Alkane oxidation in pure cultures and natural microbial communities from geothermal deep-sea environments: linking diversity and function

By

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ABSTRACT OF THE DISSERTATION

Alkane oxidation in pure cultures and natural microbial communities from geothermal deep-sea environments: linking diversity and function

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At deep-sea hydrothermal vents, the flux of energy is mediated by microbial oxidations, through the conversion of the chemical energy stored in reduced compounds (e.g., sulfide, hydrogen, hydrocarbons) into biochemical energy. Natural hydrocarbons are largely formed by the thermal decomposition of organic matter (thermogenesis) or by microbial processes. However, hydrocarbons can also have an abiotic origin and may form, in hydrothermal systems, by water-rock interactions, for example involving Fisher-Tropsch reactions and the serpentinization of ultramafic rocks. Despite the observation that hydrocarbons are enriched in deep-sea hydrothermal vents, our knowledge of the taxonomic and functional diversity of alkane-oxidizing bacteria from these environments remains very limited. In this dissertation, I investigated the diversity of alkane oxidizing bacteria and the genes *alkB*, *CYP153* and *almA* by performing enrichment cultures for hydrocarbon oxidizing microorganisms from fluid samples and biomass collected from experimental microcolonizers that were deployed on diffuse flow vents on the East Pacific Rise at 9°N and in the Guaymas Basin. These enrichments led to the successful isolations of pure cultures of aerobic, mesophilic organisms capable of using *n*-alkanes as

their carbon sources. Our isolates were, for the most part, *Gammaproteobacteria* of the genus *Acinetobacter* and *Alcanivorax*, but some rare occurring bacteria that were numerically relevant in the environments were also isolated. The PCR amplification of the *alkB*, *CYP153* and *almA* gene fragments from these isolates, and a phylogenetic analysis of these genes was carried out. The *alkB, CYP153* and *almA* genes encode for the alkane hydroxylase, cytochrome P450 and flavin binding monooxygenase respectively, which are enzymes that catalyze the first reaction in the stepwise oxidation of *n*-alkanes. Furthermore, transcripts of the *alkB* gene were detected in two model organisms from the laboratory culture collection, *Alcanivorax* sp. strain EPR 7 and *Acinetobacter* sp. strain EPR 111. Reverse Transcription PCR (RT-PCR) experiments showed that *alkB* transcripts could be detected in the presence of dodecane but not in acetate, confirming that, in these strains, the *alkB* gene is induced. Finally, a functional gene survey of *alkB* genes in vent natural microbial populations showed that the majority of the detected sequences derived from *Gammaproteobacteria* and *Alphaproteobacteria*.

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Dedication

I would like to dedicate this thesis to my parents, José Raúl Cruz González and Mayda Matos Ortiz, and my husband Mark N. Keddis. To my parents, for their unparalleled support and encouragement throughout the years to follow my heart and work hard for what I want in life. To my husband, for his eternal support and patience and for being my strength when I didn't have any for myself. I am eternally grateful to have all of you in my life, close or far in distance, but in my heart you are always there.

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Chapter 1

Introduction

Mid-oceanic ridges and deep-sea hydrothermal vents

Mid-oceanic ridges occur where tectonic plates diverge (spreading centers) and form a continuous underwater mountain relief that has been estimated to be around 60,000 km in length (Fig 1.1) (Martin, et al., 2008, Ramirez-Llodra, et al., 2007). Spreading centers are classified by the velocity of the activity from slow (20-50 mm yr^{-1}) to superfast (130-170 mm yr^{-1}). The site of study is the East Pacific Rise, which is a mid-oceanic ridge located between the Nazca and Pacific Plates. Most vents occur along mid-oceanic ridges, where the cycling of seawater through the earth's crust allows the reaction of seawater with crustal rocks at high temperatures, enriching the hydrothermal fluids with reduced organic and inorganic chemical species. At deep-sea vents, the flux of energy is mediated by microbial oxidations, through the conversion of the chemical energy stored in reduced compounds (e.g., sulfide, hydrogen, hydrocarbons) into biochemical energy. Microorganisms mediate the flux of energy from the chemical source to the higher trophic levels. While chemosynthetic bacteria and archaea have been the main focus in studies of hydrothermal systems, Holger Jannasch indicated that there was potential for heterotrophic aerobic oxidation in these systems even though, at that time, there was no evidence of it (Jannasch, 1995).

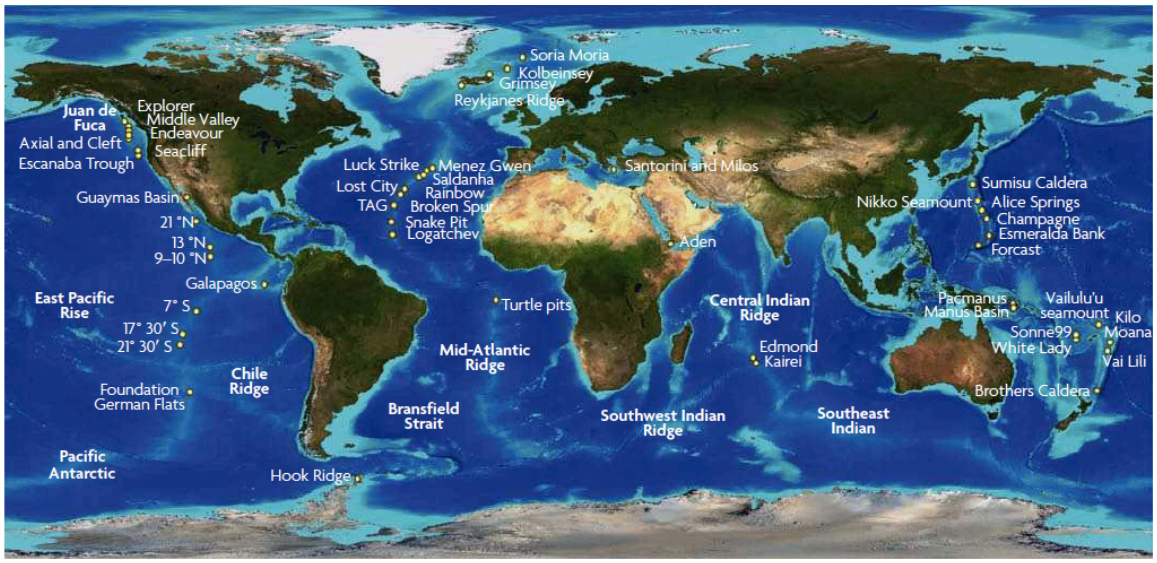

Fig. 1.1: Global distribution of hydrothermal vents (Martin, et al., 2008).

Hydrocarbons in deep-sea hydrothermal vents

Since the discovery of deep-sea hydrothermal vents in 1977, the presence of hydrocarbons has been noted, but their sources remained elusive (Jannasch and Mottl, 1985). In 1988, the maturation of organic matter in these systems was investigated (Brault, et al., 1988a). Maturation of organic matter is the process by which sedimentary organic matter buried in the basins is exposed to increasingly high subsurface temperatures over time. The organic matter then undergoes physical and chemical transformations at high temperature which determine the type of end products (may it be methane, petroleum or natural gas) (Alfred V. Hirner*, 1989, J. Connan, 1980). At hydrothermal vents, petroleum generation occurs by pyrolisis of immature organic matter (Brault, et al., 1989). This dissertation will concentrate on two hydrothermal vent systems: Guaymas Basin, located in the Gulf of California, and the section of the East Pacific Rise (EPR) located at approximately 9° 50'N, 104° 17'W, between the Clipperton and Kane fracture zones and about 500 nautical miles off the western coast of Mexico,

(Fig. 1.1). Guaymas Basin is a very unique hydrothermal system located in center of the Gulf of California, and is part of a rift zone that links the East Pacific Rise in the south with the San Andreas Fault in the north. It is a semi-enclosed system that receives sediment from terrestrial and pelagic sources. The basin is covered by a thick layer of sediments consisting of diatomaceous ooze and terrigenous mud (Didyk and Simoneit, 1989). The interactions between the photosynthetically produced organic matter in the sediments and the high temperature conditions allow the formation of petroleum-like hydrocarbons (Bazylinski, et al., 1989). In early studies, the release of oil globules from the sediments was observed in several sites within the Guaymas Basin hydrothermal system (Didyk and Simoneit, 1989). In contrast, the East Pacific Rise is a fast spreading ridge that is characterized by exposed basalt (bare lava rock hydrothermal system) and by the presence of focused flow fluids released via "black smokers", or chimney structures made of polymetallic sulfides. While both systems have similar macrofauna (tubeworms, clams), a distinct aspect of the Guaymas Basin system is the presence of large "mats" of filamentous bacteria of the genus *Beggiatoa* (Teske, et al., 2002).

Kawka and Simoneit (1987) noted that in the Guaymas Basin system, organic matter underwent maturation generating petroleum-like hydrocarbons (Kawka and Simoneit, 1987). In an early study, Brault, et al., 1988a) found that hydrocarbons, including *n*-alkanes, were 100 to 400 times more concentrated in warm fluids from unsedimented deep-sea vents on the East Pacific Rise than in seawater. Furthermore, a study revealed the presence of thermogenic hydrocarbons in hydrothermal vents off the coast of New Zealand caused by microbial activity (biogenic) or catagenetic decomposition of organic matter (Botz, et al., 2002). Natural hydrocarbons in deep-sea hydrothermal vents are

largely formed by the thermal decomposition of organic matter (thermogenesis) or by microbial processes. Water-rock interactions at hydrothermal vents enable the abiotic formation of hydrocarbons by Fisher-Tropsch reactions, the serpentinization of ultramafic rocks in the presence of several different catalysts (Foustoukos and Seyfried, 2004, Ji, et al., 2008, Thomas M. McCollom, et al., 1999, McCollom and Seewald, 2007, Proskurowski, et al., 2008).

Hydrocarbons with a biogenic origin (methane for the most part) are derived from the microbial degradation of organic matter in anoxic sediments, whereas hydrocarbons with a thermogenic origin are derived from the transformation of organic matter caused by high temperatures and hydrothermal pyrolisis of immature sedimentary organic matter (Brault, et al., 1988a, Brault, et al., 1989). Methane presence has been widely studied in hydrothermal vents and in other marine environments such as cold seeps. Most of the studies looking at the origin of hydrocarbons have been focused on the production of methane (Brault, et al., 1989, Foustoukos and Seyfried, 2004, Holm and Charlou, 2001, Ji, et al., 2008, Thomas M. McCollom, et al., 1999, McCollom and Seewald, 2007, Proskurowski, et al., 2008). Based on previous work showing the presence of high concentrations of hydrocarbons in marine geothermal environments, I chose to investigate the presence and activity of hydrocarbonoclastic bacteria (from the Greek *klastos*, which means broken in pieces: Bacteria capable of breaking down hydrocarbons) in these unique environments.

Alkane oxidation by bacteria

Aerobic and anaerobic bacterial alkane oxidation has been extensively studied in pure cultures and in pelagic seawater, in relation to the oxidation of a range of *n*-alkanes (Harayama, et al., 2004, Rojo, 2009, L. Wang, et al., 2010, Wentzel, et al., 2007, Xu, et al., 2008). Alkanes, also known as paraffins, are quite inert, and require an activator to become available for degradation. In anaerobic environments, activation occurs with the addition of a C_1 or a C_4. In the presence of oxygen, however, the activation step is carried out by several oxygenases. The main focus of this dissertation is on aerobic processes and, more specifically, on the degradation of medium *n*-alkanes (C_5- C_{13}). Aerobic degradation of *n*-alkanes containing two or more carbons typically starts with the oxidation of a terminal methyl group to generate a primary alcohol which is further oxidized to the corresponding aldehyde and finally converted into a fatty acid, as shown in Fig. 1.2 (Rojo, 2009). Enzymes involved in the aerobic and anaerobic oxidation of *n*-alkanes of various lengths have been studied and, in some cases, the mechanism for aerobic alkane oxidation has been thoroughly characterized (Beilen, et al., 2003). The key enzymes involved in aerobic *n*-alkanes oxidation in medium to long chain are: the AlkB alkane hydroxylase, encoded by the *alkB* gene, the cytochrome P450, encoded by the *CYP153* gene (degrades hydrocarbons that range in length from C_5-C_{11}), and the flavin binding monooxygenase AlmA, encoded by the *almA* gene (degrades hydrocarbons that are C_{18} and longer). These enzymes are involved in the alkane degradation mechanism using a monooxygenase to supply the inert molecule with oxygen, which creates a reactive oxygen species (Beilen, et al., 2003, Rojo, 2009).

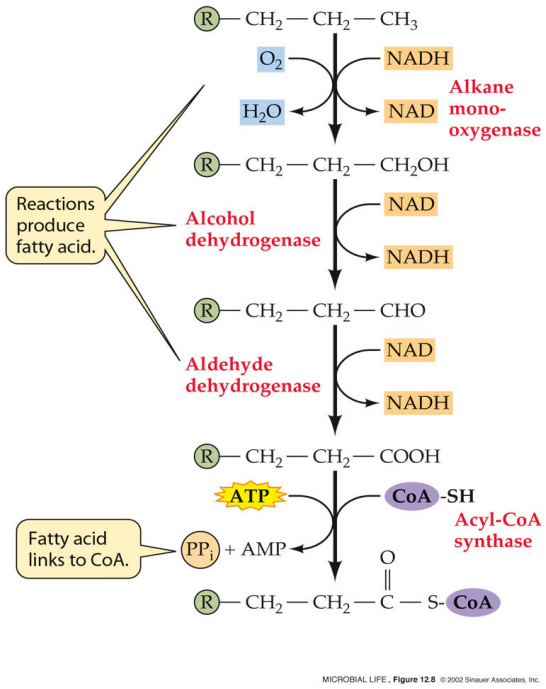

Fig 1.2: Aerobic *n*-alkane oxidation in bacteria from Perry et al., 2002.

- Alkane hydroxylase, *alkB*

These enzymes are known as a class of particulate alkane hydroxylases (pAHs). The AlkB enzymes are integral membrane diiron non-heme monooxygenases that allow a wide range of *Proteobacteria* and *Actinomycetales* to grow on *n*-alkanes with carbon chain lengths from C_5 – C_{16}. Some bacteria, such as *Pseudomonas putida,* can degrade propane and *n*-butane as well as medium chain alkanes (Rojo, 2009). The best characterized alkane degradation pathway is the one encoded by the OCT plasmid of *P. putida* GPo1 (Beilen, et al., 2001). Here, the AlkB protein functions in a complex with two electron transfer proteins, a dinuclear iron rubredoxin (AlkG), and a mononuclear iron rubredoxin reductase (AlkT), channeling electrons from NADH to the active site of the alkane hydroxylase (Beilen, et al., 2003, Rojo, 2009).

- Cytochrome P450, *cyp*153

These soluble heme-containing enzymes are present in several bacteria that sometimes, similar to pAHs, are capable of degrading mid to long chain *n*-alkanes through terminal alkane hydroxylation. The first bacterial P450-encoding genes were discovered in *Acinetobacter calcoaceticus* and were assigned to the *CYP153* family. After further studies, different P450 enzymes were found in additional alkane utilizing species, including *Alcanivorax borkumensis, Sphingomonas* and *Mycobacterium* spp. (Beilen, et al., 2006, Bogaert, et al., 2011). Further tests have shown that alkanes are utilized as the physiological substrates for these enzymes (Funhoff, et al., 2006). It has been known that most alkanotrophs oxidize mixed substrates with different, often length-specific, enzymes. One such example is *Sphingomonas* sp. HXN200, which contains five *CYP153* genes. It was found that the product of three out of the five total genes showed activity for alkanes from C_5-C_{10}, whereas the product of the remaining two genes did not have affinity for these substrates. These observations suggest that these two genes are either pseudogenes, or act on different substrates such as longer chain *n*-alkanes (Beilen, et al., 2005). *Alcanivorax borkumensis* is an example for the presence of both multiple alkane hydroxylase and *CYP153* genes. It was discovered that one of its alkane hydroxylases is essential for the degradation of hexane, but no definitive activity could be found for the the cytochrome P450 enzymes. However, when a double mutant was generated, the activity for growth on C_8-C_{16} alkanes decreased. Because this organism is a dominant species in oil polluted water, it is assumed that these CYP153 enzymes are used to oxidize long chain alkanes (Bogaert, et al., 2011, Sabirova, et al., 2006)

- Flavin binding monooxygenase, *almA*

Recently, the *almA* gene was found to encode for a flavin binding monooxygenase, which is specifically involved in the degradation of *n*-alkanes with carbon chain longer than C_{30} (Wentzel, et al., 2007). This gene was first found in the bacterium *Acinetobacter* sp. Strain DSM 17874 via a mutation screening process (Throne-Holst, et al., 2007). Furthermore, Wang et. al. (2012) carried out a survey of the the *almA* gene on surface seawater and showed a wide diversity of the gene in marine bacteria presumably related to the *Alpha* and *Gammaproteobacteria*. The same authors also noted that, frequently, members of the genus *Alcanivorax* sp. contained multiple copies of these genes (Wang and Shao, 2012).

Detection of alkane oxidizing bacteria in the environment

With the increasing impact of oil spills on the environment, the role of microorganisms in the degradation of petroleum hydrocarbons has become a major topic of research. In recent years, many studies have been performed on the detection of alkane oxidizing bacteria in the environment. In 1989, Bazylinski et. al. carried out a study in the Guaymas Basin hydrothermal system, where the presence of petroleum-like hydrocarbons prompted researchers to investigate if these could be oxidized by microbes. These authors obtained aerobic mesophilic enrichment cultures that showed hydrocarbon oxidation activity, while enrichments at 55°C were negative. However, no taxonomic identification of the hydrocarbon-oxidizing microorganisms was attempted. Some of the sediments obtained in this study were covered with mats of the chemolithotrophic, sulfide-oxidizing bacterium, *Beggiatoa*. The authors speculated that the hydrocarbon-oxidizing

microorganisms could form a substratum population whose metabolic products might support the formation of the *Beggiatoa* mats. At the same time, oxygen consumption by such substratum population could create the optimal oxygen gradients required by *Beggiatoa* (Bazylinski, et al., 1989).

In recent years, several studies explored the diversity of marine alkane oxidizing bacteria from deep-sea sediments as well as surface water samples from non-hydrothermal vent systems. In a review published in 2004, the authors catalogued some marine organisms as "professional" and "non-professional" hydrocarbonoclastic bacteria. The "professional" organisms used a limited range of carbon sources, with a preference for petroleum hydrocarbons. The "professional" species belonged to the following genera: *Alcanivorax, Cycloclasticus, Marinobacter, Neptunomonas, Oleiphilus, Oleispira* and *Planococcus*. "Non-professional" bacteria are deemed those who do not use petroleum hydrocarbons as their main preference when other substrates are present (Harayama, et al., 2004). Some example of these "non-professional" hydrocarbonoclastic bacteria were listed as: *Vibrio, Pseudoalteromonas, Marinomonas* and *Halomonas* (Harayama, et al., 2004, Head, et al., 2006). In a study of uncontaminated deep-sea sediments originating from the Mediterranean Sea, hydrocarbonoclastic bacteria of the *Alcanivorax*, *Marinobacter* and *Pseudomonas* genera, as well as some Gram positive bacteria such as *Rhodococcus* spp., were isolated (Tapilatu, et al., 2010). These studies, along with others, reported the presence of *Alcanivorax* –affiliated strains in deep-sea environments (Cui, et al., 2008, Head, et al., 2006). In contrast, the majority of bacteria isolated from hydrocarbon-contaminated soils along the Mediterranean shoreline were Gram-positive bacteria that belonged to the genera *Nocardia, Rhodococcus* and *Gordonia*

(Quatrini, et al., 2008).

In addition to physiological studies on enrichment and pure cultures of alkane-oxidizing bacteria, researchers have also investigated the genes involved in alkane degradation. Molecular surveys of the transcripts of genes encoding for the alkane hydroxylase (*alkB)* and cytochrome P450 (*cyp153A)* from various environments have shown a prevalence of genes related to *Alcanivorax* and *Marinobacter* spp.*,* but also from more uncommon alkane oxidizers like *Salinisphaera* and *Bacillus* spp., among others (L. Wang, et al., 2010, Wasmund, et al., 2009). These authors also observed that many of these bacteria have multiple homologs of the *alkB* or the *CYP153* genes, or both genes co-occurring in the same organism (Beilen, et al., 2003, L. Wang, et al., 2010). It was noted that organisms that encoded for both genes could oxidize a wider range of alkanes (Beilen and Funhoff, 2007, L. Wang, et al., 2010). Furthermore, a metagenomic analysis of deep-sea sediments from the Pacific ocean showed the presence, for the first time, of alkane hydroxylase genes in deep-sea sediments, including genes related to *Pseudomonas putida* sequences and unique AlkB sequences (Xu, et al., 2008).

Because of their fundamental role in the vent food web, chemosynthetic processes have been the major focus of studies on the microbiology of deep-sea hydrothermal vents. However, heterotrophic microorganisms capable of using organic carbon sources of geothermal origin, such as *n*-alkanes, may also play a critical role in the vent ecosystem. However, while the studies described above provide some information on the occurrence and diversity of hydrocarbonoclastic bacteria in deep-sea environments, little if any data exist on microbial hydrocarbon oxidation at deep-sea hydrothermal vents. Furthermore, while there is evidence for geothermally generated hydrocarbons at deep-

sea vents, data on the concentration of dissolved medium and long chain *n*-alkanes in hydrothermal fluids are scarce. In fact, most of the current studies on microbial oxidation of alkanes in deep-sea reducing environments are focused on short-chain hydrocarbons (C_1-C_4; e.g., van Beilen et al., 2007). Hence, our knowledge of the taxonomic, physiological and metabolic diversity of mid-chain (C_{10}-C_{16}) or long-chain (>C_{16}) alkane-oxidizing bacteria from these environments remains very limited.

Outlook and Objectives of this study

The main focus of my doctoral research is the physiology and metabolism of aerobic hydrocarbonoclastic bacteria. Since these bacteria oxidize subseafloor-generated hydrocarbons to fatty acids, they effectively contribute to the recycling of organic matter at deep-sea vents.

I chose to use a combination of culture-dependent and independent approaches to investigate aerobic hydrocarbonoclastic bacteria as a model system for heterotrophic processes in deep-sea geothermal environments. These microorganisms seem to be versatile since they live at the interphase between reducing environments and seawater from the bottom and oxidize subseafloor-generated hydrocarbons to fatty acids. My experimental strategy employed a polyphasic approach to investigate the taxonomic and physiological diversity of hydrocarbon-oxidizing microorganisms from several deep-sea environments characterized by different biogeochemical regimes.

My work focused on the diversity of deep-sea alkane-oxidizing bacteria, and on the link between diversity and function in this class of organisms by targeting three key enzymes involved in the oxidation of medium to long chain *n*-alkanes. These are: the *alkB* gene, that encodes for the alkane hydroxylase (AlkB), the *cyp153/p450* gene that encodes for the cytochrome P450, and the *almA* gene that encodes for a flavin binding monooxygenase as described above. My overarching hypothesis is that the metabolic activities of aerobic hydrocarbonoclastic bacteria at deep-sea hydrothermal vents contribute to the detoxification of the environment by the degradation of naturally occurring hydrocarbons and therefore facilitate macrofaunal colonization.

Chapter 2

Detection and diversity of alkane-oxidizing bacteria in deep-sea hydrothermal vents in the East Pacific Rise and Guaymas Basin.

Introduction

Of all environments on Earth at which life flourishes, deep-sea hydrothermal vents are among the most extreme. During the cycling of seawater through the earth's crust along the mid-oceanic ridge system, where most deep-sea vents occur, the reaction of seawater with crustal rocks at high temperatures enriches the hydrothermal fluids with reduced organic and inorganic chemical species. At deep-sea vents, the flux of energy is mediated by microbial oxidations, through the conversion of the chemical energy stored in reduced compounds (e.g., sulfide, hydrogen, hydrocarbons) into biochemical energy. Therefore, in this environment microorganisms mediate the flux of energy from the chemical source to higher trophic levels.

Thermogenesis, or the thermal decomposition of organic matter, results in hydrocarbon formation as a results of abiotic interactions in hydrothermal fluids via water-rock interactions (Foustoukos and Seyfried, 2004, Ji, et al., 2008, Thomas M. McCollom, et al., 1999, McCollom and Seewald, 2007, Proskurowski, et al., 2008). Hydrocarbons, including *n*-alkanes, were found to be 100 to 400 times more concentrated in warm fluids from unsedimented deep-sea vents on the East Pacific Rise than in seawater (Brault, et al., 1988a). Furthermore, a study revealed the presence of thermogenic hydrocarbons in hydrothermal vents off the coast of New Zealand (Botz, et al., 2002).

Hydrocarbons (methane for the most part) with a biogenic origin are derived from the microbial degradation of organic matter in anoxic sediments, while hydrocarbons with a thermogenic origin are derived from transformation of organic matter caused by high temperatures (Brault, et al., 1988a). However, despite the observation that hydrocarbons are enriched in deep-sea hydrothermal vents, our knowledge of the taxonomic and functional diversity of alkane-oxidizing bacteria from these environments remains very limited.

My research focuses on two main hydrothermal vent systems: Guaymas Basin and the East Pacific Rise (EPR). Guaymas Basin is a very unique hydrothermal system located in center of the Gulf of California and is part of a rift zone that links the East Pacific Rise in the south with the San Andreas Fault in the north. The Guaymas Basin is a very nutrient-rich environment, as the system receives sediment from terrestrial and pelagic sources and its interactions with high temperatures make for petroleum like products (Bazylinski, et al., 1989). One observation made of some sites in this system was the amount of oil present in some sites released from the sediments as globules (Didyk and Simoneit, 1989). The East Pacific Rise on the other hand, is a fast spreading ridge, which is distinct by the amount of basalt and chimneys present. Unlike Guaymas, the EPR has exposed and very active fluid discharge.

Bacterial alkane oxidation has been extensively studied in pure cultures and in pelagic seawater both aerobically and anaerobically in relation to the oxidation of *n*-alkanes range (Harayama, et al., 2004, Rojo, 2009, L. Wang, et al., 2010, Wentzel, et al., 2007, Xu, et al., 2008). Here I am focusing on the aerobic degradation of medium chain length *n*-alkanes.

In recent years, several studies were published that explored the diversity of alkane degrading bacteria from deep-sea sediments as well as surface water samples from non-hydrothermal vent systems. Most of the alkane-oxidizing bacteria isolated in the course of these studies belonged to the genera *Alcanivorax, Marinobacter* and *Pseudomonas* as well as gram positive bacteria such as *Rhodococcus sp*.(Tapilatu, et al., 2010). In oil polluted marine environments, however, *Alcanivorax* spp. were the most commonly detected hydrocarbonoclastic bacteria in the early stages of the contamination process (Head, et al., 2006). To understand why *Alcanivorax* overcomes other bacteria in oil-contaminated environments, Hara et. al.(2003), performed a competition study between *Alcanivorax borkumensis* strain ST-T1 and *Acinetobacter venetianus* strain T4. In this study, the authors compared growth rates between the two bacteria coexisting in the same culture and when they were grown with three different hydrocarbons (hexadecane, pristane and crude oil). The authors concluded that the perseverance of *Alcanivorax* might be due to its ability to degrade linear and branched alkanes and, most importantly, pristine, since this hydrocarbon is ubiquitous to seawater (Hara, et al., 2003). Several studies across a variety of environments have shown that common hydrocarbonoclastic bacteria are present, such as *Alcanivorax* and *Marinobacter,* but also uncommon alkane oxidizers like *Salinisphaera* spp., *Vibrio* spp. and *Bacillus* spp., among others (Head, et al., 2006, L. Wang, et al., 2010).

Degradation of hydrocarbons is hindered by the difficulty of the bacteria to access substrates. In order to overcome that obstacle, bacteria produce biosurfactants. Biosurfactants are a diverse group of surface-active chemical compounds that reduce the surface and interstitial tensions of aqueous media. Biosurfactants have many applications,

including the remediation of metals and organics, enhanced transport of bacteria, enhanced oil recovery, among others (Bodour and Miller-Maier, 1998, Youssef, et al., 2004). For this reason it is important to determine if biosurfactants are produced by alkane oxidizing bacteria and facilitate their interaction with the hydrocarbons.

In this chapter, I will report the taxonomic and physiological diversity of alkane-oxidizing bacteria from several deep-sea vents, as well as physiological and biochemical characteristics that are essential for the utilization of hydrocarbons in geothermal environments.

Materials and Methods

Isolation and characterization of aerobic alkane oxidizing bacteria

Hydrothermal fluids, sediment samples, microbial mats, sulfides and tubeworms were collected from the East Pacific Rise hydrothermal vent system (9° 50' N, 104° 17' W) and from vents located in the Guaymas Basin, Gulf of California at (27° 00' N, 111° 24' W) in December 2006, October 2007 and January 2008. Primary enrichments for aerobic alkane oxidizing bacteria were carried out in Artificial Seawater Minimal Media which was composed of (l^{-1}): NaCl, 23.6g; KCl, 0.64g; $MgCl_2$ •$6H_2O$, 4.53g; $MgSO_4$•7 H_2O, 5.94g; $CaCl_2$ •2 H_2O, 1.3g; Na_2HPO_4 •$7H_2O$, 43.0 mg; $NaNO_3$, 0.22 g; NH_4Cl, 0.65g) supplemented with dodecane ($C_{12}H_{26}$) in the vapour phase as the sole carbon and energy source. After autoclaving, ASW MM was supplemented with 1 mM vitamin B12, 1 ml trace-element solution SL-10 and 1 ml mixed vitamin solution 141(Melitza Crespo-Medina, et al., 2009). Samples were incubated at 30°C using the setup described by Rosenberg in 2006 (Fig.2.1) (Dworkin and Falkow, 2006). Independent cultures that showed consistent growth after repeated transfers were purified by successive isolations of single colonies in solidified ASWMM with dodecane medium containing noble agar (15g l^{-1}). In addition to the direct enrichments (i.e., enrichments from undiluted inocula), dilutions of the primary inocula were also performed in a 7-fold series as a strategy to dilute out fast growing organisms and enrich for numerically abundant organisms that might be outcompeted in the undiluted cultures. Such dilutions were set up with ASW minimal media with dodecane ($C_{12}H_{26}$) as the sole carbon source delivered in the vapor phase. As with the direct enrichment isolations, cultures that showed consistent growth after repeated transfers were purified by successive isolations of single colonies in

solidified ASWMM medium with dodecane delivered in vapor phase. In addition, some isolations were done in Artificial Seawater (ASW), containing per liter: NaCl, 24g; KCl, 0.7g; $MgCl_2$, 7.0g, yeast extract, 3.0g; peptone, 2.5g and low strength ASW media (modified ASW containing 0.1 yeast extract and 0.5g peptone l^{-1}). In this case, the ability of the isolates to grow with alkanes as their sole carbon source was tested after purity was achieved.

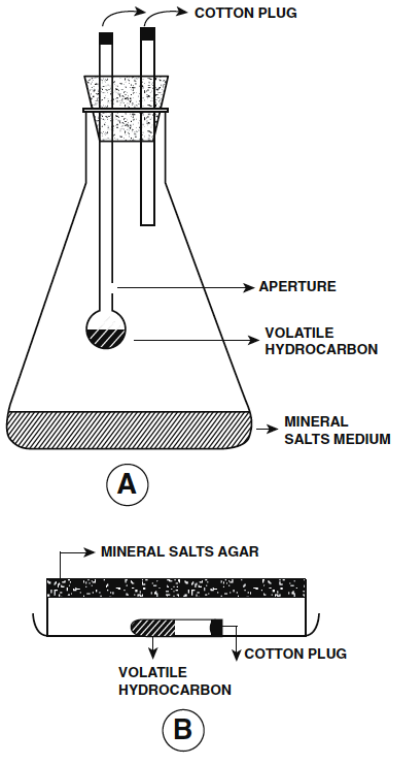

Fig.2.1: Setup for enrichments and isolations of alkane-oxidizing bacteria (Dworkin and Falkow, 2006)

Nucleic acid extraction, purification and PCR amplification of the 16S rRNA gene

Genomic DNA was extracted from cells collected by centrifugation using the UltraClean Microbial DNA isolation kit, according to manufacturer's instructions (MoBio Laboratories). The full-length 16S rRNA gene was amplified from the genomic DNA by PCR with primers 8F and 1517R generating a 1500 bp amplicon. The PCR protocol used to amplify the 16S gene was 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72°C, ending with a final extension of 7 minutes at 72°C. Sequences were determined for both strands on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

The 16S rRNA gene sequences were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). Sequences were then aligned using ClustalX v 1.8 (Thompson, et al., 1997) and manually adjusted using Seaview (Galtier, et al., 1996). Phylogenetic distances were calculated using the Observance Divergence and the neighbor joining method to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier, et al., 1996) and their robustness was tested by bootstrap analysis with 500 re-samplings.

Most Probable Number Counts

Most probable number (MPN) counts were used to estimate the proportion of heterotrophic alkane-oxidizing bacteria among the total bacterial population in hydrothermal fluids from the East Pacific Rise. Total population cell counts were performed by DAPI staining (Porter and Feig, 1980). The MPN were carried out by setting a serial ten-fold dilution of hydrothermal fluid samples under heterotrophic growth conditions using ASW minimal media with dodecane (Melitza Crespo-Medina, et al., 2009) as its sole carbon source as described above. Five replicates tubes were inoculated for each dilution and were incubated aerobically at 30°C for up to 10 days in the dark, without shaking. Growth was confirmed by direct microscope counts of acridine orange-stained cells visualized with an Olympus BX60 microscope with an oil immersion objective lens (Uplan F1 100x 1.3). The number of cells mL^{-1} and the 95 percent confidence intervals were determined using an MPN index (Alexander, 1982).

Substrate Utilization

Several alkane-oxidizing isolates belonging to different genera (*Pseudoalteromonas, Halomonas, Rhizobiales, Arenibacter, Alcanivorax, Marinobacter, Parvibaculum* and *Salinisphaera* spp.) were selected to perform a Biolog assay on a GN2 MicroPlate™ to compare the carbon utilization/oxidation profiles of the chosen isolates as described previously (Melitza Crespo-Medina, et al., 2009). In brief, confluent growth of all strains was obtained on Artificial Sea Water media (l^{-1}: NaCl, 24g; KCl, 0.7g; $MgCl_2$, 7.0g; yeast extract, 3.0g; peptone, 2.5g) solidified with 15g Bacto Agar (Becton, Dickinson and Company) overnight. Cells were collected using a sterile cotton swab and resuspended in 15 ml salt solution (l^{-1}: NaCl, 23.5g; $MgCl_2 \bullet 6H_2O$, 10.6g). The cell suspension was adjusted to an OD_{600} of 0.3±0.05, supplemented with 5mM sodium thioglycolate and dispensed (in 150 µl aliquots) to each well of one Biolog GN2 MicroPlate™, which were incubated at 35°C. A change in color, indicative of the oxidation of the substrate, was monitored for 48 hours.

Hydrocarbon Utilization

Six isolates from this study (*Acinetobacter* EPR111, EPR 144; *Alcanivorax* EPR 7, EPR 159, *Marinobacter* EPRGB229 and *Parvibaculum* EPR92) were selected to investigate utilization of hydrocarbon substrates of different lengths. Isolates were grown in ASW Minimal Media using octane (C_8H_{18}), dodecane ($C_{12}H_{26}$) or hexadecane ($C_{16}H_{34}$) as sole carbon source. Growth was determined by direct microscope counts of acridine orange-stained cells visualized with an Olympus BX60 microscope with an oil immersion objective lens (Uplan F1 100x 1.3).

Biosurfactant detection

Biosurfactant production was determined in selected isolates from this study and a biosurfactant producing strain (*Pseudomonas* H40) was used as a positive control by a semiquantitative drop-collapse method developed by Bodour and Miller-Maier, (1998). Selected isolates belonging to the genera *Marinobacter*, *Alcanivorax*, *Acinetobacter*, *Parvibaculum*, *Salinisphaera* and *Pseudomonas* were grown in three different media conditions: ASW Minimal media with 10 mM acetate as sole carbon source, ASW Minimal media with dodecane delivered as a vapor, and ASW Minimal Media with dodecane directly in the media. Cultures were harvested after 12 hours incubation at 35ºC; cell counts were done with acridine orange staining on cells visualized with an Olympus BX60 microscope with an oil immersion objective lens (Uplan F1 100x 1.3). Cultures were spun down to obtain the cell free supernatant for both studies.

To further verify production of biosurfactant, bioemulsion assays were performed by a modified version of the method developed by Cooper and Goldenberg, (1987). All strains were grown in ASW Minimal Media with 10 mM acetate as sole carbon source for 12 hours and spun down to obtain cell-free supernatant. Bioemulsion assays were performed with three different hydrocarbons: dodecane ($C_{12}H_{26}$), hexadecane ($C_{16}H_{34}$) and spent motor oil in a 1:1 ratio between the hydrocarbon utilized and the supernatant. The mixture was vortexed for 3 minutes and left at room temperature undisturbed. Measurements (length of emulsion layer) were taken 24h after the initial mixing and an emulsification index was calculated (Cooper and Goldenberg, 1987).

Results

Most Probable Number (MPN) Counts

The Most Probable Number (MPN) test was performed as an initial assessment of the abundance of aerobic alkane-oxidizing bacteria at diffuse-flow vents characterized by different temperature regimes and located on the East Pacific Rise at 9º N. MPN counts showed a broad variability in the percentage of alkane oxidizers between sites (between 0.5 to 11.5% of the total counts; Table 2.1). In fluids with comparable temperatures, the percentage of hydrocarbon-oxidizing bacteria changed considerably. For example, in 15.5°C vent fluids at the Tica vent site, the percentage of alkane oxidizers was 3.76, whereas in 12.5°C fluids from the same site the percentage went down to 0.5 (Table 2.1). MPN counts indicated that in fluids whose in-situ temperature was higher than 40°C aerobic alkane-oxidizing bacteria were undetectable (Table 2.1).

Sample/Temperature (°C)	Dodecane-Oxidizing Bacteria* (cells/ml)	Total Counts (Cells/mL)	% of alkane oxidizers
Mk119/2.5°C	5.40×10^5	1.75×10^7	3.08
Tica/15.5°C	$>1.60 \times 10^6$	4.25×10^7	3.76
Tica/12.5°C	3.50×10^5	7.00×10^7	0.5
Bottom/2°C	3.50×10^7	3.03×10^8	11.55
Mk89/8°C	1.80×10^5	1.75×10^7	1.02
Micro Mat/40-110°C	$\leq 2.0 \times 10^1$	2.41×10^8	<0.0001
Alvinella Pillar/40°C	$\leq 2.0 \times 10^1$	4.88×10^7	<0.0001

Table 2.1: MPN counts on alkane oxidizing bacteria for diffuse flow vents in the East Pacific Rise. Dilutions were done with dodecane as a carbon source

Isolation and characterization of alkane oxidizing bacteria

Enrichment cultures for aerobic alkane-oxidizing bacteria were performed by inoculating fluids, sediments and other sources from the two study sites in medium supplemented with dodecane as sole carbon source. After consecutive isolations, 51 pure cultures were obtained from fluids, sulfides, biofilms and the surfaces of vent invertebrates (Table 2.2) . Figures 2.2 shows the relative abundance of the isolates obtained in this study by taxonomic ranking at the genus level. The taxonomic assignment of each isolate was confirmed by comparative phylogenetic analyses of the 16S rRNA gene (Fig.2.3). Overall, most isolates belonged to the *Gammaproteobacteria*, and strains related to the *Alcanivorax, Acinetobacter* and *Marinobacter* genera were highly represented (30, 15 and 8 of the total number of isolates, respectively; Fig. 2.2). When looking at the comparison between diluted and undiluted isolations, it can be seen that *Alcanivorax* spp. Were mostly isolated from undiluted enrichments and the diluted enrichments generated diverse isolations (Fig 2.2).

Since the MPN data indicated that aerobic hydrocarbon-oxidizing bacteria were not detectable in fluids whose temperature exceeded 40°C, all culture experiments were carried out at 25 - 30°C in ASWMM with dodecane as sole carbon source. Although several isolates were initially isolated in rich media, they were subsequently tested for their ability to grow with dodecane as their sole carbon source (Table 2.2). Most of the frequently isolated strains (*Acinetobacter, Alcanivorax* and *Marinobacter* spp.) were obtained from low dilutions (e.g., 10^{-2}) or undiluted samples, while rare-occurring organisms, such as *Parvibaculum hydrocarbonoclasticum,* were isolated from a higher dilution series (10^{-6}; Table 2.2).

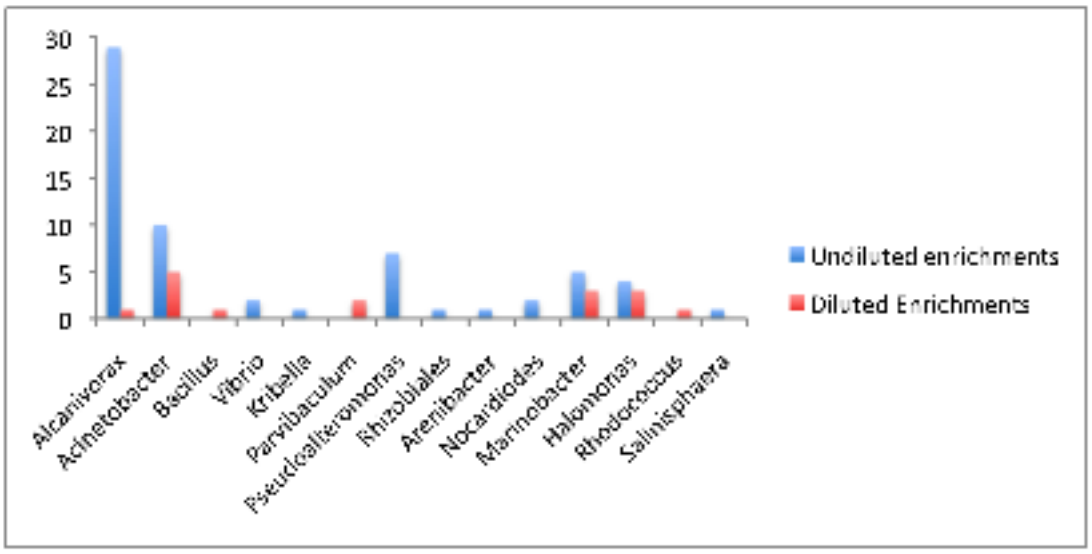

Fig. 2.2: Distribution of isolates obtained by alkane oxidizer enrichment cultures. Blue bars denote isolates done in undiluted enrichments and red bars denote isolations made from diluted enrichments

Isolate	Sample	Isolation Medium [Growth T (°C)]	Growth in ASWMM/Dodecane	Closest Relative
EPR 7	Mk 119 vent Fluid undiluted	ASW FS (37-45)	+	*Alcanivorax*
EPR 70	Mk 119 vent fluid undiluted	142-A (28)	+	*Salinisphaera*
EPR 111	MPN 10^{-2} dilution	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 92	Tica Vent fluid 10^{-6} dilution	ASWMM/Dodecane (28)	+	*Parvibaculum*
EPR 144	Microcolonizer CV9 (10^{-4} dilution)	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 145	Major	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR146	Sulfide	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR147	Microcolonizer CV6	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR148	Major 10^{-2} dilution	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 149,150	Major 10^{-4} dilution	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 159	Basalt	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 160	Major	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 161	Microcolonizer CV9	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 163	*Alvinella* worm	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 169	Major	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 170	Microcolonizer CV3 10^{-2} dilution	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 171	Major 10^{-2} dilution	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 173	Microcolonizer CV9 (from fluids)	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 174	Microcolonizer CV3 (from fluids)	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 175	Major	ASWMM/Dodecane (30)	+	*Acinetobacter*

EPR 176	Sulfides	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 177	Microcolonizer CV5	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 178	*Alvinella* worm	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 179	Sulfides mixed with fluids	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 180	*Alvinella* worm	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 181	Microcolonizer CV5	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPR 182	Major	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPRGB 224	Microbial Mat undiluted	LS ASW	+	*Vibrio*
EPRGB 225	Microbial Mat undiluted	LS ASW	+	*Vibrio*
EPRGB 229	Oiltown Slurp undiluted	ASWMM/Dodecane (30)	+	*Marinobacter*
EPRGB 230	Oiltown Slurp 10^{-1} dilution	LS ASW	-	*Bacillus*
EPR 231	Sulfides and *Alvinella* tubes	ASWMM/Dodecane (30)	+	*Marinobacter*
EPRGB 232	Oiltown slurp sediment undiluted	ASWMM/Dodecane (30)	+	*Marinobacter*
EPR 233	*Tevnia* worm and tube undiluted	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPRGB 234	Major Fluids	ASWMM/Dodecane (30)	+	*Alcanivorax*
EPRGB 235	Sediment Core undiluted	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 236	Microcolonizer biofilm	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 237	*Alvinella* tube	ASWMM/Dodecane (30)	+	*Acinetobacter*
EPR 238	*Alvinella* worm	ASWMM/Dodecane (30)	+	*Acinetobacter*

Table 2.2: List of isolates resulting from alkane oxidizing enrichments. “Major” indicates a diffuse flow sample.

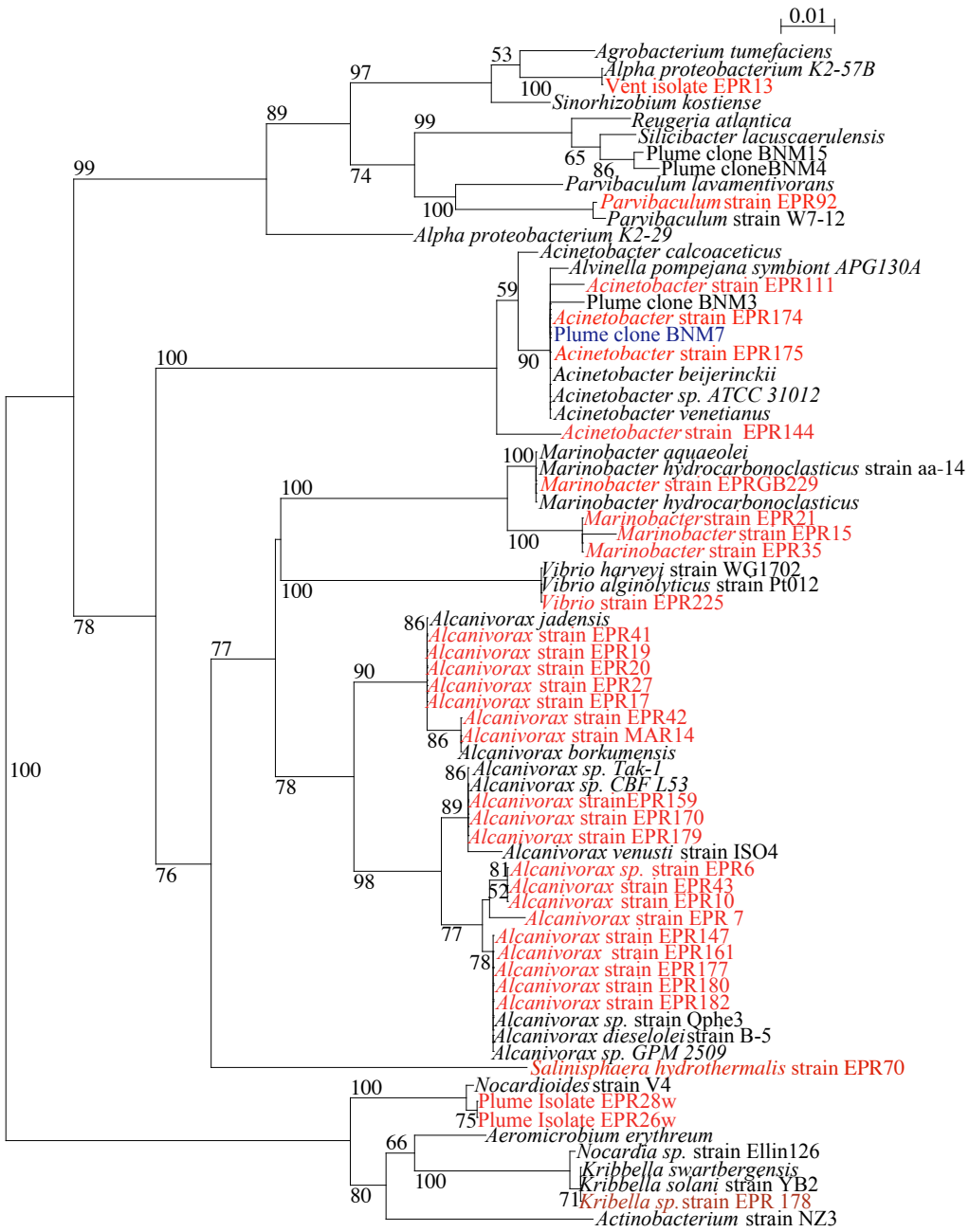

Fig.2.3: Neighbor-joining phylogenetic tree inferred from 16S rRNA gene. Bootstrap values based on 500 replications are shown as percentages at branch nodes. Bar, 0.02% estimated substitutions

Substrate and Hydrocarbon Utilization

Overall, the Biolog assays showed that the organisms tested were diverse in their substrate usage, although some isolates that belong to different phylogenetic groups utilize similar metabolic substrates, such as Krebs cycle intermediates and some glucose polymers (Table 2.3). The ability of these bacteria to utilize a wide range of substrates is a reflection of their diverse metabolic potential. All known hydrocarbonoclastic bacteria (*Alcanivorax* and *Marinobacter*) that we tested utilized both Tween 40 and Tween 80. To investigate substrate range of *n*-alkanes used by these bacteria, six representative isolates from the most frequently isolated genera were selected: two *Acinetobacter* spp., two *Alcanivorax* spp., one *Marinobacter* sp. as well as a unique isolate (*Parvibaculum* sp.). All isolates grew in octane, dodecane and hexadecane, which correspond to the range of medium to long chain hydrocarbons (Table 2.4).

Organism/ Carbon Source	*Pseudoalteromonas* EPR3	*Halomonas* EPR 11	*Rhizobiales* EPR 13	*Arenibacter* EPR14	*Alcanivorax* EPR17	*Alcanivorax* EPR 7	*Marinobacter* EPRGB229	*Parvibaculum hydrocarbonoclasticum*	*Salinisphaera hydrothermalis*
Dextrin	+		+	+					+
Tween 40					+	+	+	+	+
Tween 80					+	+	+		+
D-Cellobiose	+		+	+				+	+
i-Erythriol			+			+		+	+
D-Fructose	+	+						+	+
L-Fucose			+						
a-D-Glucose	+			+				+	+
a-D-Lactose	+		+	+					
Maltose	+		+	+					+
Sucrose	+		+	+					+
D-Trehalose	+		+	+					+
Turanose			+	+					+
Pyruvic Acid		+	+	+					+
Succinic Acid		+	+			+	+	+	+
Acetic Acid	+				+	+	+	+	+
a-Hydroxybutyric Acid					+	+			
b-Hydroxybutiryc Acid						+		+	
a-Ketobutyric Acid	+	+			+	+	+	+	
a-Ketoglutaric Acid			+	+				+	+
Sebacic Acid	+		+	+		+	+	+	
Bromosuccinic Acid					+	+		+	
Succonasmic Acid		+	+			+		+	
L-Alanine	+	+	+						+
L-Alanylglycine	+		+						
L-Aspartic Acod	+								
L-Glutamic Acid	+	+							
Urocanic Acid	+	+		+			+	+	
a-Ketovaleric Acid			+						
L-Proline							+	+	+

Table 2.3: Metabolic profiles of different carbon sources via BIOLOG GN2 MicroPlate^{TM} by diverse alkane oxidizing isolates. + denotes growth while blank denotes that no growth was observed.

Strain/Carbon source	*Acinetobacter* EPR 111	*Acinetobacter* EPR 144	*Alcanivorax* EPR7	*Alcanivorax* EPR159	*Marinobacter* EPRGB229	*Parvibaculum hydrocarbonoclasticum* EPR92
Octane	+	+	+	+	+	+
Dodecane	+	+	+	+	+	+
Hexadecane	N/A	+	N/A	+	+	+

Table 2.4: Hydrocarbon utilization by select alkane oxidizing isolates; + denotes presence of turbidity in media indicating growth. N/A signifies the test was not performed

Biosurfactant detection

Eight isolates were selected as representative isolates for biosufactant detection. Frequently isolated strains (*Acinetobacter* EPR11 and EPR144*, Alcanivorax* EPR7 and EPRGB233, *Alcanivorax* EPR8, and *Marinobacter* EPRGB229) as well as unique strains (*Parvibaculum* EPR92 and *Salinisphaera* EPR70) were selected for this experiment. Two different tests were performed to look at the presence of biosurfactants since the sensitivity of these test varies (Youssef, et al., 2004). The drop collapse test was done as a preliminary test to determine the broad level of biosurfactant production. Subsequently, a bioemulsion assay was performed to measure the presence and emulsifying efficiency of the biosurfactant towards a specific hydrocarbon (Youssef, et al., 2004). Results from the drop collapse study showed that all organisms, produced biosurfactant when grown on acetate (Table 2.5). When comparing biosurfactant production in the presence of dodecane in two different deliveries, *Alcanivorax* EPR 7 and *Alcanivorax* EPR8 produced biosurfactant only when dodecane was supplied in mycellic form. The bioemulsion assay showed that the production of biosurfactants varied in response to the type of hydrocarbon used in the assay. While most bacteria tested showed a higher emulsification

index in the presence of spent motor oil (*Pseudomonas* H40, *Acinetobacter* EPR111, *Alcanivorax* EPRGB233, *Marinobacter* EPRGB229 and *Salinisphaera* EPR70) than in pure *n*-alkanes, some of the isolates did not produce biosurfactants when exposed to hexadecane (e.g., *Acinetobacter* EPR111 and *Alcanivorax* EPRGB233).

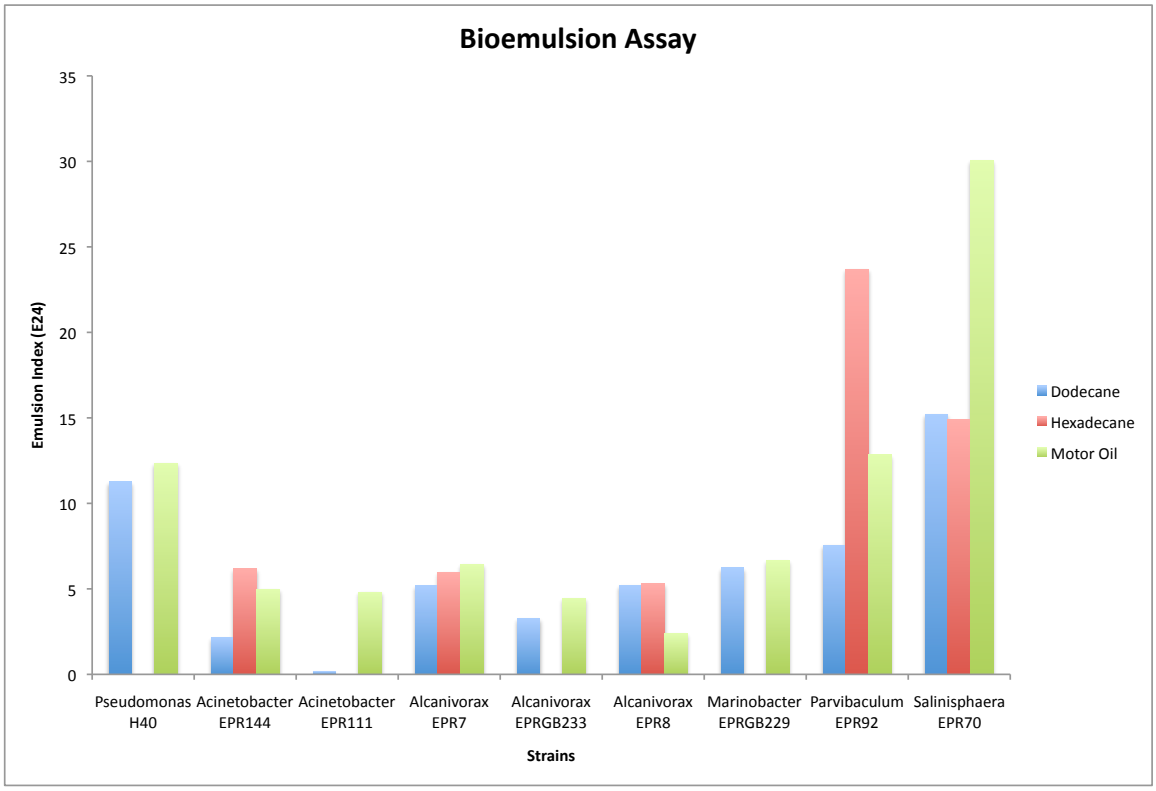

Fig. 2.4: Emulsion Index of representative isolates after 24 h bioemulsion assay with dodecane, hexadecane and spent Motor Oil

Strain/Carbon Source	*Acinetobacter* EPR 144	*Acinetobacter* EPR111	*Alcanivorax* EPR 7	*Alcanivorax* EPR 8	*Marinobacter* EPRGB 229	*Parvibaculum EPR 92*	*Salinisphaera* EPR 70
Acetate	+	+	+	+	+	+	+
Dodecane Mycellic Delivery	-	-	+	+	-	-	-
Dodecane Vapor Delivery	-	-	-	-	-	-	-

Table 2.5: Drop Collapse test performed on various representative isolates on different carbon sources/ delivery

Discussion

The MPN test indicated that the relative abundance of aerobic alkane-oxidizing bacteria in the East Pacific Rise diffuse flows varied in between sites and mainly in response to different temperature regimes (Table 2.1). This can be observed between the Tica vent sample at 15.5°C, where alkane oxidizers accounted for 3.76% of the total population; in contrast, less than 0.01% in the *Alvinella* pillar at 40°C of the population were alkane oxidizers (Table 2.1). An enrichment for aerobic hydrocarbon-oxidizing bacteria at temperature above 45°C was also attempted, without success. Overall, these results suggest that hydrocarbon-oxidizing bacteria from deep-sea hydrothermal vents tend to be mesophilic and are undetectable in fluids exceeding 40°C. This observation must be taken in the context of the specific experimental conditions used in this work, as thermophilic bacteria that can oxidize hydrocarbons anaerobically have been reported beforehand (Kniemeyer, et al., 2007, Rueter, et al., 1994).

Enrichments for alkane oxidizing bacteria in diffuse flow vents in the East Pacific Rise and Guaymas Basin led to the isolation of diverse bacteria (Table 2.2). The majority of our isolates belonged to the *Gamma*- and *Alphaproteobacteria*, and more specifically to the genera *Alcanivorax, Marinobacter* and *Acinetobacter* (Fig.2.2, 2.3). The *Acinetobacter* strains reported in my study are the first isolated from deep-sea hydrothermal vents. In an early study, Durand, et al., (1994) reported the isolation of putative *Acinetobacter* spp. from the Lau Basin vents in southwestern Pacific. In this study, the authors isolated several strains under heterotrophic sulfur-oxidizing conditions and then identified them on the basis of the API20NE metabolic panel. However, no further taxonomic identification of these strains was carried out.

The other common isolates obtained in this study, *Alcanivorax* and *Marinobacter* spp., are well known hydrocarbonoclastic bacteria which are ubiquitous in marine environments (Head, et al., 2006). In addition to the isolation of these organisms, several unconventional alkane oxidizers were isolated, including *Bacillus* sp., *Vibrio* sp. and *Nocardia* sp., a genus that was also reported in other studies from surface seawater and other environments (Harayama, et al., 2004, Head, et al., 2006, Quatrini, et al., 2008, Tapilatu, et al., 2010, W. Wang, et al., 2010). In my experiments *Alcanivorax* spp. was often isolated from undiluted vent fluids. This observation suggests that *Alcanivorax* is a fast-growing bacterium under the experimental conditions that I used and that this bacterium is often present in marine environments where there is oil present (Harayama, et al., 2004, Satpute, et al., 2010). Moreover, the observation that *Alcanivorax* spp. were not isolated from serial dilutions of vent fluids, indicates that, while present, these bacteria are not numerically abundant in this environment. In contrast, the isolation of various organisms belonging to the *Acinetobacter* genera from diluted vent fluids (up to 10^{-4}) suggests that this bacterium is numerically abundant in diffuse flow vents (Fig.2.2, Fig. 2.3, Table 2.2). In a competition study performed by Hara et al. (2003), an *Alcanivorax* strain was grown in a co-culture with an *Acinetobacter venetianus* strain to study the rate of growth and the preference in substrates. The authors found that even though *Acinetobacter* grew more rapidly in the presence of linear *n*-alkanes than *Alcanivorax*, the latter could grow faster and better in the presence of branched alkanes and pristane. The authors concluded that the broad substrate specificity for alkane degradation of *Alcanivorax* spp. allows this bacterium to prevail over *Acinetobacter* spp. (Hara, et al., 2003).

I was also able to isolate *Marinobacter* spp, from diluted fluids (10^{-2}) and *Parvibaculum hydrocarbonoclasticum* (see chapter 3) from a 10^{-6} dilution. The latter was never isolated from undiluted fluids, suggesting that, while numerically abundant, it was outcompeted by organisms with a broader substrate specificity and/or an ability to grow faster, such as *Alcanivorax* spp. These results suggest that both *Marinobacter* spp, and *Parvibaculum* spp. are numerically abundant and possibly key to the degradation of *n*-alkanes in diffuse flow hydrothermal vents and underscore the importance of using alternative cultivation strategies.

When looking at the utilization of different length hydrocarbons we can see that all isolates tested can degrade all three chosen hydrocarbons (Table 2.4). All isolates came from different sites in both East Pacific Rise and Guaymas Basin, and despite that, all of them possess the same degradation potential. van Beilen et. al. suggested that the potential to degrade multiple length hydrocarbons might come from the presence of multiple copies of alkane hydroxylases (AlkB) and different monoxygenases optimized to degrade different chains than AlkB (such as cytochrome P450 and most recently the AlmA protein) (Beilen and Funhoff, 2007, Beilen, et al., 2003, Beilen, et al., 2005, Wentzel, et al., 2007). Having a diverse metabolic versatility may be of importance in deep-sea vents since this is a very active and fluid environment. Conditions and carbon sources rapidly change and metabolic plasticity is a trait that may be advantageous for the survival of hydrocarbonoclastic bacteria.

Biosurfactant detection in our selected isolates provided a view at the dynamic production of these molecules. The drop collapse test results revealed that hydrocarbon delivery in these isolates affects biosurfactant production (Table 2.5). Delivery of the hydrocarbon in a vaporized phase rather than as micelles inhibited the production of biosurfactants. Because the hydrocarbon in its vapor phase diffuses through the media, it might be more bioavailable for utilization. The alkane delivered as a vapor is dispersed and small enough to diffuse through the membrane. Hence, the bacteria are capable of acquiring it without the need to produce biosurfactants (Olivera, et al., 2009). Although our results are not definitive, they provide a foundation for future research investigating biosurfactant production.

This work was performed with assistance from Ronald Wong, Jasmine Ashraf

Chapter 3

Parvibaculum hydrocarbonoclasticum sp. nov., a mesophilic, alkane-oxidizing alphaproteobacterium isolated from a deep-sea hydrothermal vent on the East Pacific Rise.

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Introduction

At deep-sea hydrothermal vents, natural hydrocarbons are largely formed by the thermal decomposition of organic matter (thermogenesis) or by microbial processes (*e.g.,* reduction of fatty acyl-CoA). However, hydrocarbons can have an abiotic origin and may form, in hydrothermal systems, by water-rock interactions, for example involving Fisher-Tropsch reactions and the serpentinization of ultramafic rocks (Berndt, et al., 1996, T. M. McCollom, et al., 1999). Despite the observation that hydrocarbons, including *n*-alkanes, are enriched in deep-sea hydrothermal vents (Brault, et al., 1988b), our knowledge of the taxonomic and functional diversity of alkane-oxidizing bacteria from these environments remains very limited. In this study, we describe a novel aerobic, alkane-oxidizing *alphaproteobacterium* that was isolated from hydrothermal fluids collected at a deep-sea vent on the East Pacific Rise (EPR) at 9º N.

Materials and Methods

Hydrothermal fluids (*in-situ* temperature: 15 °C) were collected from the Tica vent on the EPR at a depth of 2513 m during R/V Atlantis cruise AT 11-10 (April, 2004). The fluids were collected using titanium samplers operated by the manipulator of the Deep-Submergence Vehicle *Alvin*. In the laboratory, 1 ml of fluid was inoculated into 10 ml of Artificial Sea Water Minimal Medium (ASW MM which was composed of (L^{-1}): NaCl, 23.6g; KCl, 0.64g; $MgCl_2$ •$6H_2O$, 4.53g; $MgSO_4$•7 H_2O, 5.94g; $CaCl_2$ •2 H_2O, 1.3g; Na_2HPO_4 •$7H_2O$, 43.0 mg; $NaNO_3$, 0.22 g; NH_4Cl, 0.65g). After autoclaving, ASW MM was supplemented with 1 mM vitamin B12, 1 ml trace-element solution SL-10 and 1 ml mixed vitamin solution 141 (M. Crespo-Medina, et al., 2009) and serially diluted in the same medium to a factor of 10^{-8}. Each tube was then supplemented with dodecane ($C_{12}H_{26}$) in the vapor phase as the only carbon and energy source and it was incubated at 28 °C. Subsequently, a pure culture was isolated from the highest dilution (10^{-6}) that showed growth by successive transfers of single colonies on ASW MM/dodecane solidified with 15g of Noble agar (l^{-1}) (Sigma). Long term stocks of the new isolate were prepared by adding 150µl of sterile Glycerol (Fisher Scientific) to 1 ml culture and were stored at -80 ºC.

Cells were routinely stained in 0.1 % acridine orange and visualized with an Olympus BX 60 microscope with an oil immersion objective (UPlanFl 100/1.3).

Growth rates (µ; h^{-1}) were estimated as: $\mu=(\ln N_2-\ln N_1)/(t_2-t_1)$, were N_2 and N_1 represents the number of cells ml^{-1} at time (in h) t_2 and t_1. Generation times (t_g ; h) were calculated as: $t_g=(\ln 2)/\mu$. To determine the optimal growth temperature, strain EPR92^T was incubated in ASW medium between 20 and 40 ºC (at 5 ºC intervals).

Genomic DNA was extracted from cells collected by centrifugation using the UltraClean™ Microbial DNA isolation kit, according to the manufacturer's instructions (MoBio Laboratories). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR. The 16S rRNA sequences were assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). Sequences were then aligned using ClustalX v 1.8 (Thompson, et al., 1997) and manually adjusted using Seaview (Galtier, et al., 1996). Phylogenetic distances were calculated using the Observance Divergence and the neighbor joining method to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier, et al., 1996) and their robustness was tested by bootstrap analysis with 500 re-samplings (Pérez-Rodríguez, et al., 2010, Vetriani, et al., 2004). To establish the degree of DNA similarity between strain EPR92^T and its closest validly published species, *Parvibaculum indicum*, a DNA-DNA hybridization experiment was carried out. Briefly, cells were disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by (Cashion, et al., 1977). The hybridization was carried out as described by (De Ley, et al., 1970) and (Huss, et al., 1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

The metabolic profiles of strain EPR92^T and that of *Parvibaculum lavamentivorans* were determined using a Biolog assay on a GN2 MicroPlate™ as described previously (M. Crespo-Medina, et al., 2009). Analyses of the cellular fatty acids, polar lipids and respiratory quinones of strain EPR92^T were carried out by the Identification Service of the Deutsche Sammlung für Mikroorganismen und Zellkulturen

(DSMZ), at Braunschweig, Germany, and by Dr Brian Tindall (also of the DSMZ). These analyses were based on a freeze-dried sample (200 mg) of cells that had been grown in ASW medium to early stationary phase, under optimal culture conditions.

Since both strain EPR92^T and *P. lavamentivorans* can use *n*-alkanes as their sole carbon and energy sources, the presence of genes involved in the oxidation of hydrocarbons in the genomes of these organisms was investigated. In particular, the *alkB* and *CYP153* genes were targeted, which encode for the integral membrane nonheme iron alkane hydroxylase (AlkB) and for the cytochrome P450 soluble alkane hydroxylase (of the CYP153 family), respectively. A PCR to amplify a 550 bp fragment of the *alkB* gene from the genomic DNA of strain EPR92^T was attempted utilizing primers designed by (T. H. Smits, et al., 1999) designated as TS2SF and alkdeg1RE with the PCR program as previously described (T. H. M. Smits, et al., 1999), but it did not yield any amplification product. However, a 355 bp fragment of the gene encoding for cytochrome P450 was successfully amplified from the genomic DNA of strain EPR92^T, utilizing the primers designed by van Beilen et.al., 2006 based on the cytochrome p450 of several known hydrocarbonoclastic bacteria designated as p450Fw1 and P450rev3 following the PCR protocol used by them (van Beilen, et al., 2006). The fragment was then sequenced and the deduced amino acid sequences were translated using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq). The amino acid sequences were then aligned with homologous protein sequences obtained from GenBank (including the P450 sequence from *P. lavamentivorans*) using ClustalX v 1.8 (Thompson, et al., 1997) and manually adjusted using Seaview (Galtier, et al., 1996). Phylogenetic distances were calculated using the PAM matrix and the neighbor joining method to evaluate tree topologies.

Phylo_win was utilized to plot tree topologies (Galtier, et al., 1996) and their robustness was tested by bootstrap analysis with 1,000 re-samplings (Pérez-Rodríguez, et al., 2010, Vetriani, et al., 2004).

Results

The pure culture obtained using from these enrichment was designated as strain EPR92^T. EPR92^T cells were Gram-negative rods, approximately 1.4 μm in length and 0.4 μm in width (Fig. 3.1a). A polar flagellum was visible in electron micrographs of platinum-shadowed cells grown in ASW MM/dodecane (Fig. 3.1b). Flagella were not visible in electron micrographs of platinum-shadowed cells grown in ASW medium. The presence of endospores was not observed.

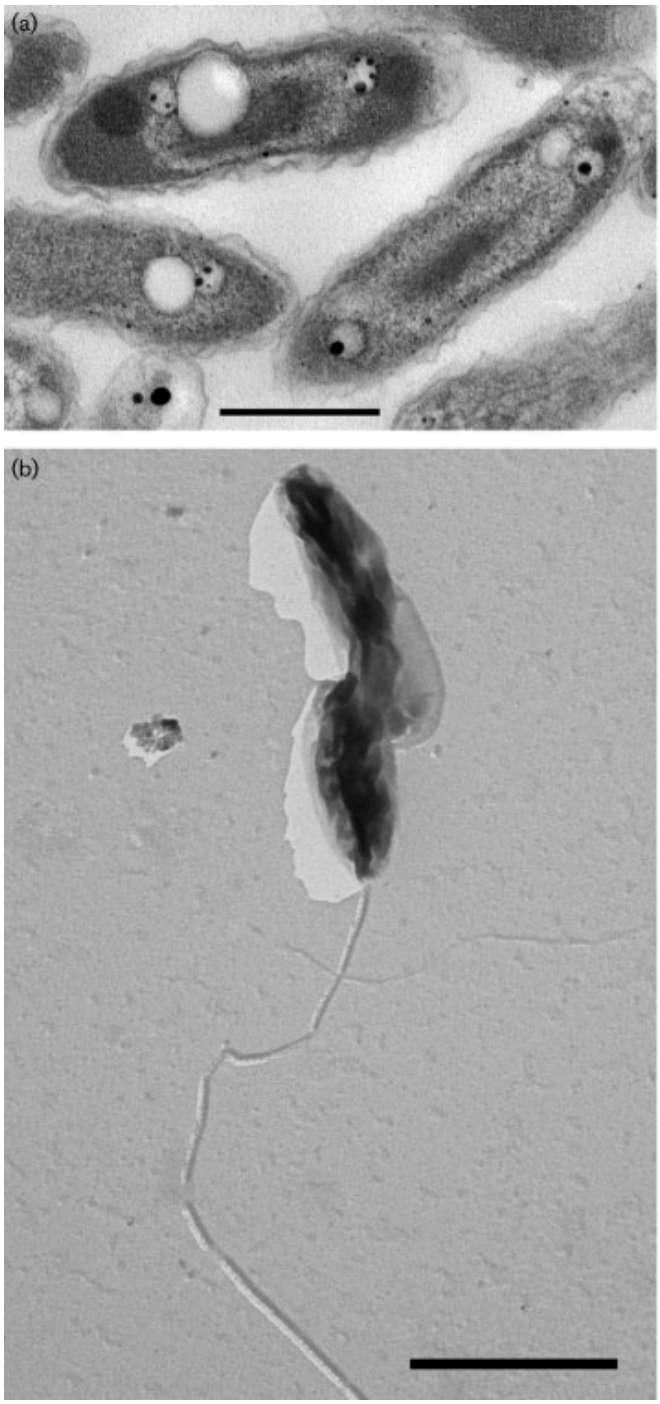

Fig 3.1:Electron micrographs of a thin section of cells of strain EPR92^T (a) and a platinum-shadowed cell of strain EPR92^T grown in dodecane-supplemented ASW minimal medium, showing the presence of a polar flagellum (b). Bars, 0.5 mm (a) and 1.0 mm (b).

Strain EPR92^T was a heterotrophic, aerobic bacterium which grew in Artificial Seawater (ASW) medium and in low-strength ASW (LS ASW) medium supplemented with yeast extract (3.0 and 0.1 g l^{-1}, respectively) and peptone (2.5 and 0.5g l^{-1}, respectively) (M. Crespo-Medina, et al., 2009) and in ASW MM supplemented with octane (C_8H_{18}), dodecane ($C_{12}H_{26}$) or hexadecane ($C_{16}H_{34}$). Growth was not observed in ASW MM supplemented with 46 mg l^{-1} $NaHCO_3$ or in anaerobic ASW medium supplemented with 7.3 mM nitrate. Strain EPR92^T exhibited catalase activity, detected by the formation of gas bubbles after concentrated cells were resuspended in 70 μl of a 3 % solution of H_2O_2 at room temperature. Transmission electron micrographs were obtained as previously described (Vetriani, et al., 2004). The presence of cytochrome C, a component of the cytochrome oxidase system, was detected in strain EPR92^T according to the protocol described in (Kovacs, 1956). The optimal growth temperature of strain EPR92^T was 35 °C (Fig. 3.1a), and all subsequent experiments were carried out in ASW medium at this temperature. Growth of strain EPR92^T occurred at concentration of NaCl between 10 and 50 g 1^{-1}, with an optimum at 25 g 1^{-1} (Fig. 3.1b). The optimal pH for growth was determined as previously described (Voordeckers, et al., 2005). Growth of strain EPR92^T occurred between pH 4.0 and pH 8.5, with an optimum at pH 7.5 (Fig. 3.1c). Under optimal conditions, the generation time of strain EPR92^T was 63 min.

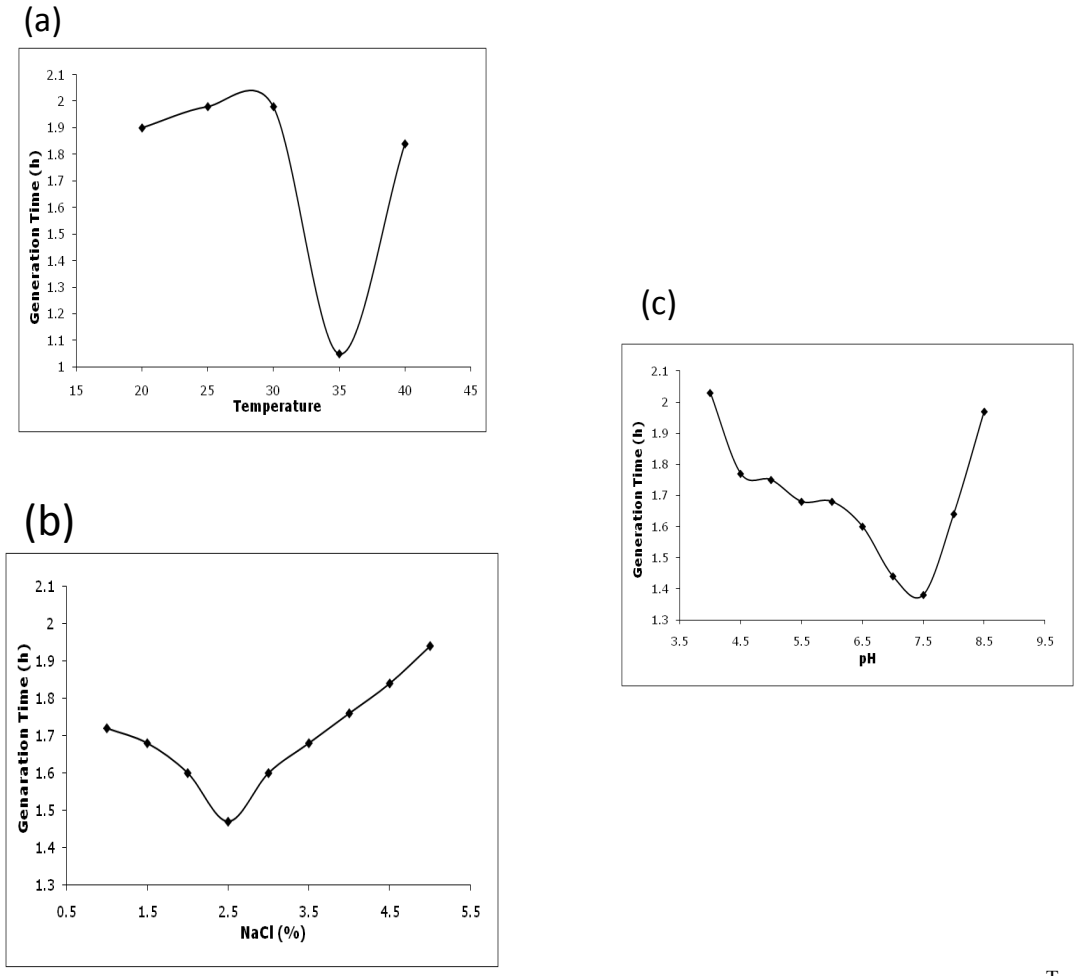

Fig. 3.2. Generation time (in h) of *Parvibaculum hydrocarbonoclasticus* sp. nov. EPR92^T under different growth conditions. (a) Temperature, (b) NaCl concentrations; (c) pH.

Phylogenetic analysis of the 16S rRNA gene sequence placed strain EPR92^T within the class '*Alphaproteobacteria*' (Fig. 3.3). Strain EPR92^T was related to four strain of the recently described genus *Parvibaculum*, of which *Parvibaculum lavamentivorans* DSM 13023^T and *Parvibaculum indicum* are the only validly published species (95 and 98 % sequence identity to strain EPR92^T, respectively) (Lai, et al., 2011, Schleheck, et al., 2007). *Parvibaculum indicum*, along with *Parvibaculum* sp. strain

psc10 (99 % sequence identity to strain EPR92^T) and strain W7-12 (98 % sequence identity) (Wang, et al., 2008), were all isolated from marine environments (Fig. 3.3). The G+C content of the genomic DNA of the strain EPR92^T, determined by HPLC analysis of the deoxyribonucleosides (Mesbah, et al., 1989), was 60.7 mol%, while that of *P. lavamentivorans* and *P. indicum* were 64.5 and 62.1 mol%, respectively (Lai, et al., 2011, Schleheck, et al., 2007). The results showed that the DNA-DNA similarity between strain EPR92^T and *P. indicum* was 47.7 %, indicating that the two strains do not belong to the same species. Table 3.1 shows a comparative analysis of the metabolic profiles of the two organisms. Furthermore, comparative growth analyses between EPR92^T and *P. lavamentivorans* showed that both bacteria grew in ASW, LS ASW, and ASW MM supplemented with octane, dodecane or hexadecane.

Comparative phylogenetic analysis placed the P450 enzymes from strain EPR92^T and *P. lavamentivorans* (73 % amino acid identity) in a discrete cluster related to the P450 enzyme of *Polaromonas* sp. strain JS666 (71 %amino acid identity), a *Betaproteobacterium* that can biodegrade petroleum hydrocarbons and chlorinated ethenes (Mattes, et al., 2008) (Fig. 3.4).

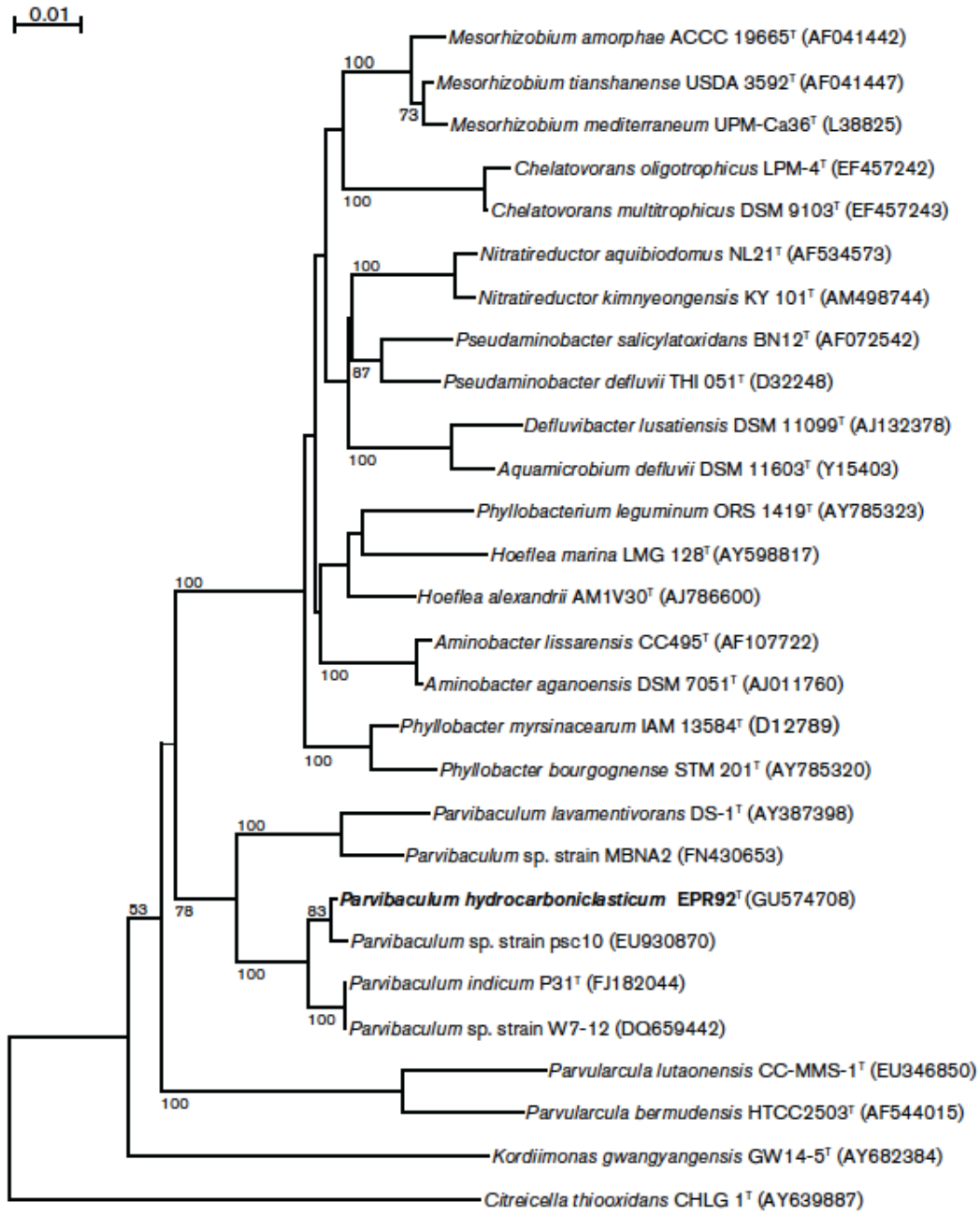

Fig.3.3: A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain EPR92^T and related taxa. Percentage bootstrap values > 50% (based on 1000 replications) are shown at branch points. Bar, 0.01 substitution per nucleotide position.

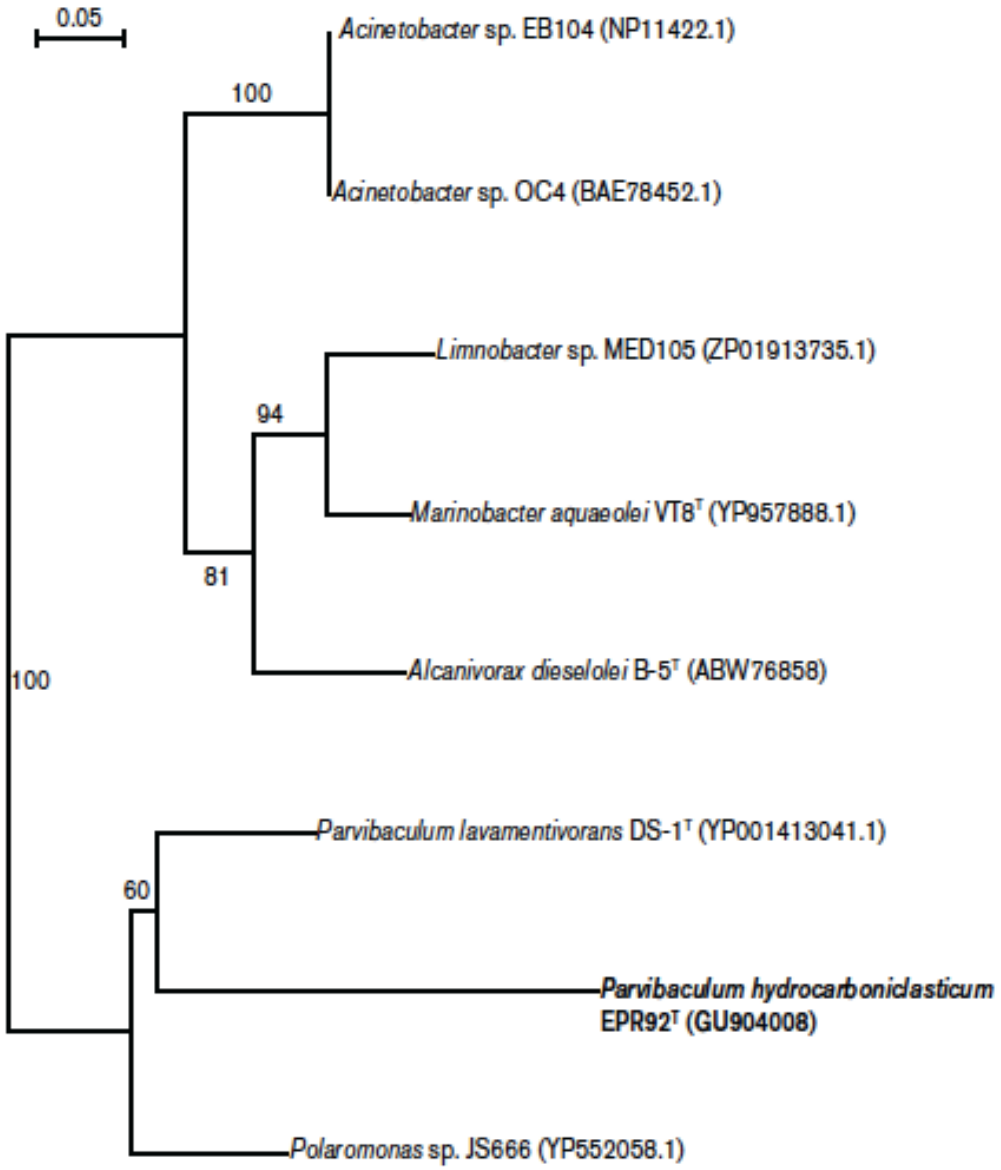

Fig 3.4: A neighbour-joining phylogenetic tree based on amino acid sequences for cytochrome P450 alkane hydroxylase, showing the relationships between strain EPR92^T and related taxa. Percentage bootstrap values > 50% (based on 1000 replications) are shown at branch points. Bar, 0.05 substitutions per site.

The major cellular fatty acids of strain EPR92^T were analyzed, as the methyl ester derivatives, using the Sherlock Microbial Identification System (MIDI) and a 6890N GC (Agilent)(Kuykendall, 1988). The fatty acid profile of the novel strain was similar to those of *P. indicum* and *P. lavamentivorans* (Lai, 2011, Schleheck, 2004) with C18 : 1v7c (48.71 %), C19 : 0 cyclo v8c (17.89%) and 11-methyl C18 : 1v7c (7.93 %) predominating (Table 3.3). The predominant polar lipids of strain EPR92^T, identified by TLC (Tindall, 1990a, Tindall, 1990b), were similar to those identified in *P. lavamentivorans* (Schleheck, 2004), and included phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine as well as two unidentified aminolipids (AL2 and AL3) that migrated between phosphatidylglycerol and phosphatidylcholine on the TLC plates (Fig. 3.5). Two additional aminolipids, one (AL1) detected between phosphatidylethanolamine and phosphatidylglycerol and the other (AL4) below phosphatidylcholine, were detected in strain EPR92^T (Fig. 3.5) but not in *P. lavamentivorans* (Schleheck, 2004). Analysis of the respiratory lipoquinones of strain EPR92^T, by TLC and then HPLC of the eluted products(Tindall, 1990a, Tindall, 1990b) ,indicated that only Q-10 was present. In contrast, *P. lavamentivorans* and *P. indicum* were each found to have Q-11 as their sole respiratory quinone. Table 3.2 summarizes the features that differentiate strain EPR92^T from the type strains of *P. indicum* and *P. lavamentivorans.*

Physiological, phylogenetic and genetic analyses indicated that strain EPR92^T is not related to *P. lavamentivorans* at the species level, and therefore strain EPR92^T represents a novel species within the genus *Parvibaculum*, for which we propose the name *Parvibaculum hydrocarbonoclasticum*.

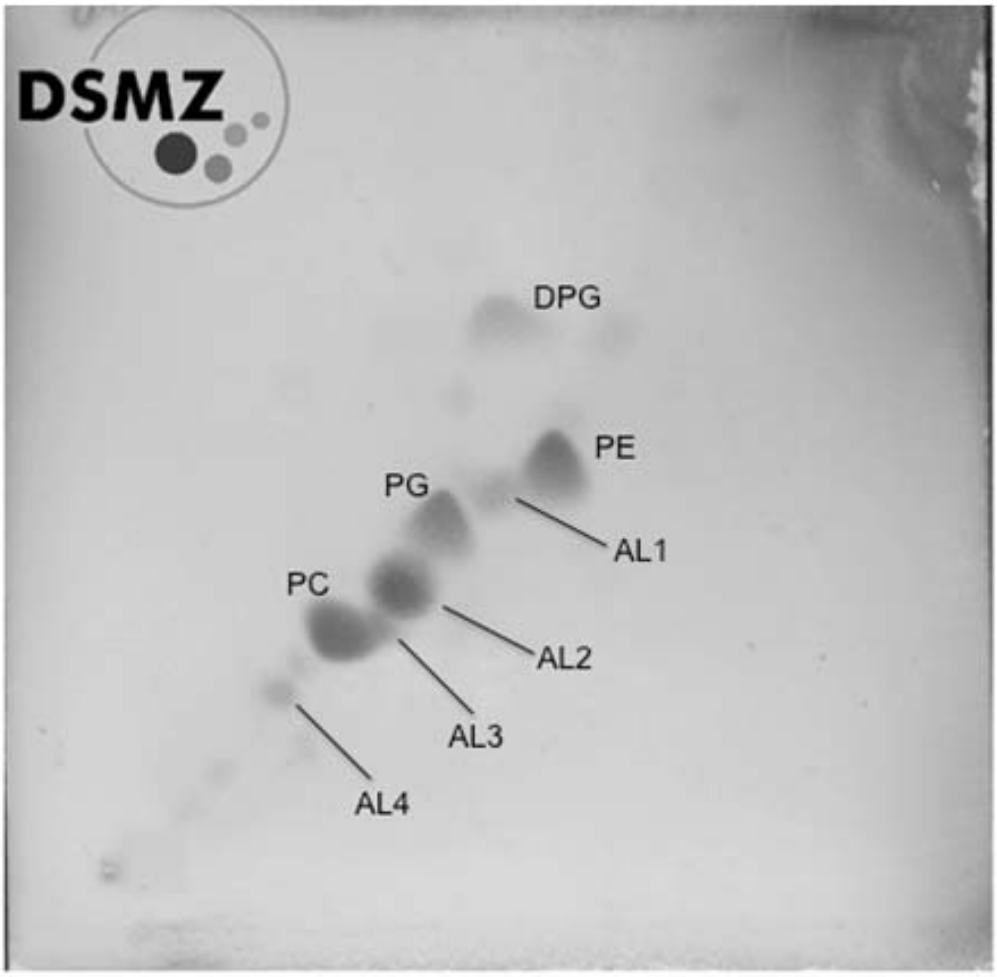

Fig 3.5: Two-dimensional thin layer chromatogram of a polar lipid extract from of strain EPR92^{T}. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; AL1–AL4, unidentified aminolipids

Carbon Source	1	2	3
Glycogen	+	-	-
Tween 40	+	+	-
D-Arabitol	+	-	-
D-Cellobiose	+	-	-
i-Erythritol	+	-	-
m-Inositol	-	-	+
D-Mannitol	+	-	-
D-Mannose	+	-	-
β-Methyl-D-Glucoside	+	+	-
Citric Acid	-	-	+
D-Galacturonic Acid	-	-	+
D-Gluconic Acid	-	-	+
α-Hydroxybutyric Acid	+	-	-
β-Hydroxybutyric Acid	+	+	-
γ-Hydroxybutyric Acid	+	+	-
Itaconic Acid	-	-	+
α-Ketovaleric Acid	+	-	-
Propionic Acid	+	-	-
Bromosuccinic Acid	+	-	-
L-Alaninamide	-	-	+
L-Glutamic Acid	+	-	-
Hydroxy-L-Proline	-	-	+
γ-Aminobutyric Acid	+	-	-
Uridine	-	-	+
Glycerol	+	+	-
Cis-Acotinic Acid	+	-	+
D,L- Lactic Acid	+	-	+
Succinic Acid Mono –Methyl Ester	+	-	+
γ-Aminobutyric Acid	+	-	+
α-Ketoglutaric Acid	+	-	+
Sebacic Acid	+	-	+
D,L,α-Glycerol Phosphate	+	+	-

Strains: 1, EPR92^T; 2, *P. indicum* P-31^T; 3, *P. lavamentivorans* DS-1^T. All data from this study.

+, Positive (source utilized); 2, negative (source not utilized)

Table 3.1: Utilization of carbohydrates, as sole carbon sources, by strain EPR92^T and type strains of the two most closely related species in the genus *Parvibaculum*

Characteristic	1	2	3
Oxidase	+	-	+
Respiratory quinones	Q_{10}	Q_{11}	Q_{11}
Principal fatty acids	$C_{18:1\omega7c}$ $C_{19:0}$ cyclo $_{\omega8c}$ 11-methyl $C_{18:1\omega7c}$ putative aldehyde $C_{12:0}$	$C_{18:1\omega7c}$ $C_{18:0}$ $C_{19:0}$ cyclo $_{\omega8c}$ 11-methyl $C_{18:1w7c}$	$C_{18:1\omega7c}$ $C_{18:0}$ $C_{19:0}$ cyclo $_{\omega8c}$ 2-OH $C_{19:0}$ cyclo
16S rRNA gene identity (%)	100	98.7	95.8
DNA-DNA similarity (%)	100	47.7	ND
DNA G+C Content (mol%)	60.7	62.1	64

Strains: 1, EPR92^T (data from this study); 2*, P. indicum* P-31^T (Lai et al., 2011); 3, *P. lavamentivorans* DS-1^T (Schleheck et al., 2004).+, Positive; 2, negative; ND, no data available.

Table 3.2: Features that differentiate strain EPR92^T from the two most closely related species in the genus *Parvibaculum*.

Fatty acid	
$C_{13:1}$	0.13
$C_{14:0}$	0.16
Iso $C_{15:0}$	0.11
Anteiso $C_{15:0}$	0.41
$C_{16:0}$	2.46
3-OH $C_{15:0}$	0.32
$C_{17:1\omega6c}$	0.13
$C_{17:0}$	0.54
2-OH $C_{16:0}$	0.13
3-OH $C_{16:0}$	2.25
$C_{18:1\omega9c}$	0.73
$C_{18:1\omega5c}$	0.27
$C_{18:0}$	3.32
11-methyl $C_{18:1\omega7c}$	7.93
3-OH $C_{17:0}$	0.40
Cyclo $C_{19:0\omega8c}$	17.89
2-OH $C_{18:1}$	1.20
10-methyl $C_{19:0}$	0.51
3-OH $C_{18:0}$	2.19
$C_{20:1\omega7c}$	0.20
$C_{20:0}$	1.29
Summed Feature 1 (Iso H $C_{15:1}$/3-OH $C_{13:0}$)	0.16
Summed Feature 2 (Aldehyde $C_{12:0}$/Unknown)	7.35
Summed Feature 3 ($C_{16:1\omega7c}$/$C_{16:1\omega6c}$)	1.22
Summed Feature 8* ($C_{18:1\omega7c}$/$C_{18:1\omega6c}$)	48.71

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 contained iso-C15: 1 H and/or C13:0 3-OH; summed feature 2 contained C12:0 aldehyde and/or an unknown fatty acid; summed feature 3 contained C16: 1ω7c and/or C16: 1ω6c; summed feature 8 contained C18: 1ω7c and/orC18: 1ω6c.

Table 3.3: Cellular fatty acid content (%) of strain EPR92^T

Description of *Parvibaculum hydrocarboniclasticum* sp. nov.

Parvibaculum hydrocarboniclasticum [hy.dro.car.bo.ni.clasti. -cum. N.L. n. hydrocarbonum hydrocarbon; N.L. adj. clasticus -a -um (from Gr. adj. klastos -eˆ -on broken in pieces) breaking; N.L. neut. adj. hydrocarboniclasticum hydrocarbonoclastic, breaking hydrocarbon]. Cells are aerobic, Gram-staining-negative rods (1.460.4 mm). A polar flagellum was observed on cells grown in ASW minimal medium supplemented with dodecane. Catalase and oxidase-positive. Growth occurs at 20–40 °C (optimum 35 °C), with 1.0–5.0% (w/v) NaCl (optimum 2.5%), and at pH 4.0–8.5 (optimum pH 7.5). Aerobic heterotrophic growth occurs with *n*-alkanes as sole carbon and energy sources, and in complex ASW medium. Nitrate is not used as an electron acceptor. The predominant fatty acids are C18: 1v7c, C19: 0 cyclo v8c and 11-methyl C18: 1v7c. The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and four unidentified aminolipids. Q-10 is the major respiratory quinone. The type strain, EPR92^{T} (5DSM 23209T5JCM 16666 T), was isolated from hydrothermal vent fluids collected from the East Pacific Rise (at 9° 509 N 104° 179 W). The genomic DNA G+C content of the type strain, as determined by HPLC, is 60.7 mol%.

Chapter 4

Detection and diversity of alkane hydroxylases in hydrocarbonoclastic bacteria from deep-sea hydrothermal vents

Introduction

Ocean oil pollution has become a topic of debate over the past years. The latest of an unfortunate series of oil spills occurred in April of 2010 when, after the explosion of the Deepwater Horizon drilling rig, an estimated 4,9 billion barrel of oil (equivalent to 2.1 x 10^8 US gallons) were released in the Gulf of Mexico from a depth of about 1,500 m, causing extensive contamination of the region. As a consequence of events like these, there has been an increasing interest in the application of bioremediation to enhance oil degradation and thereby mitigate the ecological damage done by these spills (McKew, et al., 2007). McKew, et al., (2007) posed a very important question by observing that, though the bacteria responsible for degrading petroleum were present, it was not until the addition of a rhamnolipid that degradation was increased. Rhamnolipids are glycolipid biosurfactants produced by *Pseudomonas aeruginosa* that is the best-characterized bacterial surfactant to date (Desai and Banat, 1997). As stated in chapter 2, biosurfactants aid the bacteria in the sequestration of hydrocarbons and facilitate access into the bacterial cell.

A general approach to assess the natural distribution of hydrocarbonoclastic bacteria in the environment - and their potential for the degradation of petroleum hydrocarbons - is to use the genes that encode for the alkane hydroxylases as diagnostic markers. A study done by Paisse et al (2011), however, suggested that the use of some alkane hydroxylase-encoding genes (in this case *alkB*) as markers to assess the potential

for biodegradation was not very effective. The author mentioned the need for broad and specific *alkB* probes, which results in costly and time intensive work as well as the disappearance of signal early on even though degradation continues. Furthermore, since such diagnostic genes can be detected also in pristine environments, the authors concluded that the approach was not specific for contaminated sites.

Hence, the mechanism of alkane oxidation in bacteria must be studied and understood as well as the potential for oil degradation. Bacterial alkane oxidation has been extensively studied in pure cultures and in pelagic seawater communities both aerobically and anaerobically in relation to the oxidation of a range of *n*-alkanes (Beilen, et al., 2003, Harayama, et al., 2004, Heiss-Blanquet, et al., 2005, Luz, et al., 2004, Quatrini, et al., 2008). Here, we are focusing on aerobic processes and more specifically in the degradation of medium chain *n*-alkanes. By having a working knowledge of all enzymes involved in alkane oxidation, it may be possible to design a multi-marker approach that can be utilized as a way to monitor biodegradation in oil polluted environments.

The key enzymes involved in aerobic *n*-alkane mechanism are: the alkane hydroxylase known as *alkB*, *cyp153* that encodes for cytochrome P450 (degrades hydrocarbons that range C_5-C_{11}) and *almA* which is involved in the degradation of long chain hydrocarbons. All of these genes function as monooxygenases, in which they supply the hydrocarbon molecule with oxygen that creates a reactive oxygen species (Rojo, 2009). The AlkB enzymes are a known class of particulate alkane hydroxylases (pAHs). Several surveys have been carried out in different environments, the most common being in surface seawater. For instance, W. Wang, et al. (2010) found a large recurrence of *alkB* gene from a diverse group of bacteria, such as *Salinisphaera* spp. and *Alcanivorax* spp., among others, most of which belonged to the *Gammaproteobacteria*.

The *cyp153* gene encodes for a soluble heme-containing enzyme called the cytochrome P450. This enzyme is present in all organisms in the tree of life mainly as one that catalyzes the oxidation of organic substances from lipids, drugs and other toxic chemicals. P450 is the major enzyme involved in drug metabolism and bioactivation in most organisms (Meunier, et al., 2004). In some bacteria however, its function is to degrade mid to long chain *n*-alkanes (Funhoff, et al., 2006). The specific type of P450 enzyme has been found in an array of different bacteria, mostly belonging to *Proteobacteria* such as *Parvibaculum* spp., *Bulkholderia* spp. and *Marinobacter* spp (L. Wang, et al., 2010).

The flavin binding monooxygenase, encoded by the *almA* gene, is involved in the degradation of long chain hydrocarbons (Wentzel, et al., 2007). In a recent environmental survey, multiple and diverse genes ranging from well known alkane-oxidizing bacteria,

such as *Acinetobacter* spp., to more recently characterized ones, such as *Salinisphaera* spp. were detected in the water column at various depths and temperatures in the South China Sea, Indian Ocean, Pacific Ocean and several sites in the Atlantic Ocean (Wang and Shao, 2012). Wang et. al. (2010) and Beilen, et al., 2003, observed that many of these bacteria have multiple homologs of the *alkB*, *cyp153* and *almA* genes, which in some cases co-occur in the same genome. It was also observed that the bacteria that encoded for different alkane hydroxylases could degrade a broader range of alkane substrates (Beilen and Funhoff, 2007, L. Wang, et al., 2010). A study that investigated the reaction mechanisms of AlkB and P450 in alkane hydroxylases co-occurring in the *Gammaproteobacterium*, *Hydrocarboniphaga effuse,* using the diagnostic substrate norcarane, revealed that the two enzymes are mechanistically distinguishable and that this type of assays can identify previously unidentified active hydroxylases (Rozhkova-Novosad, et al., 2007).

To understand the distribution of alkane hydroxylases in hydrocarbonoclastic bacteria from deep-sea hydrothermal vents, we surveyed the presence of *alkB*, *cyp153* and *almA* genes in pure cultures and natural microbial communities from the East Pacific Rise and Guaymas Basin.

Materials and Methods

Alkane hydroxylase (*alkB),* cytochrome P450 (*CYP153)* and flavin binding monooxygenase (*almA)* detection in pure cultures

Genomic DNA extracted from the pure cultures isolated in the laboratory as described in chapter 2 were screened for both *alkB, cyp153,* and *almA* using the primers shown in Table 4.1.The protocol to amplify the *alkB* was as follows: 5 minutes at 94°C; 30 cycles of 45 seconds at 30°C, 1 minute at 40°C, and 1 minute at 72°C with a final extension of 7 minutes at 72°C. The protocol to amplify for the *cyp153* gene was: 4 minutes at 95°C; 25 cycles of 45 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C ending with a final extension of 5 minutes at 72°C. For the amplification of the *almA* gene the following protocol was used: initial denaturation for 5 minutes at 94°C, 30 cycles with 30 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C, followed by a final elongation step for 10 minutes at 72°C.

PCR amplicons of the right size were purified using the EXOSAP-IT enzyme (Affymetrix) and sequenced (Table 4.1). The *alkB, cyp153* and *almA* sequences were translated into the amino acid sequence using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/) and assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). Sequences were then aligned using ClustalX v 1.8 (Thompson, 1997) and manually adjusted using Seaview (Galtier, et al., 1996). Phylogenetic distances were calculated using the PAM matrix and the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier, 1996) and their robustness was tested by bootstrap analysis with 500 re-samplings.

Primers used	Sequence	Expected fragment size (bp)
TS2SF (*alkB)(T. H. Smits, et al., 1999)*	5'-AAYAGAGCTCAYGARYTRGGTCAYAAG-'3	550
alkdeg1RE (*alkB)(T. H. Smits, et al., 1999)*	3'-GTRAGICTRGTRGTRCGCTTAAGGTG-'5	550
p450fw1 (*cyp153)(Beilen, et al., 2006)*	5'-GTSGGCGGCAACGACACSAC -3'	330
p450rev3(*cyp153)(Beilen, et al., 2006)*	5'-GCASCGGTGGATGGCGAAGCCRAA-3'	330
AlmAdf (*almA)(Wang and Shao, 2012)*	5'GGNGGNACNTGGGAYCTNTT-3'	1100
AlmAdR (*almA)(Wang and Shao, 2012)*	5'-ATRTCNGCYTTNAGNGTCC-3'	1100

Table 4.1: PCR primers used in this study to amplify the *alkB, cyp153* and *almA* genes.

Alkane hydroxylase (*alkB)* and cytochrome P450 (*CYP153)* detection in natural microbial assemblages

For environmental screening, genomic DNA was extracted from biomass collected from vent sites with the MoBio Microbial DNA Extraction Kit (MoBio Laboratories) following the manufacturers' instructions and the *alkB* and *CYP153* genes were amplified by PCR. PCR products were cloned into pCR4-TOPO plasmid vectors, and the ligation products were transformed into competent *E. coli* Oneshot cells (Invitrogen, Inc., Carlsbad, CA, USA). Clones were screened for correct insertion and restriction fragment length polymorphism (RFLP) was performed to identify unique restriction patterns. Enzymatic digestion were performed with restriction endonucleases HaeIII (Promega)

and MspI (Promega), incubated for 2 hours at 37°C and run on a 3% metaphor agarose gel for 1.5 h at 75V at 4°C. Unique restriction patterns were visualized under UV light. Plasmid extraction was performed with QIAPrep® Spin Miniprep Kit (QIAGEN) and plasmids were submitted to sequencing. Sequences were translated into the amino acid sequence using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/) and assembled using the AutoAssembler Program (Applied Biosystems, Foster City, CA). Sequences were then aligned using ClustalX v 1.8 (Thompson, et al., 1997) and manually adjusted using Seaview (Galtier, et al., 1996). Phylogenetic distances were calculated using the neighbor joining method was used to evaluate tree topologies. Phylo_win was utilized to plot tree topologies (Galtier, et al., 1996) and their robustness was tested by bootstrap analysis with 500 re-samplings. (Melitza Crespo-Medina, et al., 2009).

Detection of transcripts of the *alkB* (alkane hydroxylase), *cyp153* (cytochrome P450) and *almA* (flavin binding monoxygenase) genes

Representative isolates (*Acinetobacter* EPR111, *Alcanivorax* EPR7, *Acinetobacter* EPR144) were selected to investigate the expression of the alkane hydroxylase, cytochrome P450 and flavin binding monooxygenase when the organisms were grown with or without *n*-alkanes of different chain lengths (dodecane, hexadecane or octane). Cultures were grown to mid-exponential phase in Artificial Seawater (ASW) medium supplemented with yeast extract and peptone; in ASW minimal medium supplemented with acetate and in ASW minimal medium supplemented with octane (C_8H_{18}), dodecane ($C_{16}H_{34}$) and hexadecane ($C_{16}H_{34}$) as a sole carbon source in vapor phase. RNA was extracted utilizing the RNeasy Mini Kit (QIAGEN) following the manufacturers' instructions. RNA samples were subsequently treated with Turbo DNAfree (Ambion) following the manufacturers protocol. cDNA was generated for these samples using the SuperScript™ III One-Step RTPCR System with Platinum® *Taq* DNA Polymerase (Invitrogen) with the appropriate primers for *alkB, cyp153* and *almA* respectively (Table 4.1) (Funhoff, et al., 2006, T. H. Smits, et al., 1999).

Results

Detection of *alkB, cyp153* and *almA* genes in isolates and natural microbial communities

To investigate the distribution of the *alkB* and *cyp153* genes in our collection of aerobic alkane-oxidizing isolates, I carried out a systematic screening of these genes by PCR amplification (Fig.4.1 and Fig.4.2). The majority of the *Gammaproteobacteria* in our collection carried the *alkB* gene. Phylogenetic analysis of the amino acid sequence deduced from the *alkB* gene showed that the sequences amplified from several *Marinobacter* strains (*Marinobacter* spp. strain EPR21 and MAR16) were closely related to the AlkB from *Alcanivorax* spp. (Red clones in Fig. 4.1) indicating the occurrence of horizontal gene transfer.

The *alkB* gene was also amplified from genomic DNA extracted directly from natural microbial assemblages sampled from hydrothermal fluids whose temperature ranged between 25 and 40°C (EW 25°C, AP 40°C and Mk 28°C sites). Phylogenetic analysis of the amino acid sequence deduced from the *alkB* gene from natural microbial communities showed a prevalence of *Marinobacter* and *Alcanivorax*-related sequences in the 25 – 28°C fluids, while *Acinetobacter*-related sequenced were detected in the 40°C fluids (Blue clones in Fig. 4.1).

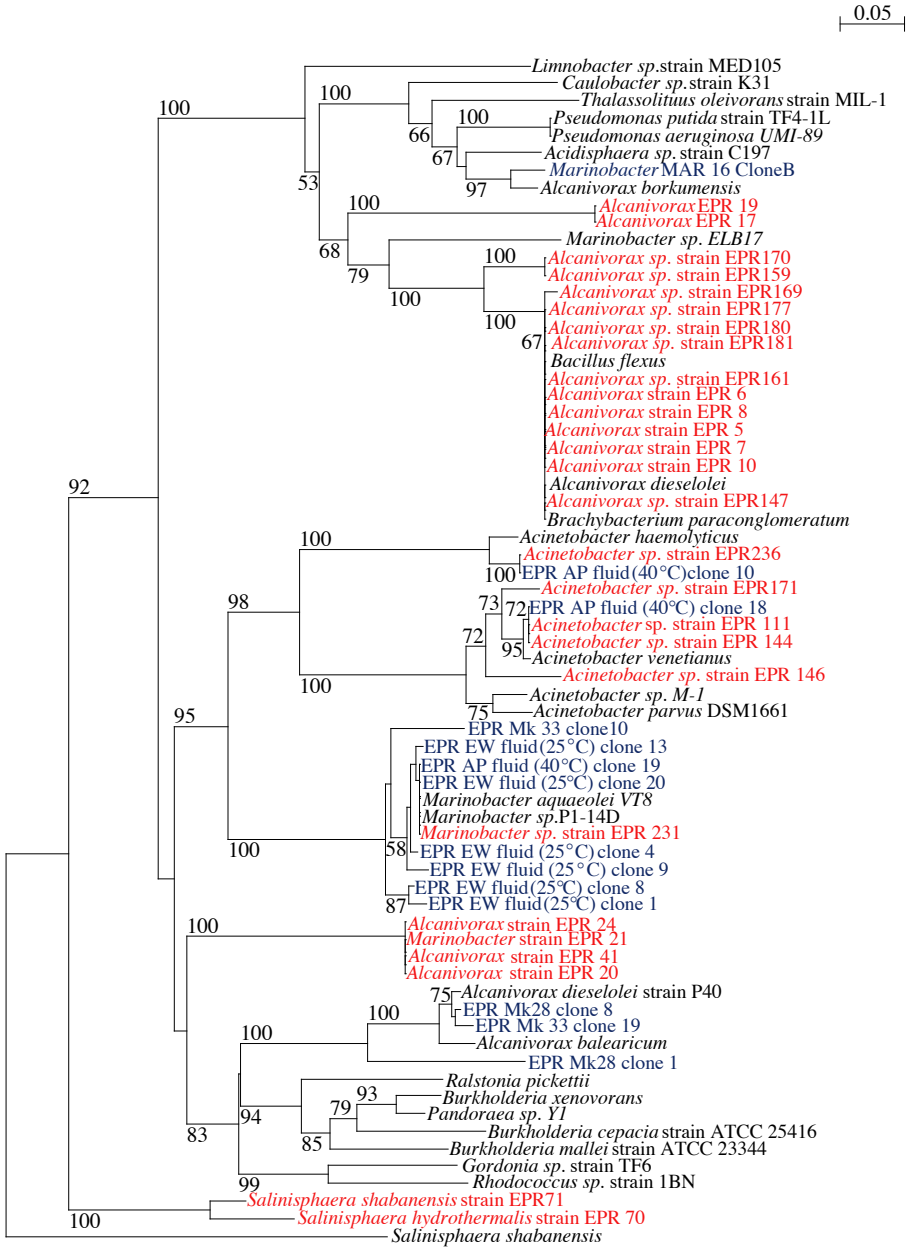

Fig. 4.1: Neighbor-joining phylogenetic tree inferred from the amino acid sequences deduced from a fragment of the *alkB* gene (encoding alkane hydroxylase AlkB protein). EW: East Wall site; AP: *Alvinella* Pillar site: Mk 28: Marker 28 site; Mk 33: Marker 33 site. Red color denotes pure cultures and blue signifies an environmental sample. Bootstrap values based on 500 replications are shown as percentages at branch nodes. Bar, 0.1% estimated substitutions.

The *cyp153* gene was amplified from various *Alcanivorax* strains, *Acinetobacter* sp. strain EPR 144 and *Parvibaculum hydrocarbonoclasticum* strain EPR 92 (red sequences in Fig. 4.2). Some of the isolates that encoded for the *cyp153* gene did not have the *alkB* gene (*Parvibaculum hydrocarbonoclasticum* EPR92 and *Alcanivorax* sp. EPR182). No amplification of the *cyp153* gene was obtained from genomic DNA extracted from the natural microbial communities.

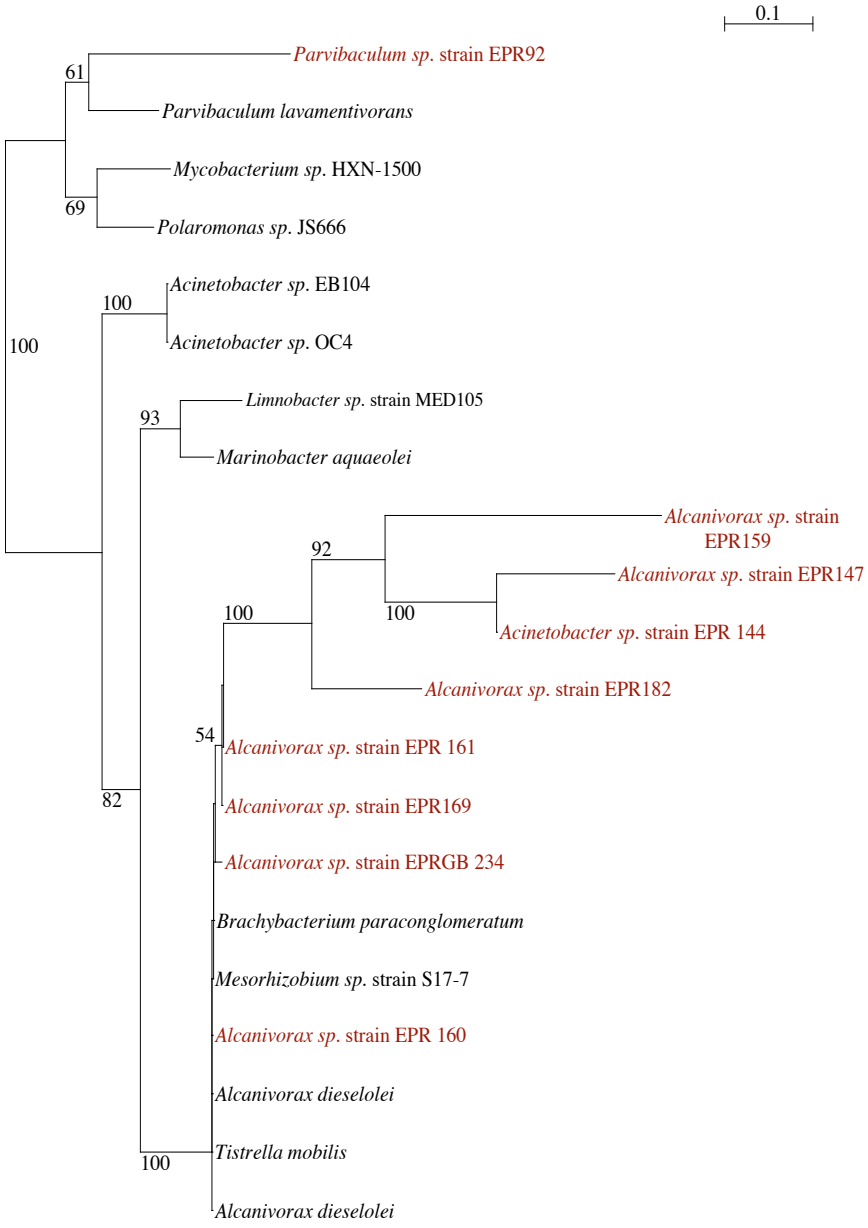

Fig 4.2: Neighbor-joining phylogenetic tree inferred from the amino acid sequences deduced from a fragment of the *cyp153* gene (encoding the cytochrome P450). Bootstrap values based on 500 replications are shown as percentages at branch nodes. Bar, 0.1% estimated substitutions.

Isolates representative of each of the three genera, *Acinetobacter*, *Alcanivorax* and *Marinobacter*, along with *Parvibaculum hydrocarbonoclasticum* strain EPR92 and *Salinisphaera hydrothermalis* strain EPR70 were selected and screened for the presence of the *almA* gene, encoding for the flavin binding monoxygenase. Phylogenetic analysis of the flavin binding monoxygenase from isolates showed that the AlmA protein detected in *Acinetobacter* sp. EPR144 is closely related to the enzyme found in *Alcanivorax venustensis* (Fig. 4.3). I was also able to detect the *almA* in *Alcanivorax* sp. EPR 7 and in *Marinobacter* sp. EPRGB229. A comparison of the phylogenetic trees for the three enzymes revealed that: 1) *Acinetobacter* sp. EPR144 encodes for all three enzymes; and 2) the CYP153 and AlmA enzymes from *Acinetobacter* sp. EPR144 are most closely related to proteins from *Alcanivorax* spp.

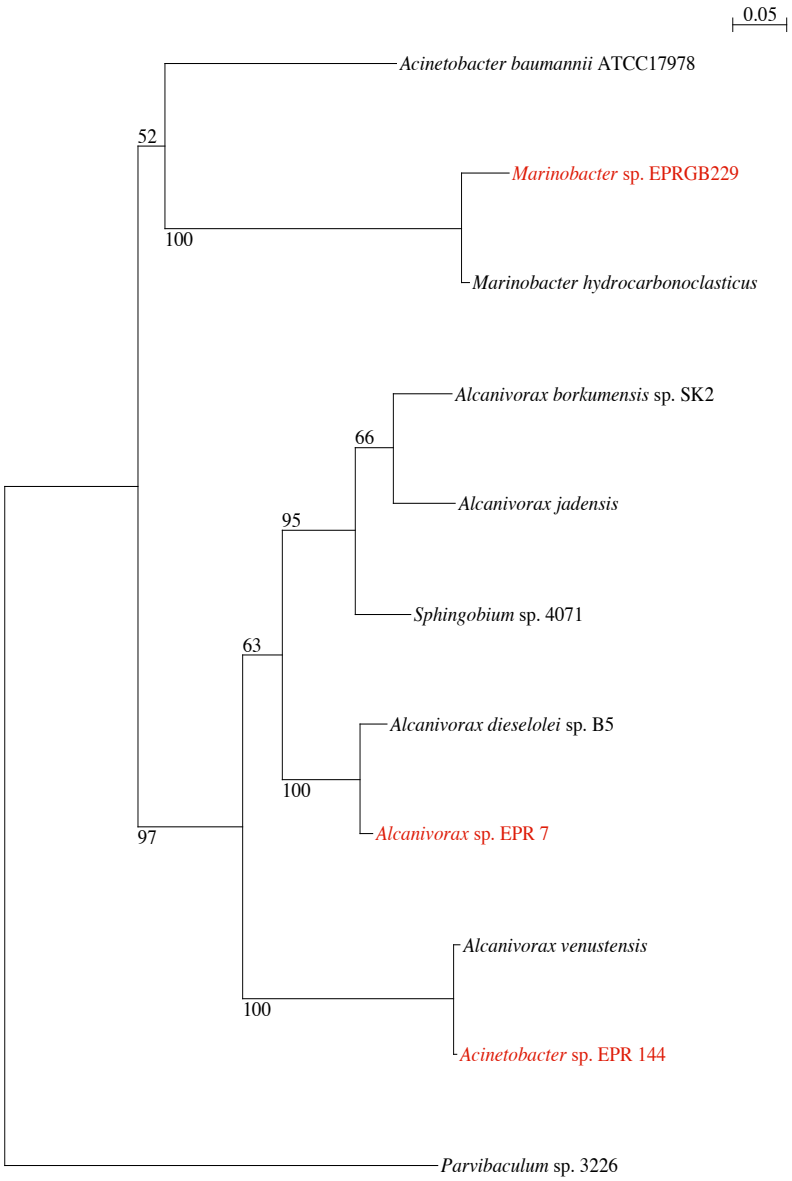

Fig. 4.3: Neighbor-joining phylogenetic tree inferred from the amino acid sequences deduced from a fragment of the *almA* gene (encoding the flavin binding AlmA protein). Bootstrap values based on 500 replications are shown as percentages at branch nodes. Bar, 0.05% estimated substitutions.

Detection of transcripts of the *alkB, cyp153* and *almA* gene in pure cultures

To further investigate the expression of *alkB,* I selected two representative isolates among the most frequently isolated genera. Thus, one member of the *Acinetobacter* genus (EPR111) and one from the *Alcanivorax* genus (EPR7) were selected and grown in three different media. Subsequently, the RNA was extracted from the culture and RT-PCR was used to assess the presence of the *alkB* gene transcript. Table 4.2 summarizes the results of these experiments: Both *Acinetobacter* EPR111 and *Alcanivorax* EPR7 expressed the *alkB* gene in the presence of yeast extract/peptone as well as dodecane. However, when both isolates were grown with acetate as the sole carbon source, the *alkB* gene transcript was not detected in either organism.

Detection of *alkB* transcript			
Organism	Yeast Extract/peptone	Acetate	Dodecane
Acinetobacter sp. EPR 111	+	-	+
Alcanivorax sp. EPR 7	+	-	+

Table 4.2: Detection of *alkB* gene transcripts in representative strains grown on different carbon sources

Acinetobacter sp. EPR144, which was found to encode for all three enzymes (Figs. 4.1-4.3), was selected to investigate if there was preferential gene expression of the *alkB, cyp153* and *almA* genes when this organisms was grown in the presence of octane (C_8H_{18}), dodecane ($C_{12}H_{26}$) and hexadecane ($C_{16}H_{34}$), respectively. This experiment revealed that, regardless of the alkane used, *Acinetobacter* sp. EPR144 transcribed only the *alkB* gene (Table 4.3).

Isolate/carbon source	*alkB*	*cyp153*
Acinetobacter sp. EPR144/octane	+	-
Acinetobacter sp. EPR144/dodecane	+	-
Acinetobacter sp. EPR144/hexadecane	+	-

Table 4.3: Detection of *alkB* and *cyp153* gene transcripts in *Acinetobacter* sp. Strain EPR 144 grown on *n*-alkane of different chain lengths as carbon sources

In a related experiment to investigate the expression of the flavin binding monoxygenase, *Acinetobacter* sp. EPR111 and *Alcanivorax* sp. EPR7 were grown with dodecane ($C_{12}H_{26}$) and *Acinetobacter* sp. EPR144 with hexadecane ($C_{16}H_{34}$). Transcripts of the *almA* gene were not detected in any of the strains or conditions tested (Table 4.4).

Isolate/ Carbon source	*almA*
Acinetobacter sp. EPR111/ dodecane	-
Alcanivorax sp. EPR 7/dodecane	-
Acinetobacter sp. EPR 144/hexadecane	-

Table 4.4: Detection of *almA* gene transcripts in representative isolates grown in different alkane chain lengths.

Discussion

In this study I wanted to determine if alkane-oxidizing bacteria isolated from deep-sea hydrothermal vents possessed the genetic potential for the oxidation of medium to long chain alkanes in particular. Furthermore, I investigated the presence of the *alkB* gene in vent natural microbial communities and detected the presence of transcripts of the *alkB, cyp153* and *almA* genes in representative isolates grown in the presence of different substrates.

PCR amplification of the *alkB* gene from alkane-oxidizing bacteria isolated from hydrothermal fluids revealed that the majority of the *Gammaproteobacteria* in Dr. Costantino Vetriani's laboratory culture collection encoded for the AlkB alkane hydroxylase. AlkB sequences were also amplified from the genomic DNA extracted from several vent natural microbial assemblages. Most AlkB sequences recovered from the natural communities were closely related to the AlkB enzymes from pure cultures belonging to the *Alcanivorax*, *Marinobacter* and *Acinetobacter* genera, indicating that these bacteria are well represented in hydrothermal fluids (Comparing red and blue sequences in Fig. 4.1). An interesting isolate is the facultatively autotrophic, thiosulfate-oxidizing bacterium, *Salinisphaera hydrothermalis*, which was found to grow on dodecane as the sole carbon source and to encode for the *alkB* gene (Melitza Crespo-Medina, et al., 2009). The detection by Wang et al. (2010) of *alkB* genes related to *Salinisphaera hydrothermalis* in bacteria isolated from the surface ocean implies a widespread distribution of this genus in marine environments.

The clustering of AlkB enzymes from several *Marinobacter* strains (*Marinobacter* spp. strain EPR21 and MAR16) with enzymes from *Alcanivorax* spp.

suggests that horizontal gene transfer between these two genera have occurred (Fig. 4.1). When trying to look if patterns emerged from the particular AlkB clustering sequences and their corresponding 16S identity, it is seen that although some of them cluster together in both phylogenetic analyses (*Alcanivorax* sp. EPR 19 and *Alcanivorax* sp. EPR17 for example) (Figs. 2.3, 4.1) it is not constant with all of our isolates (in the case of *Acinetobacter* EPR111 and *Acinetobacter* EPR144) where there is different 16S clustering than the AlkB tree. The reason for this could be, that even though their 16S identity was different the cohabitation in the same environment enables them to perform horizontal gene transfer to enhance fitness in this active and fluid environment.

It is important to point out that all the surveys were done on DNA (being from isolates or total environmental genomic), looking at the presence of the gene in the bacterial genome of our isolates but not necessarily meaning that it its an active and functional protein. The AlkB clones detected in the environmental survey from fluids whose temperature was 40°C clustered for the most part with *Acinetobacter* enzymes, whereas the clones from fluids whose temperature was lower (25°C) clustered mostly with *Marinobacter* AlkB. Moreover, several *Alcanivorax*-related enzymes were detected. These results indicate that the culture-dependent and independent approaches gave consistent results, in that the most commonly isolated genera were also represented in the AlkB surveys. Overall, I can conclude that hydrocarbonoclastic bacteria related to the genera *Alcanivorax, Acinetobacter* and *Marinobacter* occur frequently in diffuse flow hydrothermal vents. My results are in line with a paper published by L. Wang, et al., (2010), in which the presence of *alkB* and *cyp153* genes related to *Pseudomonas, Alcanivorax, Acinetobacter* spp., as well *Salinisphaera* and *Idiomarina* spp. was reported

from various sites in the surface ocean. Whether these hydrocarbonoclastic bacteria are active or not in their respective habitats remains to be seen. To this end, a transcriptomic approach would reveal if the alkane hydroxylase-encoding genes were expressed *in-situ*.

A screening of all three genes in the *Alphaproteobacterium, Parvibaculum hydrocarbonoclasticum* EPR92, revealed that this organism possesses only the *cyp153* gene. A lack of amplification of the *alkB* and *almA* genes from the genomic DNA of *P. hydrocarbonoclasticum* does not necessarily mean that they are absent from the genome, but it could suggest that the PCR primers used in this experiment failed to amplify the genes. However, an experiment carried out in collaboration with the laboratory of Rachel Austin at Bates College, where we followed the oxidation of norcarane during growth of *P. hydrocarbonoclasticum,* confirmed that this bacterium used an alkane-oxidizing CYP-like protein to catalyze the oxidation (Bertrand, Keddis et al, in revision).

In my study, *Acinetobacter* sp. EPR144 was found to encode for all three alkane hydroxylases. However, while phylogenetic analysis of the AlkB from several *Acinetobacter* strains consistently placed this enzyme in a discrete cluster with other *Acinetobacter*-encoded enzymes (Fig. 4.1), the phylogeny of both CYP153 and AlmA revealed a different story. In fact, both the CYP153 and AlmA from *Acinetobacter* sp. EPR144 were closely related to enzymes from *Alcanivorax*, clearly suggesting the occurrence of horizontal gene the two genera. This is not the only event of horizontal gene transfer that may have occurred in the evolutionary history of these genes. For instance, the AlkB proteins of several *Alcanivorax* isolates, including EPR171 and EPR180 (red color) cluster with a sequence from *Bacillus flexus* (Fig. 4.1). Furthermore, in the CYP153 tree, a branch includes sequences from *Alcanivorax* EPR160, *Tristella*

mobilis and *Alcanivorax dieselolei* (Fig. 4.2). It is well established that horizontal gene transfer occurs in the natural microbial communities, often resulting in bacteria acquiring genetic material from unrelated organisms that share the same niche. The most commonly known trait acquired by horizontal gene transfer is antibiotic resistance genes, which can provide an ecological advantage to the species carrying such genes (Perry, et al., 2002). Similarly, the acquisition of a pathway for the oxidation of hydrocarbons may provide an advantage to microorganisms that have access to these substrates.

The *almA* gene was detected in *Marinobacter* EPRGB229*, Alcanivorax* EPR144 and *Alcanivorax* EPR7 (Fig 4.3). However, a recent study focused on the diversity of the flavin-binding monooxygenase, *almA*, found a wide range of organisms containing these monooxygenases, including *Parvibaculum, Salinisphaera* and *Alcanivorax* spp. (Wang and Shao, 2012, Wentzel, et al., 2007). One of the reasons our survey wasn't as successful could be the primers did not have a sufficiently broad specificity.

Once I established the presence of these genes in my isolates, I sought out to determine if the different genes were differentially expressed in response to the carbon substrate utilized during growth. Both *Acinetobacter* EPR111 and *Alcanivorax* EPR7 expressed the *alkB* gene when grown in rich medium or in minimal medium supplemented with dodecane. However, when these bacteria were grown with acetate as the sole carbon source, they did not express the *alkB* gene (Table 4.2). Therefore, expression of the alkB gene appears to be induced by the presence of alkanes. Subsequently, I tested the expression of the three enzymes in *Acinetobacter* sp. EPR144, a strain that contains all three genes. Regardless of the substrate used (octane, dodecane or hexadecane), or the organism tested, only the *alkB* gene was expressed. The reason for

this could be that both the *cyp153* and the *almA* genes are expressed in the presence of alkanes longer than C_{16} (Beilen and Funhoff, 2007, Beilen, et al., 2003). This explanation is consistent with the observation that the AlmA enzyme is involved in the oxidation of alkanes longer than C_{32} (Throne-Holst, et al., 2007). An alternative explanation is that the copy number of these transcripts was below the threshold of detection in my RT-PCR assays. Further experiments are needed to constrain the expression of both the *cyp153* and the *almA* gene.

Chapter 5

Conclusions

Hydrothermal vents systems are a unique and active ecosystem that has brought many questions to the scientific community, one of them being how such dense and unique macrofaunal populations can thrive in such a hostile and toxic environment (Ramirez-Llodra, et al., 2007). Many studies since the discovery of deep-sea vents have been focused on chemosynthetic processes. The reason for this is that, without light at the depths chemosynthesis is the basis for carbon fixation. While chemosynthetic primary productivity has been widely studied in deep-sea geothermal environments, not much attention has been given to heterotrophic processes (Jannasch, 1995, Jannasch and Mottl, 1985, Ramirez-Llodra, et al., 2007). Hydrocarbons at deep-sea vents represent a geothermally generated organic carbon source (Brault, et al., 1988a, Brault, et al., 1989) (Berndt, 1996, Botz, et al., 2002), Hence, the study of hydrocarbonoclastic bacteria, which can oxidize *n*-alkanes to fatty acids, warrant attention. As petroleum is the number one energy source in the planet, rises in demand have expanded oil prospecting and extraction. With increased oil demand, oil spills have dramatically increased in the past 30 years. Therefore, it is imperative to understand the organisms that are present in the ocean and that, being exposed to a natural source of hydrocarbons, evolved the ability to use them as carbon and energy sources. By understanding the role of bacteria in the oxidation of crude oil components such as *n*-alkanes, the scientific community can design and implement novel bioremediation approaches that are less harmful and expensive as the present ones.

Studying how microbial communities respond to the presence of hydrocarbons naturally in hydrothermal vents might gives us an insight into their diversity and function. My work is focused on the medium chain *n*-alkanes, since they comprise of the majority of petroleum hydrocarbons. Moreover, this is one of the very few studies of hydrocarbonoclastic bacteria in deep-sea geothermal environments.

In this dissertation, I show the presence of aerobic *n*-alkane oxidizers in hydrothermal vents fluids from different diffuse flow deep-sea vents. Although the presence of these bacteria is variable between sites, isolations were successful in different dilutions (10^{-2}-10^{-6}) indicating that these organisms are segregated in warm vent environments. The isolations performed in this study coincide with some isolates from a variety of marine systems, both pristine and oil-contaminated (Cui, et al., 2008, Harayama, et al., 2004, Quatrini, et al., 2008, Tapilatu, et al., 2010, Wang, 2008, W. Wang, et al., 2010). For instance, the isolation of *Acinetobacter* species in my study at such a high number (20% of my isolates) in both undiluted and diluted enrichments (up to 10^{-4}) shows that this bacterium is an abundant member of this system.

In this dissertation, I presented the isolation and characterization of a novel alkane-oxidizing bacterium, *Parvibaculum hydrocarbonoclasticum*, from deep-sea hydrothermal vents. *Parvibaculum hydrocarbonoclasticum* seems to be abundant in vent environments since it was isolated from10^{-6} dilution tubes. *Parvibaculum* was found to contain a copy of the *cyp153* gene, encoding for cytochrome p450 that mediates aerobic alkane oxidation. Another characteristic found in this specific bacterium was the production of biosurfactant in the presence of hexadecane, dodecane and spent motor oil.

A big hurdle for alkane-oxidizing bacteria is their accession and proximity to the hydrocarbons to be able to degrade them. For this reason, I tested the production of biosurfactant in representative isolates, including some of the most frequently isolated spp, such as *Acinetobacter, Alcanivorax* and *Marinobacter*, as well as unique ones, such as *Parvibaculum hydrocabronoclasticum* and *Salinisphaera hydrothermalis*. My work has shown that alkane delivery might have an impact on biosurfactant production. In the case of *Alcanivorax* sp. EPR7 and *Alcanivorax* sp. EPR8, biosurfactant production was detected when dodecane was delivered via micelles and not as vapor. Alkane chain length might also play a role in biosurfactant production. This can be seen in the bioemulsion assays (Fig.2.4), where all isolates had very variable emulsion indexes in the presence of different hydrocarbons. More work towards the characterization of these biosurfactants is necessary and critical in the understanding of their mechanisms and efficiency.

In the course of my study, I have isolated several hydrocarbonoclastic bacteria that encode for multiple alkane hydroxylases, such as *Acinetobacter* sp. EPR144, *Alcanivorax* sp. EPR159 and *Acinetobacter* sp. EPR111. Furthermore, I demonstrated that these isolates could utilize *n*-alkanes of different chain lengths. Such substrate versatility could confer an ecological advantage in their natural habitat and, at the same time, make them excellent candidates for bioremediation of oil spills. As their presence was detected, my work showed that the *alkB* gene was being actively transcribed in *Alcanivorax* EPR111 and *Acinetobacter* EPR7, but no activity for *cyp153* and *almA* was detected. While *cyp153* and *almA* were not detected, it does not mean they are not active under other circumstances. In the case of *almA*, it could be that the *n*-alkanes utilized as carbon sources were not long enough to induce the expression of these genes, since the

range for this enzyme is thought to be from C_{32} and longer (Throne-Holst, et al., 2007, Wentzel, et al., 2007). For *cyp153*, it could be that the substrate range is different than the *alkB* or that it is being transcribed in low copy numbers and the sensitivity of the method used cannot detect it.

Only the *alkB* gene was detected in the environmental survey of alkane hydroxylases (Fig. 4.1). The inability to detect the other genes (*cyp153* and *almA*) might have been because of technical limitations of the taken approach. More comprehensive and deeper sequencing techniques might succeed at detecting genes present in low numbers.

My work has shown that alkane-oxidizing bacteria are present in deep-sea hydrothermal vents and posses the capability of using the medium chain alkanes as a carbon source, possibly producing biosurfactants to access the substrates. Heterotrophic growth of these bacteria by alkane oxidation may be a relevant process to mediate the transfer of energy and carbon from the geothermal source to the higher trophic levels, detoxifying the environment and possibly facilitating the colonization of vent invertebrates (fig. 5.1).

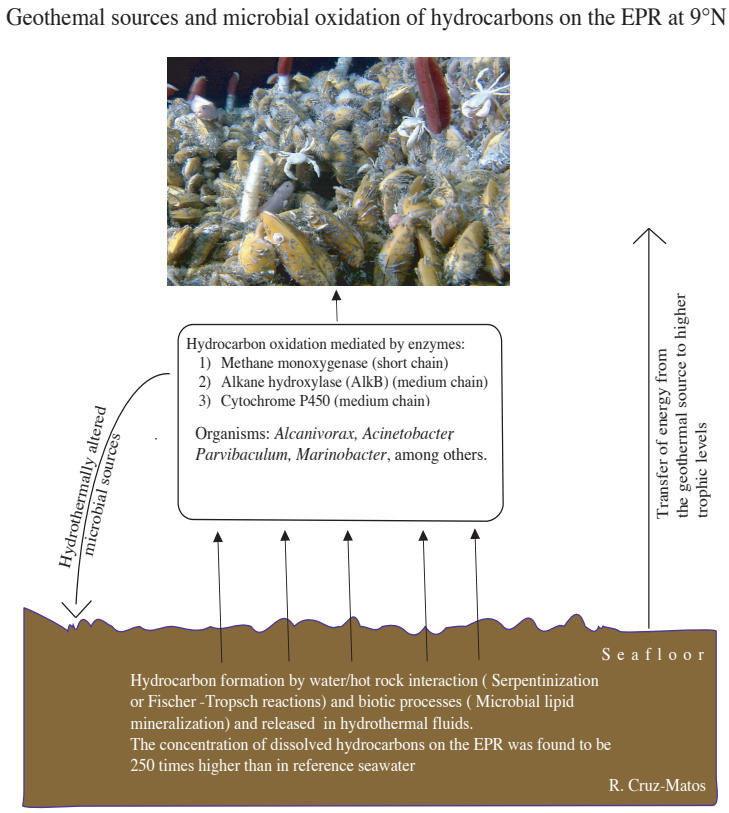

Fig.5.1: Hypothetical role of hydrocarbonoclastic bacteria in the ecology of deep-sea hydrothermal vents.

Appendix:

Isolate	Source	Media/Temperature (°C)	Closest Relative
EPR 1	3397 NM Bio Vent Plume	ASW HS (20-37)	*Pseudoalteromonas*
EPR 2	3400 NF P-Vent Plume	ASW HS (20-37)	*Pseudoalteromonas*
EPR 3	3402 NM Bio Vent Plume	ASW HS (20-37)	*Pseudoalteromonas*
EPR 5	3402 NM Bio 9 Vent Plume	ASW HS (37-45)	*Alcanivorax*
EPR 6	3398 TS2 Mk 119 Vent	ASW HS (37-45)	*Alcanivorax*
EPR 7	3398 TS2 Mk 119 Vent	ASW HS (37-45)	*Alcanivorax*
EPR 8	3398 TS2 Mk 119 Vent	ASW HS (37-45)	*Alcanivorax*
EPR 9	3398 TS1 Mk 119 Vent	ASW LS (20-45)	*Bacillus*
EPR 10	3398 NM Mk 119 Plume	ASW LS/Kan (37-45)	*Alcanivorax*
EPR11	3398 TS1 Mk 119 Vent	ASW LS (28)	*Halomonas*
EPR 12	3398 TS1 Mk 119 Vent	ASW LS (28-37)	*Pseudomonas*
EPR 13	3398 TS1 Mk 119 Vent	ASW LS/Kan (37)	*Rhizobiales*
EPR 14	3398 NM Mk 119 Plume	ASW LS/Kan (28)	*Arenibacter*
EPR 15	3398 NM Mk 119 Plume	ASW LS (28)	*Marinobacter*
EPR 16	3398 TS1 Mk 119 Vent	ASW HS (10)	*Halomonas*
EPR 17	3398 TS1 Mk 119 Vent	ASW MM/dodecane (28)	*Alcanivorax*
EPR 19	3398 NM Mk 119 Plume	ASW MM/dodecane (28)	*Alcanivorax*
EPR 20	3398 NM Mk 119 Plume	ASW MM/dodecane (28)	*Alcanivorax*
EPR 21	3398 NM Mk 119 Plume	ASW MM/dodecane (28)	*Marinobacter*
EPR 24	3398 NM Mk 119 Plume	ASW MM/dodecane (28)	*Alcanivorax*
EPR 26W	3398 NM Mk 119 Plume	ASM LS (28)	*Nocardioides*
EPR 27	3398 NM Mk 119 Plume	ASM LS (28)	*Alcanivorax*
EPR 28W	3398 NM Mk 119 Plume	ASM LS (28)	*Nocardioides*
EPR 92	4002 M22 Tica Vent; from MPN (10-6) with Dodecane	ASW MM/dodecane (28)	*Parvibaculum hydrocarbonoclasticum*
EPR 93	4002 M22 Tica Vent; from MPN (10-6) with Dodecane	ASW MM/dodecane (28)	*Parvibaculum sp.*
EPR104	4112 Alvinella Pillar	142-A (30)	*Halomonas* sp.
EPR105	4112 Alvinella Pillar	142-A (30)	*Halomonas* sp.
EPR105.1	4112 Alvinella Pillar	142-A (30)	*Halomonas* sp.
EPR106	4104 Mk 89	142-A (30)	*Marinobacter* sp.
EPR107	4104 Mk89	142-A (30)	*Marinobacter* sp.
EPR108	4104 Mk89	142-A (30)	*Marinobacter* sp.
EPR109	4104 Mk89	142-A (30)	*Marinobacter* sp.
EPR110	4107 Microbial Mat, Bio9	142-A (30)	*Rhodococcus erythropolis*
EPR111	4107 Microbial Mat Bio9	ASW MM/dodecane (30)	*Acinetobacter*
EPR 70	Mk 119 vent fluid undiluted	142-A (28)	*Salinisphaera*
EPR 111	MPN 10^{-2} dilution	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 144	Microcolonizer CV9 (10-4 dilution)	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 145	Major	ASWMM/Dodecane (30)	*Acinetobacter*
EPR146	Sulfide	ASWMM/Dodecane (30)	*Acinetobacter*
EPR147	Microcolonizer CV6	ASWMM/Dodecane (30)	*Alcanivorax*
EPR148	Major 10^{-2} dilution	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 149,150	Major 10^{-4} dilution	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 159	Basalt	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 160	Major	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 161	Microcolonizer CV9	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 163	*Alvinella* worm	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 169	Major	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 170	Microcolonizer CV3 10^{-2} dilution	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 171	Major 10^{-2} dilution	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 173	Microcolonizer CV9 (from fluids)	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 174	Microcolonizer CV3 (from fluids)	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 175	Major	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 176	Sulfides	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 177	Microcolonizer CV5	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 178	*Alvinella* worm	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 179	Sulfides mixed with fluids	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 180	*Alvinella* worm	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 181	Microcolonizer CV5	ASWMM/Dodecane (30)	*Alcanivorax*
EPR 182	Major	LS ASW (30)	*Alcanivorax*
EPRGB 224	Microbial Mat undiluted	LS ASW (30)	*Vibrio*
EPRGB 225	Microbial Mat undiluted	ASWMM/Dodecane (30)	*Vibrio*
EPRGB 229	Oiltown Slurp undiluted	LS ASW(30)	*Marinobacter*
EPRGB 230	Oiltown Slurp 10^{-1} dilution	ASWMM/Dodecane (30)	*Bacillus*
EPR 231	Sulfides and *Alvinella* tubes	ASWMM/Dodecane (30)	*Marinobacter*
EPRGB 232	Oiltown slurp sediment undiluted	ASWMM/Dodecane (30)	*Marinobacter*
EPR 233	*Tevnia* worm and tube undiluted	ASWMM/Dodecane (30)	*Alcanivorax*
EPRGB 234	Major Fluids	ASWMM/Dodecane (30)	*Alcanivorax*
EPRGB 235	Sediment Core undiluted	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 236	Microcolonizer biofilm	ASWMM/Dodecane (30)	*Acinetobacter*
EPR 237	*Alvinella* tube	ASWMM/Dodecane(30)	*Acinetobacter*
EPR 238	*Alvinella* worm	ASWMM/Dodecane(30)	*Acinetobacter*

List of isolates utilized in study with sources and closest relatives

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