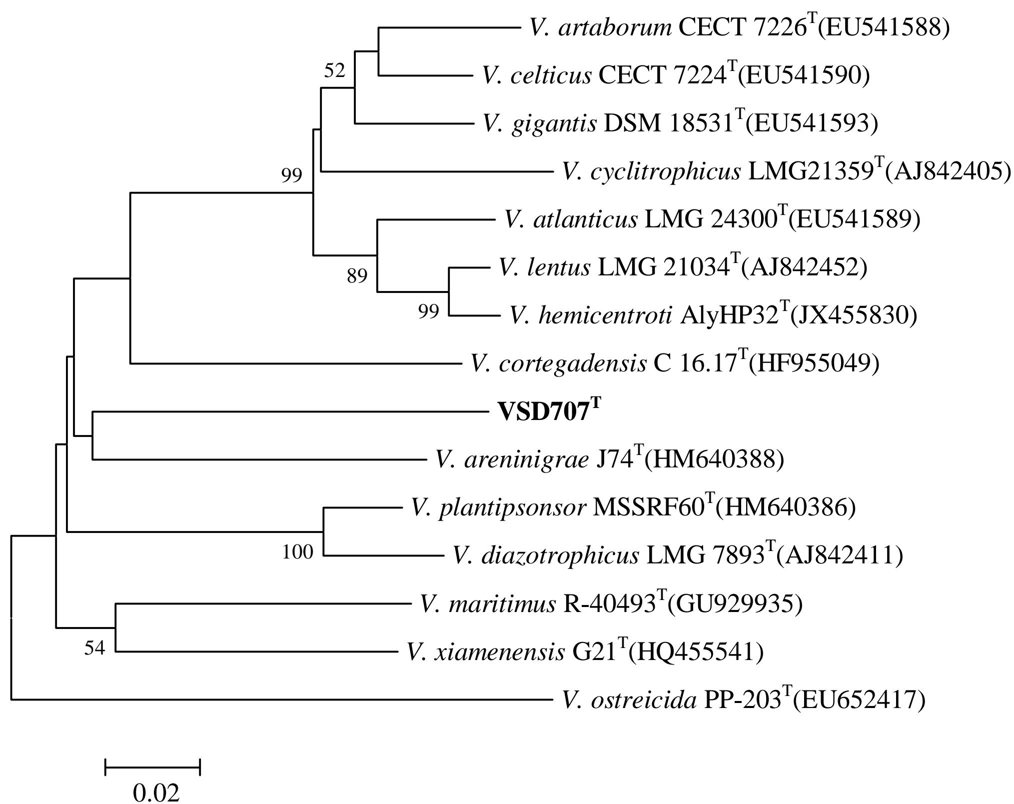


Fig. 6E.4. Phylogenetic tree based on *recA* gene sequences constructed using neighbour-joining method, showing the phylogenetic placement of strain VSD707^T and their phylogenetic neighbours of the genus *Vibrio*. Bootstrap percentages (based on 1000 replicates) are indicated at branching points (values >50 % are shown).

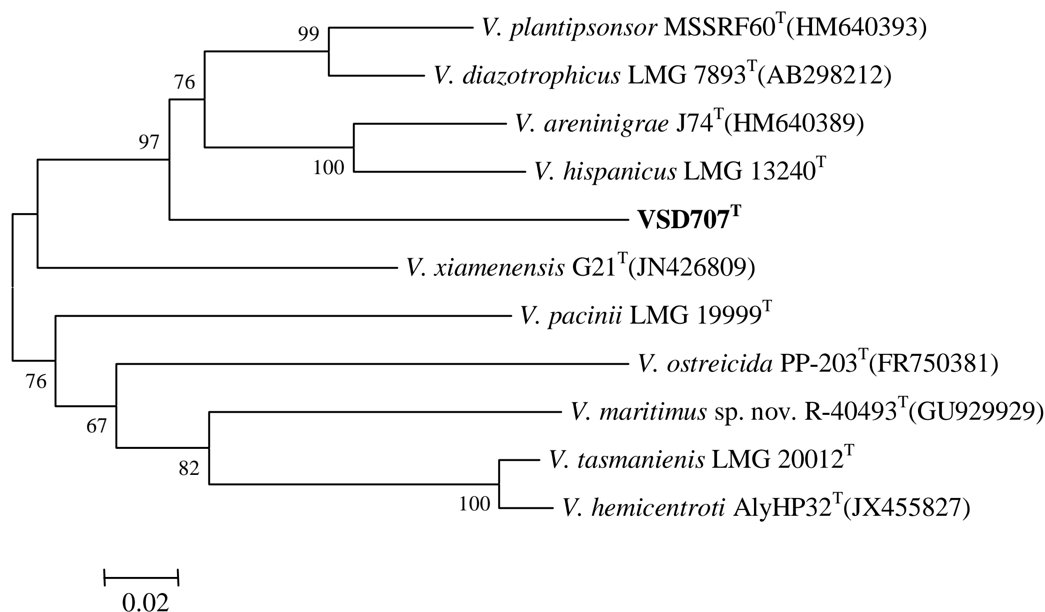


Fig. 6E.5. Phylogenetic tree based on *gyrB* gene sequences constructed using neighbour-joining method, showing the phylogenetic placement of strain VSD707^T and their phylogenetic neighbours of the genus *Vibrio*. Bootstrap percentages (based on 1000 replicates) are indicated at branching points (values >50 % are shown). Gene sequences for *V. hispanicus* LMG 13240^T and *V. pacinii* LMG 19999^T were obtained from TaxVibrio database (http://www.taxvibrio.lncc.br/)

The predominant fatty acids of strain VSD707^T is $C_{16:0}$ (26.0 %) followed by summed feature 3 (39.3 %), and summed feature 8 (14.1 %). The fatty acid profiles of strain VSD707^T was almost similar to the closest type strain *V. plantisponsor* MSSRF60^T and only minor changes were observed in proportions (Table 6E.2). The polar lipid profile consisted of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Fig. 6E.6). The G+C content of strain VSD707^T was 46.9 mol% which is little higher when compared with *V. plantisponsor* MSSRF60^T (Table 6E.1).

Table 6E.2. Fatty acid profiles of strain VSD707^T and *Vibrio plantisponsor* MSSRF60^T

Fatty acids	VSD707^T	MSSRF60^T
$C_{12:0}$	3.8	4.5
$C_{12:0}$ 3-OH	3.7	3.4
$C_{14:0}$	3.9	5.7
$C_{16:0}$	26.0	24.2
iso-$C_{16:0}$	-	2.4
$C_{18:0}$	2.0	tr
Sum in feature 2	2.0	3.3
Sum in feature 3	39.3	33.9
Sum in feature 7	1.2	tr
Sum in feature 8	14.1	17.7

Strains: VSD707^T, *Vibrio azorensis* sp. nov. (Present study); MSSRF60^T, *Vibrio plantisponsor* (Rameshkumar et al. 2011). Fatty acids are given in percentage (>1 %). -, not detected; tr, trace amount. Summed feature 2 comprises $C_{16:1}$ iso I/$C_{14:0}$ 3-OH; summed feature 3 comprises $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*; summed feature 7 is $C_{19:1}$ ω6*c*/unknown; summed feature 8 is $C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*.

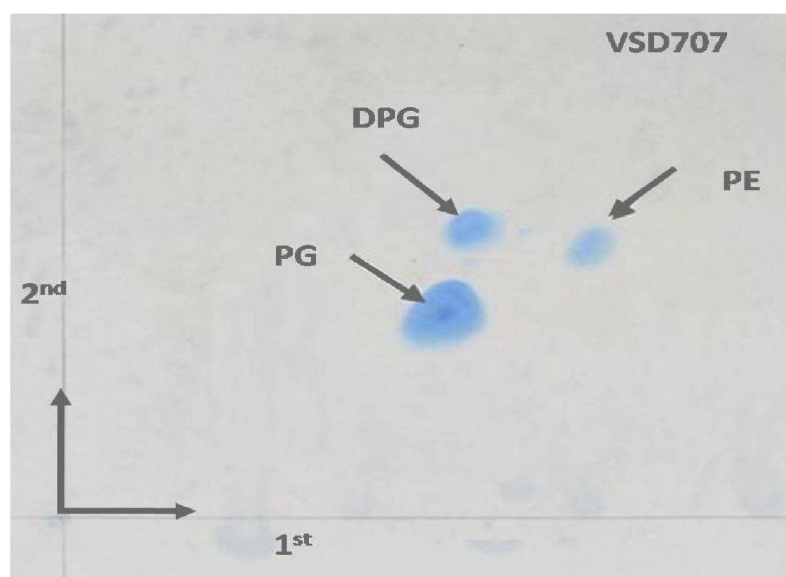


Fig. 6E.6. Two-dimensional TLC of total polar lipids of the strain VSD707^T. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol

Based on low level of similarity in 16S rRNA gene sequence (<97.0 %), chemotaxonomic data, phenotypic, and phylogenetic inference presented above, it can be concluded that strain VSD707^{T} represents a novel species of the genus *Vibrio*, for which the name *Vibrio azorensis* sp. nov. is proposed.

6E.4. Description of *Vibrio azorensis* sp. nov

Vibrio azorensis (*azo.ren'sis,* N.L. masc. adj. azorensis, after the portug. Islas dos Azores, pertaining to the Azorean Islands, the source of the sample from which the type strain was isolated).

Cells are Gram-negative, curved-rod shaped (0.4 – 0.6 × 0.7 – 2.1 μm), motile and non-spore forming. The colonies are small, non-pigmented to pale yellow, circular and smooth on MA. Growth is observed at temperature ranges from 4–32 °C, at pH 5.0–9.0 and NaCl 1-10 %. The optimum temperature and pH for growth are 25 °C and 6.0 respectively. Oxidase and catalase are positive. Nitrate is reduced to nitrite. Starch, Tween 20 and aesculin are hydrolyzed but DNA, gelatin, Tween 80, casein, tributyrin, xanthine, alginate, urea, agar, xylan and CMC are not hydrolysed. Acids produced from the substrates, L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, N-acetyl glucosamine, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, amidon (starch), gentibiose and D-arabitol. Positive (in API ZYM strips) for alkaline phosphatase, esterase C4, esterase lipase C8 (weak), lucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (weak) and β-glucosidase, but negative for lipase C14, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, N-acetyl-β-glucosaminidase and α-fucosidase. The polar lipid profile consisted of

phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol. The major fatty acids (≥10 %) are $C_{16:0}$, summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*) and summed feature 8 ($C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*). The G+C content of the type strain is 46.9 mol%.

The type strain, VSD707^T (= KCTC 32495^T = NCIM 5506^T), was isolated from shallow water hydrothermal vent sediment in Espalamaca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VSD707^T is KC534342.

Chapter 6F

Rhizobium azorensis sp. nov., isolated from a shallow hydrothermal vent (Espalamaca, Azores)

6F.1. Introduction

The genus *Rhizobium*, type genus of the family *Rhizobiaceae*, was first described by Frank (1889). *Rhizobium* is intended for a group of nodule forming, fast growing rhizobia, and it contained 70 species at the time of writing. *Rhizobium* species were frequently isolated from soil, rhizosphere, roots and nodules of several legume plants. However, some of the rhizobia members were also isolated from different sources like oil contaminated soil (Zhang et al. 2012), bioreactors (Hunter et al. 2007) and even from activated sludges (Zhang et al. 2011). Most recently, *Rhizobium subbaraonis* (Ramana et al. 2013) was isolated from beach sand. During our investigation on culturable bacterial diversity from shallow water hydrothermal vent in Azores (Chapter 2), we have isolated a novel bacterium, designated VSW331^T, belong to the genus *Rhizobium*. The main aim of the present investigation was to establish the precise taxonomic place for the strain VSW331^T by using a polyphasic taxonomic approach.

6F.2. Materials and Methods

6F.2.1. Strain isolation and culture conditions

Surface seawater sample was collected from shallow water hydrothermal vent region in Espalamaca (38°33'N; 28°39'W) during 2010. For isolating this strain, serially diluted water samples were spread plated on diluted seawater nutrient agar (dSWNA: Peptone 1.25 g L^{-1}, Beef extract 0.75 g L^{-1} and 1.5 % Agar prepared in 50 % seawater) amended with 0.5 % sodium thiosulphate. The plates were incubated for 2 days at 30 ± 1 °C. Morphologically different bacterial colonies were isolated and repeatedly quadrant streaked on the same media to get pure cultures. The strain VSW331^T was maintained at 4 °C on yeast mannitol agar (YMA) and stored as glycerol suspension -80 °C for long-

term maintenance. To compare the phenotypic differences, we purchased *Rhizobium vignae* (LMG 25447) from LMG culture collections and parallel studies were carried out along with our strain VSW331^T.

6F.2.2. Morphological and biochemical characteristics

Morphological characteristics were studied using light microscopy (Olympus BX-60) and scanning electron microscopy (Hitachi TM3000). Gram staining was carried out using standard Gram staining method (K001, Himedia) and also by KOH method (Cerny 1978). Motility was determined by hanging drop method (Collee et al. 2008) and confirmed with motility agar test. Temperature range for growth was tested on YMA at 4, 10, 20, 25, 30, 37, 40 and 50 °C. For growth at different pH, YMA medium was buffered with various buffer systems (citrate for pH 4-5; phosphate for pH 6-8 and carbonate for pH 9-10). Growth at various NaCl concentrations (0–15 %, w/v, at increments of 1.0 %) was investigated on YMA. Catalase activity was tested using 3 % (v/v) H_2O_2 (Smibert and Krieg, 1994) and oxidase activity was determined using oxidase discs (DD018, Himedia).

Hydrolytic enzyme tests were determined as described previously (section 6C.2.3) on YMA medium. Other enzyme activities were assayed using API ZYM strips (bioMérieux). Methyl red, Voges-Proskauer tests, determination of indole, H_2S production, and nitrate reduction were assessed using Hi25^{TM} Enterobacteriaceae identification kit (Himedia). Acid production from carbohydrates was determined with API 50 CH kit (bioMérieux) using API CHB/E medium.

Susceptibility to antibiotics were performed using antibiotic discs (Himedia) containing the following (μg/disc unless otherwise stated): tetracycline (10), streptomycin (300),

chloramphenicol (25), polymyxin-B (50 IU), kanamycin (30), ciprofloxacin (30), ceftazidime (30), penicillin-G (2 IU), lincomycin (10), vancomycin (5), amoxycillin (30), amikacin (30), cefadroxyl (30), bacitracin (8 IU), chlortetracycline (30), rifampicin (15), norfloxacin (10), tobramycin (10), novobiocin (30), neomycin (30) and ampicillin (25).

6F.2.3. 16S rRNA gene sequencing and phylogenetic analysis

Chromosomal DNA was extracted by using DNeasy Blood and Tissue Kit (Qiagen) according to the method described by manufacturer. The 16S rRNA gene was PCR amplified using eubacterial primers 27F and 1492R (Lane 1991). PCR amplification of *atpD* (ATP synthase gene) and *recA* (recombinase A gene) was done by following the protocols of Gaunt et al. (2001). The amplified 16S rRNA gene product was purified using QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. Sequencing of the purified 16S rRNA gene was done with automated DNA sequencer 3130xl Genetic Analyzer (Applied Biosystems). Multiple and pairwise sequence alignment were performed using Clustal W sequence alignment program (Thompson et al. 1994). The nearly complete sequence (1371 nt) was subjected to EzTaxon-e server (Kim et al. 2012a) to identify closest matching sequences. Neighbour joining analysis (Saitou and Nei 1987) was used to reconstruct the phylogenetic tree using MEGA5 software program (Tamura et al. 2011). The topology of the phylogenetic tree was evaluated by bootstrap analysis method of Felsenstein (1985) with 1,000 replications.

For DNA-DNA hybridization analysis between the strains VSW331^T and *Rhizobium vignae* CCBAU 05176^T, chromosomal DNA was extracted and purified following the method of Marmur (1961). The analysis was carried out by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with 96 well thermal cycling block in 96 well plate (Loveland-Curtze et al. 2011). The reassociation of DNA was

carried out at optimum reassociation temperature 78.0 °C [Tor = 0.51 × (% G+C) + 47.0] according to De Ley et al. (1970) and Gillis et al. (1970). Results of DNA-DNA relatedness values are the average of triplicates.

6F.2.4. Chemotaxonomic analysis

Bacterial cells were cultivated on SWNA media at 30 °C for 2 days to determine the whole cell fatty acids. Fatty acid methyl esters (FAME) were prepared as per the standard method (Sasser 1990) using MIDI Sherlock Microbial Identification System (TSBA 6.0) and analyzed using gas chromatography. Polar lipids were extracted according to the method of Collins et al. (1980) and sprays were used as described previously (Section 6C.2.4). Isoprenoid quinones were analyzed by using high performance liquid chromatography (HPLC) following the methods of Minnikin et al. (1984). The genomic DNA extraction and G+C content of the DNA was determined by reverse-phase HPLC of nucleosides according to the protocol described by Mesbah et al. (1989).

6F.3. Results and discussions

Colonies of strain VSW331^T were white, circular, convex, entire margin, opaque and 1.0 mm diameter after 2 days incubation at 30 °C on YMA. Cells stained Gram-negative, motile, non-spore forming and rod shaped (Fig. 6F.1). Temperature range for growth was observed between 4 and 40 °C (optimum 30 °C). The pH range for growth was detected between 6.0 and 9.0 (optimum 8.0) and with NaCl concentration between 0 and 10 % (optimum 4–6 % w/v). Strain VSW331^T was sensitive to the antibiotics amoxicillin, ampicillin, cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, norfloxacin, novobiocin, rifampicin, amikacin, streptomycin, tetracycline, kanamycin,

neomycin, polymyxin B, bacitracin, penicillin G and tobramycin, and resistant to vancomycin and lincomycin. The results of other phenotypic studies are shown in species descriptions and the differences noticed between strain VSW331^{T} and *Rhizobium vignae* LMG 25447^{T} are listed in Table 6F.1.

Nearly full length 16S rRNA gene sequence of strain VSW331^{T} (GenBank accession number KC534384) was compared with the sequences from the GenBank database (http://www.ncbi.nlm.nih.gov). 16S rRNA gene sequence similarity values between strain VSW331^{T} and closest validly named species of the genus *Rhizobium* in EzTaxon-e database are *R. vignae* CCBAU 05176^{T} (97.3 %), *R. alkalisoli* CCBAU 01393^{T} (96.9 %), *R. galegae* ATCC 43677^{T} (96.6 %) and *R. huautlense* S02^{T} (96.5 %). Remaining type species of the genus *Rhizobium* were less than 96.0 % sequence similarity. DNA-DNA relatedness between the strains VSW331^{T} and *Rhizobium vignae* CCBAU 05176^{T} was found to be 54.3 %. Neighbour-joining phylogenetic trees based on 16S rRNA and *atpD* genes revealed that strain VSW331^{T} formed a separate branch among other *Rhizobium* species (Fig. 6F.2 and Fig. 6F.3). Whereas, Neighbour-joining phylogenetic trees based on *recA* gene revealed that strain VSW331^{T} formed a monophyletic clade with *R. tibeticum* CCBAU 85039^{T} with low bootstrap value (54 %) (Fig. 6F.4).

Major respiratory quinone of strain VSW331^{T} was Q-10 followed by minor amount of Q-9. The major fatty acid (>10 %) found in strain VSW331^{T} was $C_{16:0}$ (17.3 %) and summed feature 8 contributed 60.4 %. Summed feature 8 contained mixture of unsaturated fatty acids $C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*. The proportion of summed feature 8 is much higher in strain VSW331^{T} compared to the closest relative *Rhizobium vignae* LMG 25447^{T} (Ren et al. 2011). In addition, the fatty acid $C_{19:0}$ cyclo ω8*c* was reported to be 37.6 % in *Rhizobium vignae* LMG 25447^{T} but the same fatty acid was observed to be

lower in strain VSW331^T. Differences observed on fatty acid analysis are given in Table 6F.2. The genomic G+C content of strain VSW331^T was found to be 64.2 mol% which is slightly higher than *Rhizobium vignae* LMG 25447^T (Ren et al. 2011).

Based on 16S rRNA phylogenetic analysis in support with DNA-DNA hybridization, housekeeping genes, phenotypic characteristics, and chemotaxonomic data, it can be concluded that strain VSW331^T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium azorensis* sp. nov. is proposed.

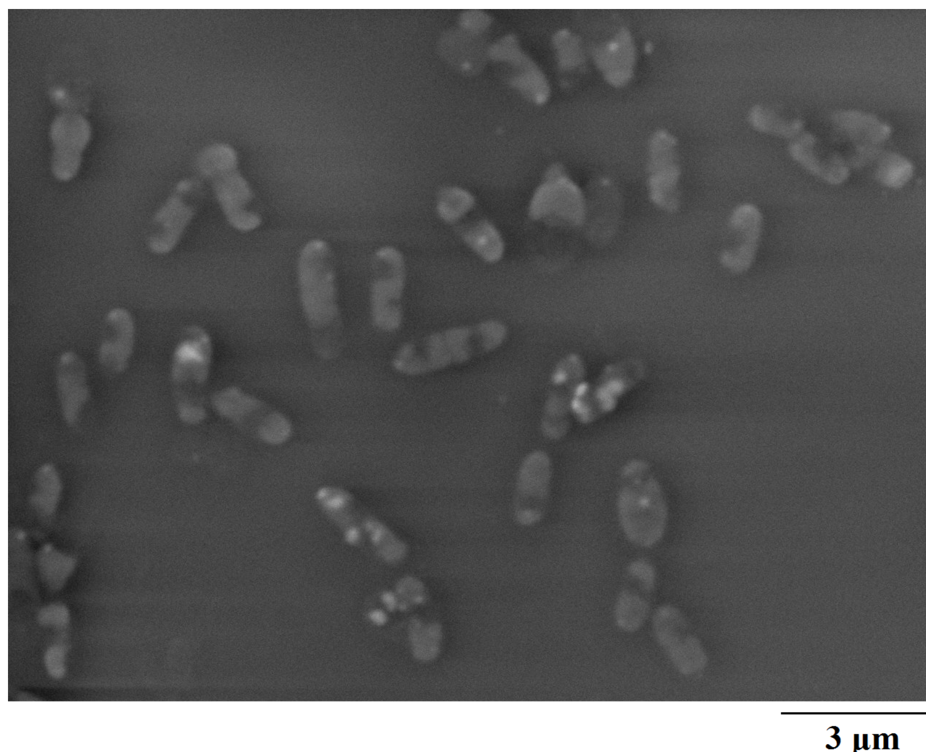


Fig. 6F.1. Scanning electron microscopic picture of strain VSW331^T

Table 6F.1. Differential characteristics of the strain VSW331^T and the type strain *Rhizobium vignae* LMG 25447^T

Characteristic	VSW331^T	LMG 25447^T
pH range	6–9	7–10*
NaCl (%)	0–10	0–2*
Nitrate reduction	-	+
Hydrolysis of:		
Tween 20	+	-
Agar	+	-
API CH50:		
D-glucose	+	-
D-fructose	+	-
D-mannose	+	-
D-lyxose	+	w
L-fucose	+	w
API ZYM:		
Esterase lipase (C8)	+	w
Acid phosphatase	w	+
Naphthol-AS-BI-phosphohydrolase	-	+
β-glucosidase	+	w
DNA G+C content (mol%)	64.2	60.8*
Antibiotic (vancomycin)	R	S
Isolation source	Shallow hydrothermal vent	nodules of legume species

Strains: VSW331^T (*Rhizobium azorensis* sp. nov.); LMG 25447^T (*Rhizobium vignae* CCBAU 05176^T). Results are obtained from this study otherwise indicated (*Ren et al. 2011). Both the strains are positive for catalase, oxidase; hydrolysis of urea; acid production from D-arabinose L-arabinose, D-ribose, D-xylose, Methyl-βD-xylopyranoside, N-acetyl glucosamine (weak), esculin, D-fucose and D-arabitol (weak); alkaline phosphatase, esterase C4, lucine arylamidase and α-glucosidase. Negative for Gram staining, hydrolysis of gelatin, Tween 80 and DNA; acid production from glycerol,

erythritol, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inocitol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melazitose, D-raffinose, amidon, glycogen, xylitol, gentibiose, D-turanose, D-tagatose, L-arabitol, potassium gluconate, potassium 2-keto gluconate and potassium 5-keto gluconate; lipase C14, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, N-acetyl-β-glucosaminidase and α-fucosidase. +, positive; -, negative; R, resistant; S, susceptible

Table 6F.2. Cellular fatty acid profiles of strain VSW331^T and type strain *Rhizobium vignae* LMG 25447^T

Fatty acid	VSW331^T	LMG 25447^T
$C_{12:0}$	1.6	-
$C_{14:0}$	1.5	-
$C_{16:0}$	17.3	19.3
$C_{18:0}$	5.9	-
$C_{16:0}$ N alcohol	-	6.0
$C_{19:0}$ cyclo ω8*c*	5.0	37.6
Summed feature 2	5.8	-
Summed feature 3	3.1	-
Summed feature 4	-	4.2
Summed feature 8	60.4	33.1

Strains: VSW331^T, *Rhizobium azorensis* sp. nov. (present study); LMG 25447^T, *Rhizobium vignae* CCBAU 05176^T (Ren et. al. 2011). Fatty acids are given in percentage (>1 %). Summed feature 2 comprises $C_{12:0}$ aldehyde/unknown ECL 10.928 and/or $C_{16:1}$ iso I/$C_{14:0}$ 3-OH, summed feature 3 comprises $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*, summed feature 4 is $C_{17:1}$ iso I/anteiso B and/or $C_{17:1}$ anteiso B/iso I, summed feature 8 is $C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*.

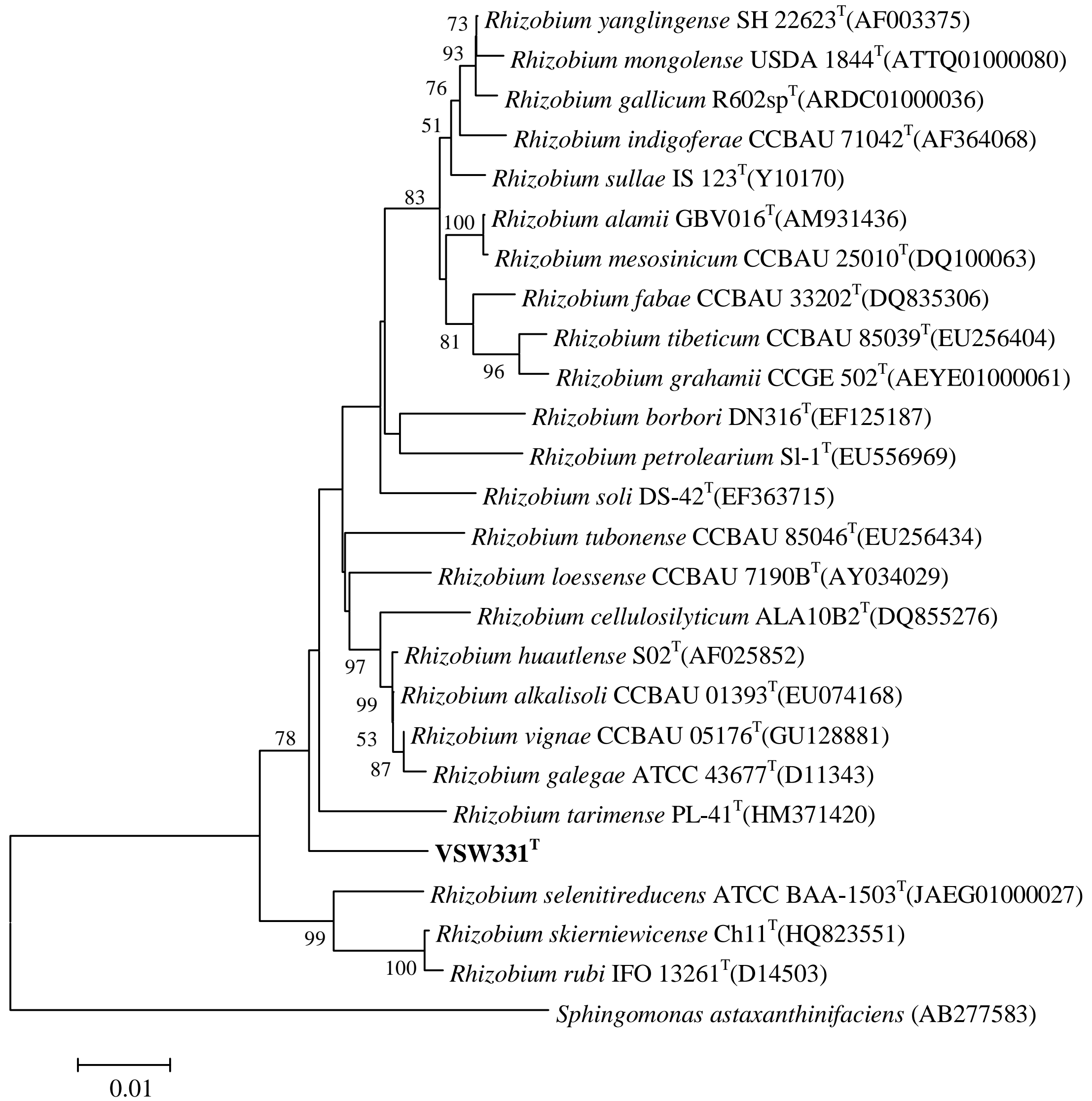


Fig. 6F.2. Neighbour-joining tree showing the phylogenetic relationship of strain VSW331^{T} and phylogenetically related species of the genus *Rhizobium* based on 16S rRNA gene sequences. Bootstrap percentages (1,000 replications) are shown at branching points and the values >50 % are shown. *Sphingomonas astaxanthinifaciens* TDMA-17^{T} (AB277583) was used as an out group. Scale bar 0.01 substitutions per nucleotide position

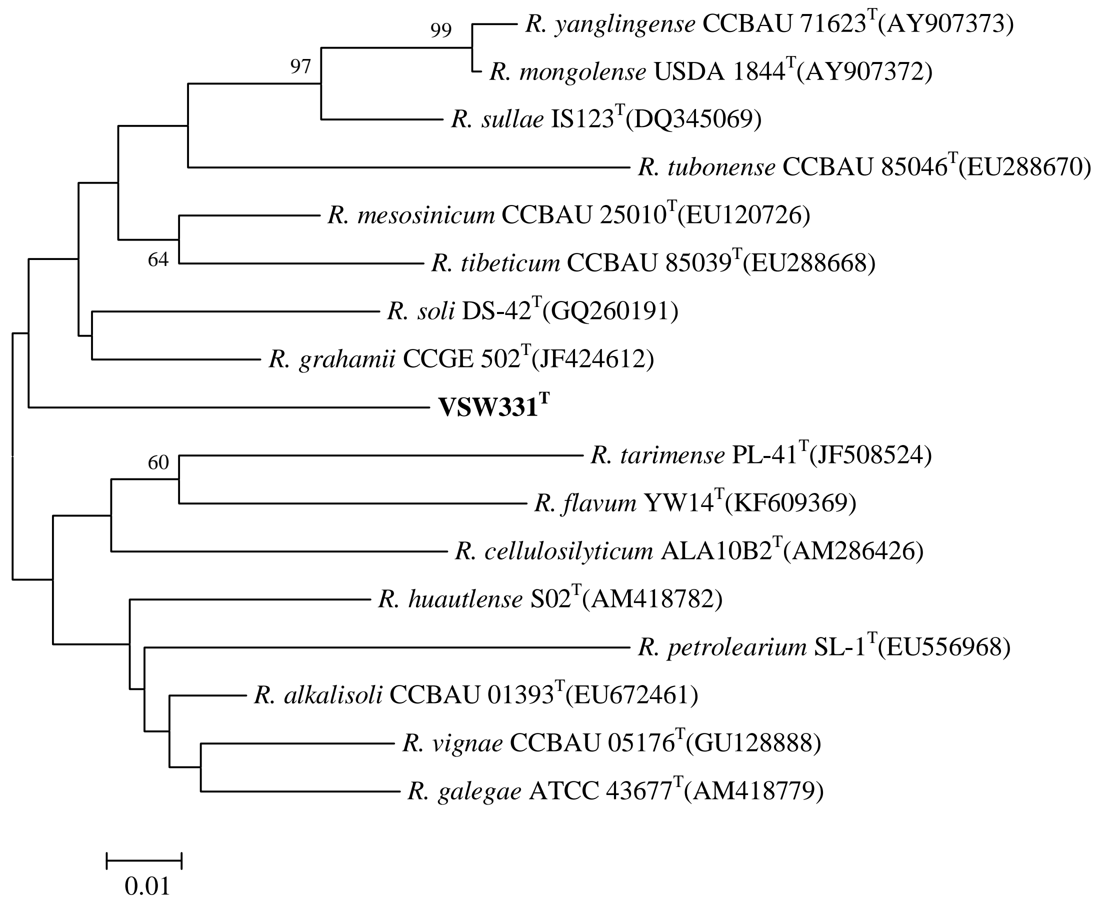


Fig. 6F.3. Neighbour-joining phylogenetic tree based on *atpD* gene sequences, showing the phylogenetic placement of strain VSW331^T and their phylogenetic neighbours of the genus *Rhizobium*. Bootstrap percentages (based on 1000 replicates) are indicated at branching points (values >50 % are shown).

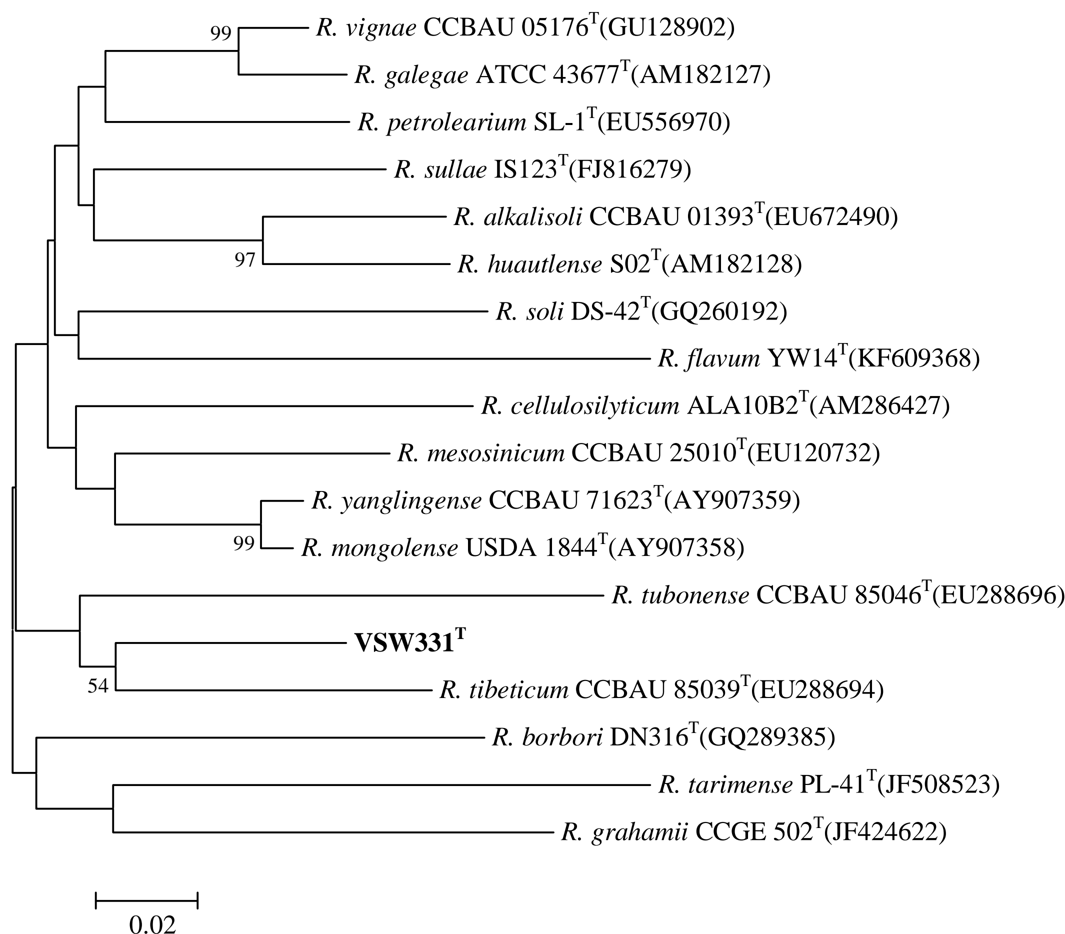


Fig. 6F.4. Phylogenetic tree based on *recA* gene sequences constructed using neighbour-joining method, showing the phylogenetic placement of strain VSW331^T and their phylogenetic neighbours of the genus *Rhizobium*. Bootstrap percentages (based on 1000 replicates) are indicated at branching points (values >50 % are shown).

6F.4. Description of *Rhizobium azorensis* sp. nov

Rhizobium azorensis (*azo.ren'sis,* N.L. masc. adj. azorensis, after the portug. Islas dos Azores, pertaining to the Azorean Islands, the source of the sample from which the type strain was isolated).

Cells are Gram-staining-negative, rod shaped (0.6–0.9 × 1.2–2.5 μm), motile and non-spore forming. The colonies are non-pigmented, 1.0 mm in diameter, circular and smooth on YMA. Growth is observed at temperature ranges from 4–40 °C, at pH 6.0–9.0 and NaCl 0-10 %. The optimum temperature and pH for growth are 30 °C and 8.0 respectively. Oxidase and catalase are positive. Nitrate is not reduced to nitrite. Tween 20, urea and agar are hydrolyzed but DNA, gelatin, starch, Tween 80, tributyrin, xanthine, alginate, aesculin, xylan and CMC are not hydrolysed. Acids produced from the substrates, D-arabinose L-arabinose, D-ribose, D-xylose, Methyl-βD-xylopyranoside, D-glucose, D-fructose, D-mannose, esculin, D-lyxose, D-fucose, L-fucose N-acetyl glucosamine (weak) and D-arabitol (weak). Positive (in API ZYM strips) for alkaline phosphatase, esterase C4, esterase lipase C8, lucine arylamidase, acid phosphatase (weak), α-glucosidase and β-glucosidase, but negative for lipase C14, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, N-acetyl-β-glucosaminidase and α-fucosidase. The major fatty acids (≥10 %) are summed feature 8 ($C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*) and $C_{16:0}$. Major respiratory quinone is Q-10 with minor amount of Q-9. The G+C content of the type strain is 64.2 mol%.

The type strain, VSW331^T (=KCTC 32415 = MTCC 11811), was isolated from surface seawater at a shallow water hydrothermal vent in Espalamaca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VSW331^T is KC534384.

Chapter 7

Summary

- A Shallow water hydrothermal vent field has been discovered during summer 2010 in the Faial-Pico channel off the Espalamaca headland (Faial Island, Azores, NE Atlantic). The main venting area, named Espalamaca extends at approximately 35 m depth. Preliminary analyses of the gaseous discharges from the vents suggest that they are mainly composed of CO_2, with low concentrations of methane, temperature as high as 35 °C and pH 5.7.
- Low-temperature hydrothermal vents, which form by mixing of high-temperature hydrothermal fluids and seawater, are omnipresent in recent submarine hydrothermal systems. Molecular approaches for the investigation of hydrothermal vent microbial communities based the genes of smaller subunit ribosomal RNA have led to insights into the community diversity and structure of microbial systems. In addition, resistance to heavy metals and producing various enzymes by hydrothermal vent microbes may possibly have an immense potential in biotechnology industries.
- Culture dependent bacterial diversity analyzed with 16S rRNA gene from this vent resulted a total of 113 bacterial phylotypes (74 phylotypes from the venting region, 18 phylotypes from the non-vent and 21 phylotypes which are common in both sites).
- *Proteobacteria* (α, β and γ) was found to be predominant phylum followed by *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Rarefaction curve analysis clearly indicated that the vent area harbors more number of phylotypes than non-vent region. Culture dependent analysis revealed more than 30 novel bacterial taxa and majority of the bacterial groups obtained from this study were not reported earlier in any of the shallow water vents.

- Culture independent bacterial diversity was investigated based on metagenomic clone library approach. The 16S rRNA gene sequences of the hydrothermal vent clones were affiliated with *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Gemmatimonadetes*, *Ignavibacteriae*, *Planctomycetes*, *Proteobacteria* (includes α, γ, ε, δ, ζ subdivisions), *Spirochaetes* and *Verrucomicrobia*.
- Among the *Proteobacteria*, γ-*Proteobacteria* was predominant in this study area which is contrast to other shallow water hydrothermal vent regions where ε-*Proteobacteria* was reported to be higher.
- The presence of methanol dehydrogenase (*mxaF*), carbonic anhydrase and sulphur oxidizing (*soxB*) genes from the micobes of Espalamaca vent sediments indicating the involvement and active role in biogeochemical cycles of the hydrothermal vent.
- *Proteobacteria* and *Bacteroidetes* are the dominant groups in both culture dependent and culture independent methods. Merely, 18.6 % of culture dependent and 15.5 % of culture independent bacterial phylotypes are common between venting and non-venting sites. Remaining around 80 % of the organisms which may be of specialized groups, their activities are inevitable in various processes involved in the vent Espalamaca.
- More than 10 phylogenetic groups were obtained in this study area which is higher while comparing with other shallow water vents except South Tonga Arc in the South Pacific Ocean.
- Phylogenetic groups *Proteobacteria* (ε, δ, ζ subdivisions), *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Gemmatimonadetes*,

Ignavibacteriae, *Planctomycetes*, *Spirochaetes* and *Verrucomicrobia* obtained from non-culturable diversity were not detected in culture dependent analysis.

- *Roseovarius*, *Photobacterium*, *Vibrio* and *Pseudoalteromonas* are the common genera observed in both culture dependent and culture independent analysis.
- Bacterial isolates from this study area could tolerate up to 50 mM manganese, 7.5 mM lead and 1 mM iron; most of the isolates were able to grow even with 7 % of thiosulphate.
- Forty six bacterial strains were able to oxidize soluble Mn(II) to insoluble Mn(III/IV) and most of the isolates were not reported previously for Mn(II) oxidation. One of the isolate (*Citreicella* sp), VSW210 could tolerate up to 50 mM $MnCl_2$ and also oxidize soluble Mn(II).
- Esterase, esterase lipase, lipase, leucine arylamidase, phosphatases, naphthol-AS-BI-phosphohydrolase, catalase and oxidase were the predominant enzymes produced by most of the Espalamaca isolates.
- VSW306 which belong to the genus *Cellulophaga* could synthesize up to 15 enzymes. Bacterial isolates in this vent were mostly mesophilic and moderately halophilic; able to grow at 30 °C and pH 6 – 8. Enzymes obtained from the above conditions may have better applications to use in detergent, animal feed, wine, fruit and starch processing industries.
- Six novel bacterial taxa were proposed from this study which includes *Nioella nitratireducens* gen. nov., sp. nov. (Strain SSW136^T), *Roseovarius azorensis* sp. nov. (Strain SSW084^T), *Vitellibacter nionensis* sp. nov. (Strain VBW088^T), *Citreicella manganoxidans* sp. nov. (Strain VSW210^T), *Vibrio azorensis* sp. nov. (Strain VSD707^T) and *Rhizobium azorensis* sp. nov. (Strain VSW331^T). Former four novel taxa were published and the later two are under review.

- The sequences obtained from this study are available in GenBank database with accession numbers from KC534142 to KC534459 (culture-dependent) and from KP303396 to KP303589 (culture-independent).
- Present investigation gives a clear picture of bacterial community structure of the shallow water hydrothermal vent Espalamaca and it forms the first report.

References

Adams LF, Ghiorse WC (1987) Characterization of an extracellular Mn^{2+}-oxidizing activity and isolation of Mn^{2+}-oxidizing protein from *Leptothrix discophora* SS-1. J Bacteriol 169: 1279–1285

Aiuppa A, Dongarra G, Capasso G, Allard P (2000) Trace elements in the thermal groundwaters of Vulcano Island (Sicily). J Volcanol Geotherm Res 98: 189–207

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiol Rev 59: 143–169

Amend JP, Rogers KL, Shock EL, Gurrieri S, Inguaggiato S (2003) Energetics of chemolithoautotrophy in the hydrothermal system of Vulcano Island, southern Italy. Geobiology 1: 37–58

Arnosti C, Jørgensen BB, Sageman J, Thamdrup B (1998) Temperature dependence of microbial degradation of organic matter in marine sediments: polysaccharide hydrolysis, oxygen consumption, and sulfate reduction. Mar Ecol Prog Ser 165: 59–70

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl Environ Microbiol 71: 7724–7736

Asker D, Beppu T, Ueda K (2007) *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family *Flavobacteriaceae*, isolated from seawater off Enoshima Island, Japan. Int J Syst Evol Microbiol 57: 837–843

Azam F (1998) Microbial control of oceanic carbon flux: the plot thickens. Science 280: 694–696

Azam F, Fenchel T, Gray JG, Meyer-Reil LA, Thingstad F (1983) The ecological role of water-column microbes in the sea. Mar Ecol Prog Ser 10: 257–263

Baross JA, Hoffman SE (1985) Submarine hydrothermal vents and associated gradient environments as sites for the origin and evolution of life. Orig Life 15: 327–45

Bartram AK, Lynch MD J, Stearns JC, Moreno-Hagelsieb G, Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. Appl Environ Microbiol 77(11): 3846–3852

Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ (2010) Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs. Environ Microbiol Rep 2: 34–43

Bernardet JF, Nakagawa Y, Holmes B (2002) Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. Int J Syst Evol Microbiol 52: 1049–1070

Bertoldo C, Grote R, Antranikian G (2002) Extremophiles: Life in extreme environments. Encyclop Environ Microbiol 6: 1232–1237

Beukes LS, Schmidt S (2012) Isolation and characterization of a manganese-oxidizing bacterium from a biofiltration system for the treatment of borehole water in KwaZulu-Natal (South Africa). Eng Life Sci 12(5): 544–552

Biebl H, Allgaier M, Lunsdorf H, Pukall R, Tindall BJ, Wagner-Dobler I (2005) *Roseovarius mucosus* sp. nov., a member of the *Roseobacter* clade with trace amounts of bacteriochlorophyll *a*. Int J Syst Evol Microbiol 55: 2377–2383

Bintrim SB, Donohue TJ, Handelsman J, Roberts GP, Goodman RM (1997) Molecular phylogeny of Archaea from soil. Proc Natl Acad Sci USA 94: 277–282

Boetius A (1995) Microbial hydrolytic enzyme activities in deep-sea sediments. Helgolander Meeresunters 49: 177–187

Boettcher KJ, Geaghan KK, Maloy AP, Barber BJ (2005) *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. Int J Syst Evol Microbiol 55: 1531–1537

Borremans B, Hobman JL, Provoost A, Brown NL, Lelie DV (2001) Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. J Bacteriol 183(19): 5651–5658

Brinkhoff T, Sievert SM, Kuever J, Muyzer G (1999) Distribution and diversity of sulfur-oxidizing *Thiomicrospira* spp. at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece). Appl Environ Microbiol 65: 3843–3849

Brooks D (2006) Tropical Marine Ecology. Hays Cummings. June 5, 2006. Hydrothermal Vents

Brouwers GJ, De Vrind JPM, Corstjens PLAM, Cornelis P, Baysse C, De Vrind-De Jong EW (1999) *cumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida* GB-1. Appl Environ Microbiol 65: 1762–1768

Brouwers GJ, Vijgenboom E, Corstjens PLAM, De Vrind JPM, De Vrind-De Jong EW (2000) Bacterial Mn^{2+} oxidizing systems and multicopper oxidases: an overview of mechanisms and functions. Geomicrobiol J 17: 1–24

Campbell BJ, Engel AS, Porter ML, Takai K (2006) The versatile Epsilonproteobacteria: key players in sulphidic habitats. Nat Rev Microbiol 4: 458–468

Cardigos F, Colaco A, Dando PR, Avila SP, Sarradin P-M, Tempera F, Conceicao P, Pascoal A, Santos RS (2005) Shallow water hydrothermal vent field fluids and communities of the D. Joao de Castro Seamount (Azores). Chem Geol 224: 153–168

Cerny G (1978) Studies on aminopeptidase for the distinction of Gram-negative from Gram-positive bacteria. Eur J Appl Microbiol Biotechnol 5: 113–122

Chamberlin WS, Dickey TD (2008) Exploring the World Ocean. McGraw-Hill Higher Education

Cho J, Giovannoni SJ (2004) Cultivation and growth characteristics of a diverse group of oligotrophic marine Gammaproteobacteria. Appl Environ Microbiol 70(1): 432–440

Choi EJ, Lee HJ, Kim JM Jeon CO (2013) *Roseovarius lutimaris* sp. nov., isolated from amarine tidal flat. Int J Syst Evol Microbiol 63: 3835–3840

Colaco A, Raghukumar C, Mohandass C, Cardigos F, Santos RS (2006) Effect of shallow-water venting in Azores on a few marine biota. Cah Biol Mar 47(4): 359–364

Collee JG, Fraser AG, Marmion BP, Simmons A (2008) Mackie and McCartney Practical Medical Microbiology (14th edn). Elsevier Private Limited, India

Collins MD, Jones D (1980) Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4-diaminobutyric acid. J Appl Bacteriol 48: 459–470

Colwell RR (2006) A global and historical perspective of the genus *Vibrio*. In: Thompson FL, Austin B, Swings J (Eds) The biology of Vibrios. American Society for Microbiology, Washington, DC, pp 3–11

Copley J (2002) All at sea. Nature 415: 572–574

Corliss JB, Dymond J, Gordon LI, Edmond JM, von Herzen RP, Ballard RD, Green K, Williams D, Bainbridge A, Crane K, van Andel TH (1979) Submarine thermal springs on the Galapagos Rift. Science 203: 1073–1083

Cornec L, Robineau J, Rolland JL, Dietrich J, Barbier G (1998) Thermostable esterases screened on hyperthermophilic archaeal and bacterial strains isolated from deep-sea hydrothermal vents: Characterization of esterase activity of a hyperthermophilic archaeum, *Pyrococcus abyssi*. J Mar Biotechnol (1998) 6: 104–110

Ćrepeau V, Cambon Bonavita M-A, Lesongeur F, Randrianalivelo H, Sarradin P-M, Sarrazin J, Godfroy A (2011) Diversity and function in microbialmats from the Lucky Strike hydrothermal vent field. FEMS Microbiol Ecol 76: 524–540

Dando P, Hughes J, Leahy Y, Niven S, Taylor L, Smith C (1995) Gas venting rates from submarine hydrothermal areas around the island of Milos, Hellenic Volcanic Arc. Cont Shelf Res 15: 913–929

Dando PR, Hooper LE (1997) Hydrothermal Thioploca-more unusual mats from Milos. BRIDGE Newsl 97: 45–47

Dang H, Zhu H, Wang J, Li T (2009) Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. World J Microbiol Biotechnol 25: 71–79

Das S, Lyla PS, Ajmal Khan S (2006) Marine microbial diversity and ecology: importance and future perspectives. Curr Sci 90(10): 1325–1335

Das SK, Mishra AK, Tindall BJ, Rainey FA, Stackebrandt E (1996) Oxidation of thiosulfate by a new bacterium, *Bosea thiooxidans* (strain BI-42) gen. nov., sp. nov.: Analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing. Int J Syst Bacteriol 46: 981–987

Dastager SG, Deepa CK, Li W, Tang S, Pandey A (2011) *Paracoccus niistensis* sp. nov., isolated from forest soil, India. Antonie van Leeuwenhoek 99: 501–506

Davis RE, Moyer C (2008) Extreme spatial and temporal variability of hydrothermal microbial mat communities along the Mariana Island Arc and southern Mariana back-arc system. J Geophys Res 113: 1–17

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12: 133–142

De Vrind JPM, De vrind-De jong EW, De Voogt J-WH, Westbroek P, Boogerd FC, Rosson RA (1986) Manganese oxidation by spores and spore coats of a marine *Bacillus* species. Appl Environ Microbiol 52(5): 1096–1110

Dell'Anno A, Danovaro R (2003) Extracellular DNA plays a key role in deep-sea ecosystem functioning. Science 309: 2179

Demirjian D, Moris Varas F, Cassidy CS (2001) Enzymes from Extremophiles. Curr Opin Chem Biol 5: 144–151

Desbruyeres D, Segonzac M, Bright M (2006) Handbook of Deep-Sea Hydrothermal Vent Fauna. Linz: Medieninhaber und Herausgeber

Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R, McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM (2008a) Genomic insights into Mn(II) oxidation by the marine Alphaproteobacterium *Aurantimonas* sp. strain SI85-9A1. Appl Environ Microbiol 74(9): 2646–2658

Dick GJ, Torpey JW, Beveridge TJ, Tebo BM (2008b) Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. Appl Environ Microbiol 74(5): 1527–1534

Dieguez AL, Beaz-Hidalgo R, Cleenwerck I, Balboa S, De Vos P, Romalde JL (2011) *Vibrio atlanticus* sp. nov. and *Vibrio artabrorum* sp. nov., isolated from the clams *Ruditapes philippinarum* and *Ruditapes decussates*. Int J Syst Evol Microbiol 61: 2406–2411

Diels L, Sadouk A, Mergeay M (1989) Large plasmids governing multiple resistances to heavy metals: a genetic approach. Ecol Environ Chem 23: 79–89

Dobrinski KP, Boller AJ, Scott KM (2010) Expression and function of four carbonic anhydrase homologs in the deep-sea chemolithoautotroph *Thiomicrospira crunogena*. Appl Environ Microbiol 76(11): 3561–3567

Douka CE (1980) Kinetics of manganese oxidation by cell-free extracts of bacteria isolated from manganese concretions from soil. Appl Environ Microbiol 39: 74–80

Durand P, Benyagoub A, Prieur D (1994) Numerical taxonomy of heterotrophic sulphur-oxidizing bacteria isolated from southwestern Pacific hydrothermal vents. Can J Microbiol 40: 690–697

Edwards KJ, Rogers DR, Wirsen CO, McCollom TM (2003) Isolation and characterization of novel psychrophilic, neutrophilic, Fe-oxidizing,

chemolithoautotrophic alpha- and gamma-proteobacteria from the deep sea. Appl Environ Microbiol 69: 2906–2913

Elshahed MS, Youssef NH, Luo Q, Najar FZ, Roe BA, Sisk TM, Buhring SI, Hinrichs K-U, Krumholz LR (2007) Phylogenetic and metabolic diversity of Planctomycetes from anaerobic, sulfide and sulphur rich Zodletone Spring Oklahoma. Appl Environ Microbiol 73: 4707–4716

Emerson D, Moyer CL (2002) Neutrophilic Fe-oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. Appl Environ Microbiol 68: 3085–3093

Emerson D, Rentz JA, Lilburn TG, Davis RE, Aldrich H, Chan C, Moyer CL (2007) A novel lineage of Proteobacteria involved in formation of marine Fe-oxidizing microbial mat communities. PLoS ONE 2: e667

Erra-Pujada M, Chang-Pi-Hin F, Debeire P, Duchiron F, O'Donohue MJ (2001) Purification and properties of the catalytic domain of the thermostable pullulanase type II from *Thermococcus hydrothermalis*. Biotechnol Lett 23: 1273–1277

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39: 224–229

Farmer JJ, Hickman-Brenner FW (2006) The genera *Vibrio* and *Photobacterium*. In Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (Eds) The Prokaryotes, a Handbook on the Biology of Bacteria (3rd edn). Springer, New York, pp 508–563

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368–376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–789

Ferguson RL, Buckley EN, Palumbo AV (1984) Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 47: 49–55

Fernandes SO, Krishnan KP, Khedekar VD, Loka Bharathi PA (2005) Manganese oxidation by bacterial isolates from the Indian Ridge System. BioMetals 18: 483–492

Fitch WM (1971) Toward defining the course of evolution: minimum change for specified tree topology. Syst Zool 20: 406–416

Francis CA, Co EM, Tebo BM (2001) Enzymatic manganese(II) oxidation by a marine α-proteobacterium. Appl Environ Microbiol 67: 4024–4029

Francis CA, Tebo BM (2002) Enzymatic manganese(II) oxidation by metabolically dormant spores of diverse *Bacillus* species. Appl Environ Microbiol 68: 874–880

Frank B (1889) Über die Pilzsysymbiose der Leguminosen. Ber Dtsch Bot Ges 7: 332–346

Fuhrman JA, McCallum K, Davis AA (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl Environ Microbiol 59: 1294–1302

Garrity GM, Bell JA, Lilburn T (2005) Family I. *Rhodobacteraceae* fam. nov. In Brenner DJ, Krieg NR, Staley JT, Garrity GM (Eds) Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2 (The *Proteobacteria*), part C (The *Alpha*-, *Beta*-, *Delta*-, and *Epsilonproteobacteria*), Springer, New York, p. 161

Garrity GM, Holt JG (2001) "Taxonomic Outline of the Archaea and Bacteria". In Boone DR, Castenholz RW (Eds) Bergey's Manual of Systematic Bacteriology Volume 1: The Archaea and the deeply branching and phototrophic Bacteria (2nd edn). Springer Verlag, New York, pp 155–166

Gaunt MW, Turner SL, Rigottier-Gois L, Lloyd-Macgilps SA, Young JPW (2001) Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int J Syst Evol Microbiol 51: 2037–2048

Gerasimchuk AL, Shatalov AA, Novikov AL, Butorova OP, Pimenov NV, Lein AY, Yanenko AS, Karnachuk OV (2010) The search for sulphate reducing bacteria in mat samples from the lost city hydrothermal field by molecular cloning. Microbiol 79: 96–105

Gerhardt P, Murray RGE, Costilov RN, Nester EW, Wood WA, Kreig NR, Philips GB (1981) Manual methods for general bacteriology. American Society for Microbiology, Washington DC: 409–443

Gillis M, De Ley J, De Cleene M (1970) The determination of molecular weight of bacterial genome DNA from renaturation rates. Eur J Biochem 12: 143–153

Giovannelli D, d'Errico G, Manini E, Yakimov M, Vetriani C (2013) Diversity and phylogenetic analyses of bacteria from a shallow-water hydrothermal vent in Milos island (Greece). Front Microbiol 4: 84 DOI: 10.3389/fmicb.2013.00184

Gold TH (1992) The deep, hot biosphere. Proc Natl Acad Sci USA 89: 6045–6049

Gómez-Pereira PR, Schüler M, Fuchs BM, Bennke C, Teeling H, Waldmann J, Richter M, Barbe V, Bataille E, Glöckner FO, Amann R (2012) Genomic content of uncultured Bacteroidetes from contrasting oceanic provinces in the North Atlantic Ocean. Environ Microbiol 14(1): 52–66

Gonzalez JM, Covert JS, Whitman WB, Henriksen JR, Mayer F, Scharf B, Schmitt R, Buchan A, Fuhrman JA, Kiene RP, Moran MA (2003) *Silicibacter pomeroyi* sp. nov. and *Roseovarius nubinhibens* sp. nov., dimethylsulfoniopropionate-demethylating bacteria from marine environments. Int J Syst Evol Microbiol 53: 1261–1269

Gotelli NJ, Entsminger GL (2004) Ecosim: null models software for ecology, version 7. Acquired Intelligence, Inc. and Kesey-Bear, Burlington, Vermout, USA

Greene AC, Madgwick JC (1991) Microbial formation of manganese oxides. Appl Environ Microbiol 57(4): 1114–1120

Guezennec J (2002) Deep-sea hydrothermal vents: A new source of innovative bacterial exopolysaccharides of biotechnological interest? J Ind Microbiol Biotechnol 29: 204–208

Gugliandolo C, Lentini V, Spanó A, Maugeri TL (2012) New bacilli from shallow hydrothermal vents of Panarea Island (Italy) and their biotechnological potential. J Appl Microbiol 112: 1102–1112

Gugliandolo C, Maugeri TL (1998) Temporal variations in heterotrophic mesophilic bacteria from a marine shallow hydrothermal vent off the Island of Vulcano (Eolian Islands, Italy). Microb Ecol 36: 13–22

Gugliandolo C, Maugeri TL, Costa F, Caccamo D (1998) Isolation and characterization of thermotolerant hydrocarbon-utilizing bacteria from marine, shallow hydrothermal vents. Rapp Comm Int Mer Médit 35: 348–349

Gummersheimer BS, Giblin T (2003) Identification of lead resistant bacteria from a heavily contaminated site. Bios 74(2): 48–54

Gupta RS (2000) The phylogeny of Proteobacteria: relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol Rev 24: 367–402

Hammond SR (1997) Offset caldera and crater collapse on Juan de Fuca ridge-flank volcanoes. Bull Volcanol 58: 617–627

Hansel CM, Francis CA (2006) Coupled photochemical and enzymatic Mn(II) oxidation pathways of a planktonic *Roseobacter*-like bacterium. Appl Environ Microbiol 72(5): 3543–3549

Hirayama H, Sunamura M, Takai K, Nunoura T, Noguchi T, Oida H, Furushima Y, Yamamoto H, Oomori T, Horikoshi K (2007) Culture-dependent and independent characterization of microbial communities associated with a shallow submarine hydrothermal system occurring within a coral reef off Taketomi Island, Japan. Appl Environ Microbiol 73(23): 7642–7656

Hoaki T, Nishijima M, Miyashita H, Maruyama T (1995) Dense community of hyperthermophilic sulfur-dependent heterotrophs in a geothermally heated shallow submarine biotope near Kodakara-jima Island, Kagoshima, Japan. Appl Environ Microbiol 61: 1931–1937

Holden JF, Adams MWW (2003) Microbe–metal interactions in marine hydrothermal environments. Curr Opin Chem Biol 7: 160–165

Hoope HG (2003) Phosphatase activity in the sea. Hydrobiologia 493: 187–200

Huber JA, Cantin HV, Huse SM, Mark Welch DB, Sogin ML, Butterfield DA (2010) Isolated communities of Epsilonproteobacteria in hydrothermal vent fluids of the Mariana Arc seamounts. FEMS Microbiol Ecol 73: 538–549

Hughes MN, Poole RK (1989) Metals and Micro-organisms. Chapman and Hall, London

Hugler M, Gartner A, Imhoff JF (2010) Functional genes as markers for sulfur cycling and CO_2 fixation in microbial communities of hydrothermal vents of the Logatchev field. FEMS Microbiol Ecol 73: 526–537

Hunter WJ, Kuykendall LD, Manter DK (2007) *Rhizobium selenireducens* sp. nov., a selenite reducing alpha-proteobacteria isolated from a bioreactor. Curr Microbiol 55: 455–460

Hunter-Cevera J, Karl D, Buckley M (2005) Marine Microbial Diversity: The key to Earth’s habitability. American Academy of Microbiology, Washington, DC

Jannasch HW, Mottl MJ (1985) Geomicrobiology of deep-sea hydrothermal vents. Science 229: 717–725

Jaysankar D, Ramaiah N, Vardanyan L (2008) Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. Marine Biotech 10(4): 471–477

Jeng MS, Ng NK, Ng PKL (2004) Feeding behaviour: Hydrothermal vent crabs feast on sea ‘snow’. Nature 432: 969

Johnson BD, Bacelar-Nicolau P, Okibe N, Thomas A, Hallberg KB (2009) *Ferrimicrobium acidiphilum* gen. nov., sp. nov. and *Ferrithrix thermotolerans* gen. nov., sp. nov.: heterotrophic, iron-oxidizing, extremely acidophilic Actinobacteria. Int J Syst Evol Microbiol 59: 1082–1089

Jones ML (1985) Hydrothermal vents of the Eastern Pacific: An overview. 6th ed. Vienna, VA: INFAX Corporation

Jorgensen NOG, Kroer N, Coffin RB, Lee C (1993) Dissolved free amino acids, combined amino acids, and DNA as source of carbon and nitrogen to marine bacteria. Mar Ecol Prog Ser 98: 135–148

Jung YT, Lee JS, Oh KH, Oh TK, Yoon JH (2011) *Roseovarius marinus* sp. nov., isolated from seawater. Int J Syst Evol Microbiol 61: 427–432

Jung YT, Park S, Yoon JH (2012) *Roseovarius litoreus* sp. nov., isolated from seawater of southern coast of Korean peninsula. Antonie van Leeuwenhoek 102: 141–148

Kamenev GM, Fadeev VI, Selin NI, Tarasov VG, Maalakhov VV (1993) Composition and distribution of macro- and meiobenthos around sublittoral hydrothermal vents in the Bay of Plenty, New Zealand. New Zeal J Mar Fresh 27: 407–418

Kämpfer P, Buczolits S, Albrecht A, Busse H-J, Stackebrandt E (2003) Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. Int J Syst Evol Microbiol 53: 893–896

Karl DM (1995) Ecology of free-living, hydrothermal vent microbial communities. In Karl DM (Ed.), Microbiology of deep-sea hydrothermal vents. CRC Press, Inc., Boca Raton, Fla

Karl DM (2002) Microbiological oceanography: hidden in a sea of microbes. Nature 415: 590–591

Kaye JZ, Sylvan JB, Edwards KJ, Baross JA (2011) *Halomonas* and *Marinobacter* ecotypes from hydrothermal vent, sub seafloor and deep-sea environments. FEMS Microbiol Ecol 75: 123–133

Kelley DS, Baross JA, Delaney JR (2002) Volcanoes, fluids, and life at mid-ocean ridge spreading centers. Annu Rev Earth Planet Sci 30: 385–391

Khandeparker R, Meena RM, Deobagkar D (2014) Bacterial diversity in deep-sea sediments from Afanasy Nikitin Seamount, Equatorial Indian Ocean. Geomicrobiol J 31: 942–949

Khandeparker R, Verma P, Meena RM, Deobagkar DD (2011) Phylogenetic diversity of carbohydrate degrading culturable bacteria from Mandovi and Zuari estuaries, Goa, west coast of India. Estuar Coast Shelf Sci 95: 359–366

Kim BS, Kim OS, Moon EY, Chun J (2010) *Vitellibacter aestuarii* sp. nov., isolated from tidalflat sediment, and an emended description of the genus *Vitellibacter*. Int J Syst Evol Microbiol 60: 1989–1992

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012a) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62: 716–721

Kim YO, Kong HJ, Park S, Kang SJ, Kim WJ, Kim KK, Oh TK, Yoon JH (2012b) *Roseovarius halocynthiae* sp. nov., isolated from sea squirt *Halocynthia roretzi*. Int J Syst Evol Microbiol 62: 931–936

Kirchman DL (2002) The ecology of Cytophaga–Flavobacteria in aquatic environments. FEMS Microbiol Ecol 39: 91–100

Kluge AG, Farris FS (1969) Quantitative phyletics and the evolution of anurans. Syst Zool 18: 1–32

Kobayashi T, Koide O, Mori K, Shimamura S, Matsuura T, Miura T, Takaki Y, Morono Y, Nunoura T, Imachi H, Inagaki F, Takai K, Horikoshi K (2008) Phylogenetic and enzymatic diversity of deep subseafloor aerobic microorganisms in organics and methane rich sediments off Shimokita Peninsula. Extremophiles 12: 519–527

Kormas KA, Tivey MK, Von Damm K, Teske A (2006) Bacterial and archaeal phylotypes associated with distinct mineralogical layers of a white smoker spire from a deep-sea hydrothermal vent site (9 degrees N, East Pacific Rise). Environ Microbiol 8(5): 909–920

Krumbein WE, Altman HJ (1973) A new method for the detection and enumeration of manganese oxidizing and reducing microorganisms. Helgol Wiss Meeresunters 25: 347–356

Kuever J, Sievert SM, StevensH, Brinkhoff T, Muyzer G (2002) Microorganisms of the oxidative and reductive part of the sulphur cycle at a shallow-water hydrothermal vent in the Aegean Sea (Milos, Greece). Cah Biol Mar 43: 413–416

Kumar RS, Ferry JC (2014) Prokaryotic carbonic anhydrases of Earth's environment. Subcell Biochem 75: 77–87

Labrenz M, Collins MD, Lawson PA, Tindall BJ, Schumann P, Hirsch P (1999) *Roseovarius tolerans* gen. nov., sp. nov., a budding bacterium with variable bacteriochlorophyll *a* production from hypersaline Ekho Lake. Int J Syst Bacteriol 49: 137–147

Lai Q, Fu Y, Wang J, Chen S, Zhong H, Sun F, Shao Z (2011b) *Citreicella marina* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Microbiol 61: 728–731

Lai Q, Zhong H, Wang J, Yuan J, Sun F, Wang L, Zheng T, Shao Z (2011a) *Roseovarius indicus* sp. nov., isolated from deep-sea water of the Indian Ocean. Int J Syst Evol Microbiol 53: 2040–2044

Lane DJ (1991) 16S/23S rRNA sequencing. In Stackebrandt E, Goodfellow M (Eds) Nucleic acid techniques in bacterial systematic. John Wiley and Sons Inc, New York, pp 115–175

Lasa A, Dieguez AL, Romalde JL (2014) *Vibrio cortegadensis* sp. nov., isolated from clams. Antonie van Leeuwenhoek 105: 335–341

Lau E, Fisher MC, Steudler PA, Cavanaugh CM (2013) The methanol dehydrogenase gene, *mxaF*, as a functional and phylogenetic marker for Proteobacterial methanotrophs in natural environments. Plos One 8(2): e56993

Learman DR, Voelker BM, Vazquez-Rodriguez AI, Hansel CM (2011) Formation of manganese oxides by bacterially generated superoxide. Nat Geosci 4: 95–98

Lee S-Y, Kang C-H, Oh T-K, Yoon J-H (2012) *Virgibacillus campisalis* sp. nov., from a marine solar saltern. Int J Syst Evol Microbiol 62: 347–351

Lentini V, Gugliandolo C, Bunk B, Overmann J, Maugeri TL (2014) Diversity of prokaryotic community at a shallow marine hydrothermal site elucidated by Illumina sequencing technology. Curr Microbiol 69: 457–466

Lentini V, Gugliandolo C, Maugeri TL (2007) Identification of enzyme-producing thermophilic bacilli isolated from marine vents of Aeolian Islands (Italy). Ann Microbiol 57(3): 355–361

Li J, Peng X, Zhou H, Li J, Sun Z (2013a) Molecular evidence for microorganisms participating in Fe, Mn, and S biogeochemical cycling in two low-temperature hydrothermal fields at the Southwest Indian Ridge. J Geophys Res: Biogeosciences 118: 665–679

Li WKW, Dickie PM (1996) Distribution and abundance of bacteria in the Ocean (http://www2.mar.dfo-mpo.gc.ca/science/review/1996/Li/Li_e.html)

Li Z, Zhao R, Ji S, Shi X, Zhang XH (2013b) *Roseovarius marisflavi* sp. nov., isolated from an amphioxus breeding zone in the coastal region of the Yellow Sea, China. Antonie van Leeuwenhoek 104: 413–421

Longnecker K, Reysenbach AL (2001) Expansion of the geographic distribution of a novel lineage of epsilon-Proteobacteria to a hydrothermal vent site on the Southern East Pacific Rise. FEMS Microbiol Ecol 35: 287–293

López-García P, Duperron S, Philippot P, Foriel J, Susini J, Moreira D (2003) Bacterial diversity in hydrothermal sediment and epsilonproteobacterial dominance in

experimental microcolonizers at the Mid-Atlantic Ridge. Environ Microbiol 5(10): 961–976

Loveland-Curtze J, Miteva VI, Brenchley JE (2011) Evaluation of a new fluorimetric DNA-DNA hybridization method. Can J Microbiol 57: 250–255

Luna GM, Dell'Anno A, Danovaro R (2006) DNA extraction procedure: a critical issue for bacterial assessment in marine sediments. Environ Microbiol 8: 308–320

Malmstrom RR, Kiene RP, Cottrell MT, Kirchman DL (2004). Contribution of SAR11 bacteria to dissolved dimethylsulfoniopropionate and amino acid uptake in the North Atlantic Ocean. Appl Environ Microbiol 70(7): 4129–4135

Mandernack KW, Post J, Tebo BM (1995) Manganese mineral formation by bacterial spores of the marine *Bacillus*, strain SG-1: evidence for the direct oxidation of Mn(II) to Mn(IV). Geochim Cosmochim Acta 21: 4393–4408

Marmur J (1961) A procedure for isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3: 208–218

Maugeri TL, Gugliandolo C, Caccamo D, Panico A, Lama L, Gambacorta A, Nicolaus B (2002) A halophilic thermotolerant *Bacillus* isolated from a marine hot spring able to produce a new exopolysaccharide. Biotechnol Lett 24: 515–519

Maugeri TL, Gugliandolo C, Caccamo D, Stackebrandt E (2001) A polyphasic taxonomic study of thermophilic bacilli from shallow, marine vents. Syst Appl Microbiol 24: 572–587.

Maugeri TL, Gugliandolo C, Lentini V (2013) Diversity of prokaryotes at a shallow submarine vent of Panarea Island (Italy) by high-throughput sequencing. Atti Accad Pelorit Pericol Cl Sci Fis Mat Nat 91(2): DOI: 10.1478/AAPP.912A1

Maugeri TL, Lentini V, Gugliandolo C, Cousin S, Stackebrandt E (2010) Microbial diversity at a hot, shallow-sea hydrothermal vent in the Southern Tyrrhenian Sea (Italy). Geomicrobiol J 27: 380–390

Maugeri TL, Lentini V, Gugliandolo C, Italiano F, Cousin S, Stackebrandt E (2009) Bacterial and archaeal populations at two shallow hydrothermal vents off Panarea Island (Eolian Islands, Italy). Extremophiles 13: 199–212

McDonald IR, Murrell JC (1997) The particulate methane monooxygenase gene *pmoA* and its use as a functional gene probe for methanotrophs. FEMS Microbiol Lett 156: 205–210

McQueen DA, Schottel JL (1987) Purification and characterization of a novel extracellular esterase from pathogenic Streptomyces scabies that is inducible by zinc. J Bacteriol 169: 1967–1971

Mehta MP, Butterfield DA, Baross JA (2003) Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. Appl Environ Microbiol 69(2): 960–970

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39: 159–167

Miguez CB, Bourque D, Sealy JA, Greer CW, Groleau D (1997) Detection and isolation of methanotrophic bacteria possessing soluble methane monooxygenase (sMMO) genes using the polymerase chain reaction (PCR). Microb Ecol 33: 21–31

Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal A, Parlett JH (1984) An integrated procedure for the extraction of isoprenoid quinines and polar lipids. J Microbiol Methods 2: 233–241

Miroshnichenko ML, L'Haridon S, Schumann P, Spring S, Bonch-Osmolovskaya EA, Jeanthon C, Stackebrandt E (2004). *Caminibacter profundus* sp. nov., a novel thermophile of Nautiliales ord. nov. within the class '*Epsilonproteobacteria*', isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol. 54(1): 41–45

Miura T, Nedachi M, Hashimoto A (2002) Sulphur sources for chemoautotrophic nutrition of shallow water vestimentiferan tubeworms in Kagoshima Bay. J Mar Biol Assoc UK 82: 537–540

Mohandass C, Raghukumar C (2005) Biological deinking of inkjet-printed paper using *Vibrio alginolyticus* and its enzymes. J Ind Microbiol Biotechnol 32(9): 424–429

Mohandass C, Rajasabapathy R, Ravindran C, Colaco A, Santos RS, Meena RM (2012) Bacterial diversity and their adaptations in the shallow water hydrothermal vent at D Joao de Castro Seamount (DJCS), Azores, Portugal. Cah Biol Mar 53: 65–76

Moissl C, Osman S, La Duk, MT, Dekas A, Brodie E, DeSantis T, Venkateswaran K (2007) Molecular bacterial community analysis of clean rooms where spacecraft are assembled. FEMS Microbial Ecol 61: 509–521

Morri C, Bianchi CN, Cocito S, Peirano A, De Biasi AM, Aliani S, Pansini M, Boyer M, Ferdeghini F, Pestarino M, Dando P (1999) Biodiversity of marine sessile epifauna at an Aegean island subject to hydrothermal activity: Milos, Eastern Mediterranean Sea. Mar Biol 135: 729–739

Moutin T, Karl DM, Duhamel S, Rimmelin P, Raimbault P, Van Mooy BAS, Claustre H (2008) Phosphate availability and the ultimate control of new nitrogen input by nitrogen fixation in the tropical Pacific Ocean. Biogeosciences 5: 95–109

Moyer CL, Dobbs FC, Karl DM (1995) Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl Environ Microbiol 61(4): 1555–1562

Mudryk ZJ, Podgórska B (2006) Enzymatic activity of bacterial strains isolated from marine beach sediments. Pol J Microbiol 15(3): 441–448

Munn CB (2011) Marine Microbiology: Ecology and Applications, 2nd ed. Garland Science, Taylor and Francis Group, New York, NY

Murdock S, Johnson H, Forget N, Juniper SK (2010) Composition and diversity of microbial mats at shallow hydrothermal vents on Volcono 1, South Tonga Arc. Cah Biol Mar 51: 407–413

Nakagawa T, Nakagawa S, Inagaki F, Takai K, Horikoshi K (2004) Phylogenetic diversity of sulfate-reducing prokaryotes in active deep-sea hydrothermal vent chimney structures. FEMS Microbiol Lett 232: 145–152

Nedashkovskaya OI, Suzuki M, Vysotskii MV, Mikhailov VV (2003) *Vitellibacter vladivostokensis* gen. nov., sp. nov., a new member of the phylum Cytophaga–Flavobacterium–Bacteroides. Int J Syst Evol Microbiol 53: 1281–1286

Needham HD, Francheteau J (1974) Some characteristics of the rift valley in the Atlantic Ocean near 36°48' North. Earth Planet Sci Lett 22: 29–43

Nercessian O, Fouquet Y, Pierre C, Prieur D, Jeanthon C (2005) Diversity of Bacteria and Archaea associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. Environ Microbiol 7(5): 698–714

Nishiyama Y, Massey V, Anzai Y, Watanabe T, Miyaji T, Uchimura T, Kozaki M, Suzuki H, Niimura Y (1997) Purification and characterization of *Sporolactobacillus inulinus* NADH oxidase and its physiological role in aerobic metabolism of the bacterium. J Ferment Bioeng 84: 22–27

Oh YS, Lim HJ, Cha IT, Im WT, Yoo JS, Kang UG, Rhee SK, Roh DH (2009) *Roseovarius halotolerans* sp. nov., isolated from deep seawater. Int J Syst Evol Microbiol 59: 2718–2723

Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, De Vrind-De Jong EW, De Vrind JPM, Corstjens PLAM (1997) Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. Appl Environ Microbiol 63(12): 4793–4799

Pandey SK, Narayan KD, Bandyopadhyay S, Nayak KC, Das SK (2009) Thiosulfate oxidation by *Comamonas* sp. S23 isolated from a sulfur spring. Curr Microbiol 58: 516–521.

Park MS, Chung B-K, Lee HY, Jin HM, Lee S-S, Oh, YK, Jeon CO (2011) *Citreicella aestuarii* sp. nov., isolated from a tidal flat. Int J Syst Evol Microbiol 61: 2595–2599

Park S, Lee KC, Bae KS, Yoon J-H (2014) *Vitellibacter soesokkakensis* sp. nov., isolated from the junction between the ocean and a freshwater spring and emended description of the genus *Vitellibacter*. Int J Syst Evol Microbiol 64: 588–593

Park S, Lee MH, Yoon J-H (2013) *Oceanicola litoreus* sp. nov., an alphaproteobacterium isolated from the seashore sediment. Antonie van Leeuwenhoek 103: 859–866

Park S, Yoon J-H (2013) *Roseovarius sediminilitoris* sp. nov., isolated from seashore sediment. Int J Syst Evol Microbiol 63: 1741–1745

Peeters K, Ertz D, Willems A (2011) Culturable bacterial diversity at the Princess Elisabeth Station (Utsteinen, Sør Rondane Mountains, East Antarctica) harbours many new taxa. Syst Appl Microbiol 34: 360–367

Perner M, Gonnella G, Kurtz S, LaRoche J (2014) Handling temperature bursts reaching 464 °C: different microbial strategies in the Sisters Peak hydrothermal chimney. Appl Environ Microbiol. DOI: 10.1128/AEM.01460-14

Peterson DA, Frank DN, Pace NR, Gordon JI (2008) Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. Cell Host Microbe 3(6): 417–427

Pichler T, Veizer J, Hall GEM (1999) The chemical composition of shallow-water hydrothermal fluids in Tutum Bay, Ambitle Island, Papua New Guinea and their effect on ambient seawater. Mar Chem 64: 229–252

Podar M, Reysenbach (2006) New opportunities revealed by biotechnological explorations of extremophiles. Curr Opin Biotechnol 17: 250–255

Podgorsek L, Petri R, Imhoff JF (2004) Cultured and genetic diversity, and activities of sulfur-oxidizing bacteria in low-temperature hydrothermal fluids of the North Fiji Basin. Mar Ecol Prog Ser 266: 65–76

Polz MF, Cavanaugh C (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. Proc Natl Acad Sci USA 92: 7232–7236

Poremba K, Hoppe H-G (1995) Spatial variation of benthic microbial production and hydrolytic enzymatic activity down the continental slope of the Celtic Sea. Mar Ecol Prog Ser 118: 237–245

Prieur D, Erauso G, Jeanthon C (1995) Hyperthermophilic life at deep-sea hydrothermal vents. Planet Space Sci. 43: 115–122

Prol-Ledesma R, Canet C, Torres-Vera M, Forrest M, Armienta M (2004) Vent fluid chemistry in Bahia Concepcion coastal submarine hydrothermal system, Baja California Sur, Mexico. J Volcanol Geotherm Res 137: 311–328

Qin S, Yuan B, Zhang Y, Bian G, Tamura T, Sun B, Li W (2012) *Nocardioides panzhihuaensis* sp. nov., a novel endophytic actinomycete isolated from medicinal plant *Jatropha curcas* L. Antonie van Leeuwenhoek 102(2): 353–360

Quartau R, Curado F, Bouriak S, Monteiro JH, Pinheiro L (2003) *ProjectoGemas – Localização e distribuição de areiasemredor da ilha do Pico*. RelatórioTécnico INGMARDEP, 16/2003. Dept. de GeologiaMarinha, IGM, Lisboa, p 49

Quartau R, Curado F, Cunha T, Pinheiro L, Monteiro JH (2002) *ProjectoGemas – Localização e distribuição de areiasemredor da ilha do Faial*. RelatórioTécnico INGMARDEP, 5/2002. Dep.to de GeologiaMarinha, IGM, Lisboa, p 37

Querellou J (2003) Biotechnology of marine extremophiles. Book of Abstracts, International Conference on the sustainable development of the Mediterranean and Black Sea environment. Thessaloniki, Greece, 28 May-1 June, extended abstract

Raghukumar C, Mohandass C, Cardicos F, Costa PMD, Santos RS, Colaco A (2008) Assemblage of benthic diatoms and culturable heterotrophs in shallow water

hydrothermal vent of the D. João de Castro Seamount, Azores in the Atlantic Ocean. Curr Sci 95(12): 1715–1723

Rainey FA, Kelly DP, Stackebrandt E, Burghardt J, Hiraishi A, Katayama Y, Wood AP (1999) A reevaluation of the taxonomy of *Paracoccus denitrificans* and a proposal for the combination *Paracoccus pantotrophus* comb. nov. Int J Syst Bacteriol 49: 645–651

Ramana CV, Parag B, Girija KR, Ram BR, Ramana VV, Sasikala C (2013) *Rhizobium subbaraonis* sp. nov., an endolithic bacterium isolated from beach sand. Int J Syst Evol Microbiol 63: 581–585

Rameshkumar N, Gomez-Gil B, Spröer C, Lang E, Dinesh Kumar N, Krishnamurthi S, Nair S, Roque A (2011). *Vibrio plantisponsor* sp. nov., a diazotrophic bacterium isolated from a mangrove associated wild rice (*Porteresia coarctata* Tateoka). Syst Appl Microbiol 34: 487–493

Rassa AC, McAllister SM, Safran SA, Moyer CL (2009) Zeta-proteobacteria dominate the colonization and formation of microbial mats in low-temperature hydrothermal vents at Loihi Seamount, Hawaii. Geomicrobiol J 26: 623–638

Rathgeber C, Yurkova N, Stackebrandt E, Beatty T, Yurkov V (2002) Isolation of tellurite- and selenite-resistant bacteria from hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean. Appl Environ Microbiol 68(9): 4613–4622

Reed AJ, Dorn R, Van Dover CL, Lutz RA, Vetriani C (2009) Phylogenetic diversity of methanogenic, sulfate-reducing and methanotrophic prokaryotes from deep-sea hydrothermal vents and cold seeps. Deep-Sea Research II 56: 1665–1674

Reichenbach H (1992) The order *Cytophagales*. In *The Prokaryotes*, 2nd edn. Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (Eds.). New York: Springer 4, pp. 3631–3675

Reichenbach H, Kohl W, Böttger-Vetter A, Achenbach H (1980) Flexirubin-type pigments in *Flavobacterium*. Arch Mirobiol 126: 291–293

Reisinger K, Stoeppler M, Nurnberg HW (1981) Evidence for the absence of biological methylation of lead in the environment. Nature 29: 228–230

Ren DW, Chen WF, Sui XH, Wang ET, Chen WX (2011) *Rhizobium vignae* sp. nov., a symbiotic bacterium isolated from multiple legume species. Int J Syst Evol Microbiol 61: 580–586

Roane TM (1999) Lead resistance in two bacterial isolates from heavy metal contaminated soils. Microb Ecol 37: 218–224

Rogers AD, Tyler PA, Connelly DP, Jon T, James R, Larter RD, Linse K, Mills RA, Naveira GA, Pancost RD, Pearce DA, Polunin NVC, German CR, Shank T, Boersch-Supan PH, Alker BJ, Aquilina A, Bennett SA, Clarke A, Dinley RJJ, Graham AGC, Green DRH, Hawkes JA, Hepburn L, Hilario A, Huvenne VAI, Marsh L, Ramirez-Llodra E, Reid WDK, Roterman CN, Sweeting CJ, Thatje S, Zwirglmaier K (2012) The discovery of new deep-sea hydrothermal vent communities in the Southern Ocean and implications for biogeography. PLoS Biol 10(1): e1001234

Romanenko LA, Tanaka N, Frolova GM, Svetashev VI, Mikhailov VV (2011) *Litoreibacter albidus* gen. nov., sp. nov. and *Litoreibacter janthinus* sp. nov., members of the class *Alphaproteobacteria* isolated from the seashore. Int J Syst Evol Microbiol 61: 148–154

Rosson RA, Nealson KH (1982) Manganese binding and oxidation by spores of a marine *Bacillus*. J Bacteriol 151: 1027–1034

Roussel EG, Konn Ć, Charlou J-L, Donval J-P, Fouquet Y, Querellou J, Prieur D, Cambon Bonavita M-A (2011) Comparison of microbial communities associated with three Atlantic ultramafic hydrothermal systems. FEMS Microbiol Ecol 77: 647–665

Rusch A, Walpersdorf E, deBeer D, Gurrieri S, Amend JP (2005) Microbial communities near the oxic/anoxic interface in the hydrothermal system of Vulcano Island, Italy. Chem Geol 224: 169–182

Saha BC, Zeikus JG (1989) Novel highly thermostable pullulanase from thermophiles. TIBTECH 7: 234–239

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

Sarradin P-M, Lannuzel D, Waeles M, Crassous P, Le Bris N, Caprais J-C, Fouquet Y, Fabri MC, Riso R (2008) Dissolved and particulate metals (Fe, Zn, Cu, Cd, Pb) in two habitats from an active hydrothermal field on the EPR at 13 N. The Science of the Total Environment 392: 119–129

Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. MIDI Inc., Newark, DE

Schmid M,WalshK,Webb R,Rijpstra WI, van de Pas-SchoonenK, Verbruggen MJ, Hill T, Moffett B, Fuerst J, Schouten S, Damste JS, Harris J, Shaw P, Jetten M, Strous M (2003) Candidatus “Scalindua brodae”, sp. nov., Candidatus “Scalindua wagneri”, sp. nov., two new species of anaerobic ammonium oxidizing bacteria. Syst Appl Microbiol 26: 529–538

Serres MH, Gopal S, Nahum LA, Liang P, Gaasterland T, Riley M (2001) A functional update of the *Escherichia coli* K-12 genome. Genome Biol 2(9): 1–7

Shiba T, Simidu U, Taga N (1979) Distribution of aerobic bacteria which contain bacteriochlorophyll *a*. Appl Environ Microbiol 38(1): 43–45

Sievert SM, Brinkhoff T, Muyzer G, Ziebis W, Kuever J (1999) Spatial heterogeneity of bacterial populations along an environmental gradient at a shallow submarine hydrothermal vent near Milos Island (Greece). Appl Environ Microbiol 65: 3834–3842

Sievert SM, Huegler M, Taylor CD, Wirsen CO (2008) Sulfur Oxidation at Deep-Sea Hydrothermal Vents. In: Microbial Sulfur Metabolism. Editors: Dahl C, Friedrich CG, Springer, Berlin Heidelberg, DOI: 10.1007/978-3-540- 72682, pp. 238–258

Sievert SM, Kuever J, Muyzer G (2000b) Identification of 16S ribosomal DNA-defined bacterial populations at a shallow submarine hydrothermal vent near Milos Island (Greece). Appl Environ Microbiol 66(7): 3102–3109

Sievert SM, Ziebis W, Kuever J, Sham K (2000a) Relative abundance of Archaea and Bacteria along a thermal gradient of a shallow-water hydrothermal vent quantified by rRNA slot-blot hybridization. Microbiol 146: 1287–1293

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (Eds) Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC, pp 607–654

Sorokin DY (1991) Oxidation of reduced sulphur compounds in volcanic regions in the Bay of Plenty (New Zealand) and Matupy Harbour (New Britain, Papua-New Guinea). Proc USSR Acad Sci Ser B3: 376–387

Sorokin DY, Tourova TP, Muyzer G (2005) *Citreicella thiooxidans* gen. nov., sp. nov., a novel lithoheterotrophic sulfuroxidizing bacterium from the Black Sea. Syst Appl Microbiol 28: 679–687

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44: 846–849

Storesund JE, Øvreås L (2013) Diversity of Planctomycetes in iron-hydroxide deposits from the Arctic Mid Ocean Ridge (AMOR) and description of *Bythopirellula goksoyri* gen. nov., sp. nov., a novel *Planctomycete* from deep sea iron-hydroxide deposits. Antonie van Leeuwenhoek 104: 569–584

Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MS (1999) Missing lithotroph identified as new planctomycete. Nature 400: 446–449

Sudek LA, Templeton AS, Tebo BM, Staudigel H (2009) Microbial ecology of Fe (hydr)oxide mats and basaltic rock from Vailulu'u Seamount, American Samoa. Geomicrobiol J 26: 581–596

Suzuki MT, Rappe MS, Haimberger ZW, Winfield H, Adair N, Strobel J, Giovannoni SJ (1997) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl Environ Microbiol 63: 983–989

Takai K, Komatsu T, Inagaki F, Horikoshi K (2001) Distribution of Archaea in a black smoker chimney structure. Appl Environ Microbiol 67: 3618–29

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739

Tang K, Liu K, Jiao N, Zhang Y, Chen CTA (2013) Functional metagenomic investigations of microbial communities in a shallow-sea hydrothermal system. Plos One 8(8): e72958

Tango MSA, Islam MR (2002) Potential of extremophiles for biotechnological and petroleum applications. Energy Sources 24: 543–559

Tebo BM, Johnson HA, McCarthy JK, Templeton AS (2005) Geomicrobiology of manganese(II) oxidation. Trends Microbiol 13: 421–428

Tempera F (2009) Benthic habitats of the extended Faial island shelf and their relationship to geologic, oceanographic and infralittoral biologic features. PhD thesis, http://hdl.handle.net/10023/726

Templeton AS, Staudigel H, Tebo BM (2005) Diverse Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi Seamount. Geomicrobiol J 22: 127–139

Temuujina U, Chia W-J, Changb Y-K, Honga, S-K (2012) Identification and biochemical characterization of Sco3487 from *Streptomyces coelicolor* A3(2), an exo- and endo-type β-agarase-producing neoagarobiose. J Bacteriol 194: 142–149

Teske A, Brinkhoff T, Muyzer G, Moser DP, Rethmeier J, Jannasch HW (2000) Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. Appl Environ Microbiol 66(8): 3125–3133

Thayer JS, Brinckman FE (1982) The biological methylation of metals and metalloids. Adv Organometallic Chem 20: 313–357

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68: 403–431

Thompson FL, Swings J (2006) Taxonomy of the vibrios. In: Thompson FL, Austin B, Swings J (Eds) The biology of Vibrios. American Society for Microbiology, Washington, DC, pp 29–43

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Thornburg CC, Zabriskie TM, McPhail KL (2010) Deep-sea hydrothermal vents: potential hot spots for natural products discovery. J Nat Prod 73(3): 489–499

Trincone A (2011) Marine biocatalysts: Enzymatic features and applications. Mar Drugs 9(4): 478-499

Trudinger PA (1967) Metabolism of thiosulfate and tetrathionate by heterotrophic bacteria from soil. J Bacteriol 93: 550–559

Tunnicliffe V (1991) The biology of hydrothermal vents: ecology and evolution. Annu Rev Oceanogr Mar Biol 29: 3119–3207

Tuttle JH, Schwartz JH, Whited GM (1983) Some properties of thiosulfate-oxidizing enzyme from marine heterotroph 16B. Appl Environ Microbiol 46: 438–445

Unanue M, Ayo B, Agis M, Slezak D, Herndl G, Iriberri J (1999) Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. Microb Ecol 37: 36–48

Van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6: 213–218

Van Dover CL (2000a) The Ecology of Deep-Sea Hydrothermal Vents. Princeton University Press, New Jersey, p 424

Van Dover CL (2000b) Biogeography and ecological setting of Indian Ocean hydrothermal vents. Science 294: 818–823

Van Waasbergen LG, Hildebrand M, Tebo BM (1996) Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. J Bacteriol 178: 3517–3530

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers Y-H, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304: 66–74

Vetriani C, Chew YS, Miller SM, Yagi J, Coombs J, Lutz RA, Barkay T (2005) Mercury adaptation among bacteria from a deep-sea hydrothermal vent. Appl Environ Microbiol 71(1): 220–226

Vitolins MI, Swaby RJ (1969) Activity of sulphur-oxidizing microorganisms in some Australian soils. Austr J Soil Res 7: 171–183

Wang B, Sun F, Lai Q, Du Y, Liu X, Li G, Luo J, Shao Z (2010a) *Roseovarius nanhaiticus* sp. nov., a member of the *Roseobacter* clade isolated from marine sediment. Int J Syst Evol Microbiol 60: 1289–1295

Wang B, Tan T, Shao Z (2009) *Roseovarius pacificus* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Microbiol 59: 1116–1121

Wang H, Zhang X, Yan S, Qi Z, Yu Y, Zhang X-H (2012) *Huaishuia halophila* gen. nov., sp. nov., isolated from coastal seawater. Int J Syst Evol Microbiol 62: 223–228

Wang X, Wiens M, Divekar M, Grebenjuk VA, Schröder HC, Batel R, Müller WEG (2011) Isolation and Characterization of a Mn(II)-Oxidizing *Bacillus* strain from the Demosponge *Suberites domuncula*. Mar Drugs 9: 1–28

Wang Y, Zhang X-H, Yu M, Wang H, Austin B (2010b) *Vibrio atypicus* sp. nov., isolated from the digestive tract of the Chinese prawn (*Penaeus chinensis* O'sbeck). Int J Syst Evol Microbiol 60: 2517–2523

Ward DM, Bateson MM, Weller R, Ruff-Roberts (1992) Ribosomal RNA analysis for microorganisms as they occur in nature. Adv Microbiol Ecol 12: 219–286

Watve M, Shejval V, Sonawane C, Rahalkar M, Matapurkar A, Shouche Y, Patole M, Phadnis N, Champhenkar A, Damle K, Karandikar S, Kshirsagar V, Jog M (2000) The 'K' selected oligophilic bacteria: A key to uncultured diversity?. Curr Sci 78: 1535–1542

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Triiper HG (1987) Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37: 463–464

Whittenbury R, Phillips KC, Wilkinson TF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61: 205–218

Wirsen CO, Jannasch HW, Molyneaux SJ (1993) Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites. J Geophy Res 98: 9693–9703

Wirsen CO, Sievert SM, Cavanaugh CM, Molyneaux SJ, Ahmad A, Taylor LT, DeLong EF, Taylor CD (2002) Characterization of an autotrophic sulphide oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol 68: 316–325

Wright ES, Yilmaz LS, Noguera ER (2012) DECIPHER, a Search-Based Approach to Chimera Identification for 16S rRNA Sequences. Appl Environ Microbiol 78(3): 717–725

Yang T, Lyons S, Aguilar C, Cuhel R, Teske A (2011) Microbial communities and chemosynthesis in Yellowstone Lake sublacustrine hydrothermal vent waters. Front Microbiol 2: 1–17

Yavuz E, Gunes H, Harsa S, Yenidunya AF (2004) Identification of extracellular enzyme producing thermophilic bacilli from Balcova (Agamemnon) geothermal site by ITS rDNA RFLP. J Appl Microbiol 97: 810–817

Yoon J-H, Kang S-J, Lee J-S (2013) *Marivita geojedonensis* sp. nov., isolated from seawater. Int J Syst Evol Microbiol 63: 423–427

Yoon J-H, Kang S-J, Oh T-K (2008) *Roseovarius aestuarii* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. Int J Syst Evol Microbiol 58: 1198–1202

Yoon J-H, Lee M-H, Oh T-K (2004) *Porphyrobacter donghaensis* sp. nov., isolated from sea water of the East Sea in Korea. Int J Syst Evol Microbiol 54: 2231–2235

Yoon J-H, Lee S-Y, Kang S-J, Lee C-H, Oh T-K (2007) *Pseudoruegeria aquimaris* gen. nov., sp. nov., isolated from seawater of the East Sea in Korea. Int J Syst Evol Microbiol 57: 542–547

Zeppilli D, Danovaro R (2009) Meiofaunal diversity and assemblage structure in a shallow-water hydrothermal vent in the Pacific Ocean. Aquatic Biol 5: 75–84

Zhang GX, Ren SZ, Xu MY, Zeng GQ, Luo HD, Chen JL, Tan ZY, Sun GP (2011) *Rhizobium borbori* sp. nov., aniline-degrading bacteria isolated from activated sludge. Int J Syst Evol Microbiol 61: 816–822

Zhang X, Li B, Wang H, Sui X, Ma X, Hong Q, Jiang R (2012) *Rhizobium petrolearium* sp. nov., isolated from oil-contaminated soil. Int J Syst Evol Microbiol 62: 1871–1876

Zhou H, Li J, Peng X, Meng J, Wang F, Ai Y (2009) Microbial diversity of a sulphide black smoker in main Endeavear hydrothermal vent field, Juan de Fuca Ridge. J Microbiol 47(3): 235–247

Publications

Research papers published

Rajasabapathy R, Mohandass C, Dastager SG, Liu Q, Li W-J, Colaço A (2015) *Citreicella manganoxidans* sp. nov., a novel manganese oxidizing bacterium isolated from a shallow water hydrothermal vent in Espalamaca (Azores). Antonie van Leeuwenhoek, 108: 1433-1439

Rajasabapathy R, Mohandass C, Yoon J-H, Dastager SG, Liu Q, Khieu T-N, Son CK, Li W-J, Colaço A (2015) *Vitellibacter nionensis* sp. nov., isolated from a shallow water hydrothermal vent. International Journal of Systematic and Evolutionary Microbiology, 65(2): 692-697

Rajasabapathy R, Mohandass C, Yoon J-H, Dastager SG, Liu Q, Khieu T-N, Son CK, Li W-J, Colaço A (2015) *Nioella nitratireducens* gen. nov., sp. nov., a novel member of the family *Rhodobacteraceae* isolated from Azorean Island. Antonie van Leeuwenhoek, 107: 589-595

Rajasabapathy R, Mohandass C, Dastager SG, Liu Q, Khieu T-N, Son CK, Li W-J, Colaço A (2014) *Roseovarius azorensis* sp. nov., isolated from seawater at Espalamaca, Azores. Antonie van Leeuwenhoek, 105: 571-578.

Rajasabapathy R, Mohandass C, Colaço A, Dastager SG, Santos RS, Meena RM (2014) Culturable bacterial phylogeny from a shallow water hydrothermal vent of Espalamaca (Faial, Azores) reveals a variety of novel taxa. Current Science, 106(1): 58-69. [Cover Image from this article]

Manuscripts under review/to be communicated

Rajasabapathy R, Mohandass C, Dastager SG, Ramana VV, Shouche YS, Liu Q, Li W-J, Colaço A (2015) *Rhizobium azorensis* sp. nov., isolated from a shallow hydrothermal vent (Espalamaca, Azores) (under review in International Journal of Systematic and Evolutionary Microbiology)

Rajasabapathy R, Mohandass C, Dastager SG, Liu Q, Li W-J, Colaço A (2015) *Vibrio azorensis* sp. nov., isolated from shallow water hydrothermal vent sediment (Espalamaca, Azores) (to be submitted for peer review)

Rajasabapathy R, Mohandass C, Bettencourt R, Colaço A, Meena RM, Goulart J (2015) Culture independent bacterial community at a low temperature hydrothermal vent (Espalamaca) in Azores Island (to be submitted for peer review)

Research papers presented in conferences

Rajasabapathy R, Mohandass C, Meena RM (2013) Bacterial diversity, a comparison between the hydrothermal vent and the non-vent region of Espalamaca. Oral presentation, International Marine Biotechnology Conference-2013 (November 11-15), Brisbane, Australia

Rajasabapathy R, Mohandass C, Meena RM (2013) Novel bacterial taxa from shallow hydrothermal vent of Espalamaca (Faial, Azores). Poster presentation, Asean-India Marine Biotechnology Workshop 2013 (March 19-22), CSIR-NIO, Goa, India