

**Characterization of Bacterial Hydrocarbon Degradation Potential in the
Red Sea Through Metagenomic and Cultivation Methods**

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ABSTRACT**Characterization of Bacterial Hydrocarbon Degradation Potential in the Red Sea Through Metagenomic and Cultivation Methods**

Patrick Bianchi

Prokaryotes are the main actors in biogeochemical cycles that are fundamental in global nutrient cycling. The characterization of microbial communities and isolates can enhance the comprehension of such cycles. Potentially novel biochemical processes can be discovered in particular environments with unique characteristics. The Red Sea can be considered as a unique natural laboratory due to its peculiar hydrology and physical features including temperature, salinity and water circulation. Moreover the Red Sea is subjected to hydrocarbon pollution by both anthropogenic and natural sources that select hydrocarbon degrading prokaryotes. Due to its unique features the Red Sea has the potential to host uncharacterized novel microorganisms with hydrocarbon-degrading pathways.

The focus of this thesis is on the characterization at the metagenomic level of the water column of the Red Sea and on the isolation and characterization of novel hydrocarbon-degrading species and genomes adapted to the unique environmental characteristics of the basin. The presence of metabolic genes responsible of both linear and aromatic hydrocarbon degradation has been evaluated from a metagenomic survey and a meta-analysis of already available datasets. In parallel, water column-based microcosms have been established with crude oil as the sole carbon source, with aim to

isolate potential novel bacterial species and provide new genome-based insights on the hydrocarbon degradation potential available in the Red Sea.

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LIST OF ABBREVIATIONS

ANI	Average nucleotide diversity
BacDive	Bacterial diversity metadatabase
CDS	Coding sequence
COG	Cluster of orthologous genes
DDH	DNA-DNA hybridization
DSMZ	Deutsche sammlung von mikroorganismen und zellkulturen
DWH	Deep water horizon
FDR	False discovery rate
FSW	Filtered sea water
INDIGO	INtegrated Data Warehouse of MIcrobial GenOMes
KEGG	Kyoto encyclopedia of genes and genomes
MLSA	Multilocus sequence analysis
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PSU	Practical salinity unit
PVA	Polyvinyl alcohol
RPKM	Reads per kilobase per million
SV	Sequence variants
US EPA	United States Environmental Protection Agency

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Chapter 1:

The Red Sea as natural laboratory for environmental microbiology

1.1. Introduction

The Red Sea is a "natural laboratory" for biogeochemistry due to its unique hydrological, physical and climatic characteristics (Miller *et al.*, 1966; Thisse *et al.*, 1983; Chase *et al.*, 2006). The tidal effect in the Red Sea is relatively limited and, due to the semi-enclosed nature of the basin, the water currents down to depths around 300 m are predictable (Yao *et al.*, 2014a; Yao *et al.*, 2014b). However, unlike other semi-enclosed seawater basins such as the Mediterranean Sea or the Baltic Sea, the Red Sea receives minimal water input from precipitation or rivers; precipitation does not exceed 50-60 mm annually on average (Almazroui *et al.*, 2012) and the only significant sources that discharge land waters are a few seasonal "wadi" streams (Trommer *et al.*, 2011). Therefore, with practically only a single point of seawater entry (the strait of Bab al Mandeb in the south) and two points of exit (the strait of Bab al Mandeb and the Suez canal) and with a predictable water circulation pattern and limited vertical mixing (Yao *et al.*, 2014a; Yao *et al.*, 2014b; Qian *et al.* 2011), the Red Sea represents an interesting model to study the assembly and dispersal of microbial communities.

These constant patterns in hydrology and physical properties determine a distinct temperature/salinity gradient from North to South. The cool water from the Indian Ocean that enters the Red Sea at Bab al Mandeb is constantly getting warmer

and more saline as it travels northward (around 23-24°N), albeit forming eddies depending on the season (Yao *et al.*, 2014a; Yao *et al.*, 2014b), because of the intense heat and intense water evaporation, combined with the lack of freshwater inputs. As the water continues its northward trajectory, it cools again, increasing in salinity (i.e., reaching 40-41‰) and decreasing in temperature (i.e., reaching 27-29 °C) when it reaches the Gulfs of Aqaba and Suez (Qurban *et al.*, 2014). Such a circulation pattern causes a distinct temperature/salinity gradient in the upper water layers along the North-South axis of the basin, with an increase in salinity and a decrease in temperature from South to North.

Another characteristic gradient of the Red Sea is the gradient in primary productivity from South to North, with the central and northern parts of the basin being among of the most oligotrophic marine areas worldwide (Acker *et al.*, 2008; Raitzos *et al.*, 2013; Qurban *et al.*, 2014). The water that enters the Red Sea from Bab al Mandab is characterized by nutrient concentrations ranging between 15-23 $\mu\text{mol L}^{-1}$ of nitrate and 1.4-1.9 $\mu\text{mol L}^{-1}$ of phosphate (Souvermezoglou *et al.*, 1989). As this water travels northward, the nutrients in the upper water column get depleted because of the stratification that prevents nutrient upwelling from the deep layers and the absence of other nutrient sources, reaching values of less than 2 $\mu\text{mol L}^{-1}$ and 0.2 $\mu\text{mol L}^{-1}$ for nitrate and phosphate, respectively, in the northern Red Sea (Acker *et al.*, 2008). As a result, the primary productivity is very low ranging between 10-20 $\text{mg C m}^{-2} \text{h}^{-1}$ (Qurban *et al.*, 2014) and making this water basin 1.9 - 4.4 times less productive than similar water basins at the same latitude such as the Caribbean Sea (Taylor *et al.*, 2012), the

Gulf of Mexico (Murrell *et al.*, 2007) or the southern Gulf of California (Álvarez-Borrego, 2012). The oligotrophy in the water column is maintained despite the presence of large cities on the shores of the Red Sea such as Jeddah, Port Sudan and Jizan, where the water is enriched with organic carbon and inorganic nutrients due to anthropogenic activities (El-Sayed *et al.*, 2011; Peña-García *et al.*, 2014). Due to the above unique features, the ultra-oligotrophic system of the Red Sea is potentially an important reservoir of microbial diversity, where one could expect to find microbial taxa that are adapted within these harsh environmental gradients.

1.2. Environmental selection factors

1.2.1 Temperature

Temperature is an important environmental parameter to be considered when investigating the behavior of marine microbial communities. Generally, it has been shown that as temperature rises, microbial metabolism increases because of increased enzyme kinetics (Fuhrman & Azam, 1983; Kirchman *et al.*, 2005; López-Urrutia & Morán, 2007). It has been shown both *in-situ* and in the laboratory that temperature can affect the growth rates, respiration rates and secondary metabolisms (White *et al.*, 1991; Vazquez-Dominguez *et al.*, 2007; Rivkin *et al.*, 1996). The positive correlation between temperature and growth rate is limited to a specific range of temperature depending on the microorganisms tested. Above the optimal growth temperature, the growth of microbes decreases rapidly (Izem & Kingsolver, 2005; Chen & Shakhnovich, 2010). As the temperature affects the microbial growth and metabolism, it may remarkably affect

microbial communities and even small temperature variations can lead to dramatic shifts of their taxonomic composition (Johnson *et al.*, 2006). Although, according to time series studies, it has been observed that some dominant microbial populations are present through the entire year, other “rare” taxa can vary in abundance depending on the period of the year corresponding with specific temperature (Gilbert *et al.*, 2012; Chow *et al.*, 2013). The adaption to temperature variation is thought to involve genome-wide changes as enzymatic rates, protein folding and membranes composition are all directly affected by temperature. Since temperature can affect marine microbial communities at multiple levels, it is of scientific interest to further investigate the correlation between microbial growth and diversity and temperature.

1.2.2. Salinity

As the Red Sea contains one of the world's saltiest bodies of water with an average salinity of 40 PSU (Ngugi *et al.*, 2012), microorganisms that thrive in the basin can be considered slight (0.3 to 0.5 M NaCl) or moderate halophiles (0.5 to 3.4 M NaCl). Indeed, they are exposed to higher osmotic pressure and Na⁺ concentration than in other marine basins. Na⁺ fluxes are also implicated in pH homeostasis (Oren, 2008). Moreover, due to the presence of numerous brine pools scattered along the central axis of the basin, those microbial communities in the water column adjacent to the brine pools are exposed to high osmotic pressure variation in meter scale. Osmoregulation of the cell is critical because cell integrity and hydration are dictated by their solute contents and the osmotic pressures of their environments (Altendorf *et al.*, 2009). When

the osmotic pressure of the cell decreases, the cells are subjected to water influx, which can lead to lysis. On the contrary, water efflux and dehydration are observed if the osmotic pressure increases.

Many cellular properties are affected by water fluxes that can happen almost instantaneously. To counteract these effects, moderate halophiles developed several mechanisms of osmoregulation. The main one is accumulation of solutes via active transport or synthesis if the osmotic pressure rises. Those are released via mechanosensitive channels if this pressure falls (Wood, 2016).

1.2.3 Oil hydrocarbon pollution

The Red Sea is considered one of the world's most intriguing areas for hydrocarbon exploration (Henni, 2017). It is still largely unexplored, even though Saudi Aramco (the Saudi Arabian national petroleum and natural gas company) developed several gas fields in 2013. However due to multiple challenges, including depth, seafloor topography and geology, it was halted in 2015. Nowadays the main oil pollutions in the Red sea are due to tourism, industrialization, extensive fishing, oil processing, shipping and, pollution outbreaks (Mustafa *et al.*, 2016). The majority of the studies made on the Red Sea hydrocarbon pollution were located in the Gulf of Aqaba and on the Egyptian coast. Abdallah *et al.* (2016) monitored the polycyclic aromatic hydrocarbon pollution from Suez to Hurghada in Egypt and showed that half of the samples taken were contaminated by pyrogenic hydrocarbons while other samples contained petrogenic hydrocarbons. After being released in the environment, hydrocarbons are submitted to

chemical, physical and biological modifications known as weathering. Depending on their chemical compositions and the ability of microorganisms to degrade them, some hydrocarbons have short half-lives (e.g. n-alkanes) while others can be detected a year after the initial pollution (e.g. polycyclic aromatic hydrocarbons) (Mapelli *et al.*, 2017).

1.3 Microbial diversity of the Red Sea

1.3.1 Metagenomic studies

Even though the Red Sea has an abundance of unique characteristics that may favor the selection of unique microbial life forms, its microbiota is one of the least studied among marine environments. In the recent years systematic research is characterizing the different ecosystems of the Red Sea, including the pelagic zone, the brine pools, coastal waters, mangrove and seagrass ecosystems and coral reefs. Here, I focus on the pelagic zone. Before the advent of next-generation sequencing, studies focused on specific marine groups, especially picocyanobacteria populations near the Gulf of Aqaba (Fuller *et al.*, 2005; Zeidner *et al.*, 2005). Using 16S rRNA gene pyrosequencing, Ngugi *et al.* (2012) were able to show that the surface waters were dominated by two phyla, *Proteobacteria* and *Cyanobacteria*. As was expected, within the latter, picocyanobacteria dominated, whereas the SAR11 clade was overly represented in the *Proteobacteria* phylum. This clade was further studied along the water column from the surface to 1,500 m. Results showed that its diversity along the water column depth was dependent on physicochemical parameters. The SAR11 clade is more diverse from surface to 50 m deep than in the deeper samples (Ngugi & Stingl,

2012). Comparisons of the Red Sea microbiota with other oligotrophic marine environments indicated that its composition resembles those found in the North Pacific Ocean with respect to adaptation to high-intensity light, the Mediterranean Sea for adaptation to high salinity, and the Mediterranean and Sargasso Seas for adaptation to low phosphorus concentration (Thompson, 2013a; Thompson *et al.*, 2013b).

1.3.2 Cultivation-based studies

The ability to isolate and cultivate novel microbial species is considered one of the most challenging aspects in microbiology and microbial ecology (Staley & Konopka, 1985). Enlarging the collection of isolates readily growing in laboratory conditions is considered a critical goal of modern microbiology mainly for two reasons: i) isolating new microbes would allow us to study and characterize them in depth expanding the understanding of the biology and ecology of prokaryotes, considered crucial players in every biogeochemical cycle (Jetten, 2008; Muyzer & Stams, 2008) and ii) new microbial isolates quite possibly would represent an untapped source of novel bioactive molecules that could lead to new biotechnological applications (Lee *et al.*, 2014; Ling *et al.*, 2015). Despite this, the number of novel isolates obtained from the Red Sea is less than 20 according to the BacDive database (Söhngen *et al.*, 2015). Five of which were isolated from the numerous brine pools that are present in the Red Sea (Antunes *et al.* 2011). As an example, Fiala *et al.* (1990) isolated, from Atlantis II Deep, *Flexistipes sinusarabici*, gen. nov., sp. nov., a non-motile, non-spore forming, strictly anaerobic, halophilic bacterium. Another novel halophile was isolated from Kebrit Deep, closely related to the

genus *Halanaerobium*, whose previously identified representatives were shown to occur only in sediments from salt lakes and offshore oil fields (Eder *et al.*, 2001). More recently Antunes *et al.* isolated two novel species of halophiles from Shaban Deep: *Marinobacter salsuginis*, a facultative anaerobic strain able to reduce nitrate to nitrogen in anaerobic conditions (Antunes *et al.*, 2007), and a new order-level taxon strain, *Haloplasma contractile* (Antunes *et al.*, 2008a). Only one novel archaeal strain has been successfully isolated from the Red Sea *Halorhabdus tiamatea*, an extremely halophilic anaerobe able to use S^0 or NO_3^- as electron acceptor (Antunes *et al.*, 2008b). The other sources of isolates in the Red Sea are mucus or tissues of different species of corals (Lampert *et al.*, 2006; Ben-Dov *et al.*, 2009; Zeevi Ben Yosef *et al.*, 2008; Shnit-Orland *et al.*, 2010; Paramasivam *et al.*, 2013) and marine sponges, *e.g.*, the novel bacterium *Actinokineospora spheciospongiae*, which was isolated from *Spheciospongia vagabunda* (Kämpfer *et al.*, 2015).

Only a few studies evaluated the potential biotechnological applications of Red Sea isolates. Sagar *et al.* (2013) assessed the anticancer activity of marine microorganisms isolated from the brine-seawater interface of four Red Sea brine pools. It was shown that selected isolates, all closely related to previously cultured strains, displayed cytotoxic and apoptotic activity against breast, prostate, and cervical carcinogenic cell lines. The antimicrobial activity of twenty strains isolated from the soft coral *Sarcophyton glaucum* was also evaluated in the study by ElAhwany *et al.* (2015). The results showed that the isolates possess a broad spectrum of antimicrobial activity and could be of interest for the production of novel antimicrobial agents. Moreover, the

ability to produce antimicrobial agents, suggests a role of these strains in protecting the coral host against marine pathogens.

Few studies so far have been focused on the exploitation of Red Sea microbial communities for bioremediation approach, specifically of oil-contaminated water. For instance, El-Sheshtawy *et al.* (2014) assessed the crude oil degradation capability of isolates, obtained from a historically contaminated area in Egypt (Gemsa Bay), in pure or mixed cultures and in the presence of synthetic nanoparticles and biosurfactants. The results showed that some isolates had interesting degradation capability towards specific polyaromatic hydrocarbons and that the presence of biosurfactants and Fe_2O_3 and $\text{Zn}_5(\text{OH})_8\text{Cl}_2$ nanoparticles could enhance the degradation performances. Another study investigated the population dynamics of oil-spiked marine sediment microcosms supplied with ammonium or uric acid in different locations, including the Gulf of Aqaba in the north-west end of the Red Sea (Gertler *et al.*, 2015). Researchers showed that the marine microbial community was dominated by *Pseudomonas* spp. and *Alcanivorax* spp., which are ubiquitous hydrocarbon-degrading marine microbes independently of the geographical location (Gertler *et al.*, 2015).

With the sharp rises of oil prices in recent years, there is a strong incentive to start again to exploit the resources of the Red Sea, including hydrocarbons despite the strong physical constraints, especially the high depths. However, as I have shown the capacity of the system to adapt to episodes of hydrocarbon/chemical spill is very patchy in the Red Sea from a microbiological point of view.

In the following sections, I will present an overview of hydrocarbons typically found in oil mixture and the general mechanisms of their biotic degradation, especially for n-alkanes, branched-chain alkanes, cycloaliphatic compounds and polycyclic aromatic hydrocarbons (PAHs). I will focus on the aerobic degradation of hydrocarbons, as my framework is the water column.

1.4 Diversity of hydrocarbon-degrading bacteria

1.4.1 Type of hydrocarbons and origin

Hydrocarbons in marine environment mainly come from two sources, geogenic and anthropogenic. Depicted in Figure 1.1 is the chemical structure of relevant oil related hydrocarbons. Geogenic hydrocarbons originate from natural seeps, which are commonly found in marine environments. These seeps represent the main source of hydrocarbons for the aquatic system (Transportation Research Board and National Research Council, 2003) and potential sources of microbial degraders as well (Scoma *et al.*, 2017). However, in the past years, anthropogenic activities such as oil extraction, transportation, and general industrial activities have significantly increased the level of release of hydrocarbons in the environment. In this context, an important example is the Deepwater Horizon (DWH) Blowout in 2010 that caused the release of the highest amount ever recorded of oil hydrocarbons in the deep ocean environment (Ramseur, 2010). Even though, the main source of hydrocarbons is natural, during the DWH oil spill the microbiome was not able to degrade all the oil released, *i.e.* slicks of oil heavily enriched with PAHs reached shores and were detected for at least a year requiring

cleanup interventions (Allan *et al.*, 2012). This indicates that further research is needed to investigate the metabolisms of degradation of hydrocarbons and a possible way to enhance or modify it for bioremediation purposes.

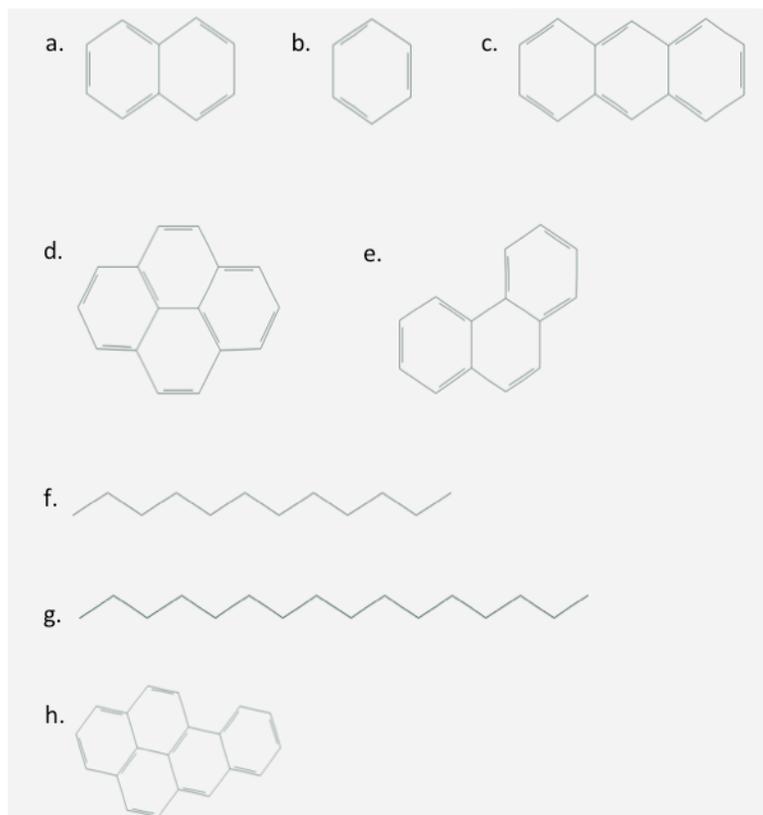


Figure 1.1 Chemical structure representation of various hydrocarbons susceptible to microbial degradation. a. Naphthalene, b. Benzene, c. Anthracene, d. Pyrene, e. Phenanthrene, f. Dodecane, g. Hexadecane, h. Benzo(α)pyrene.

1.4.2 Alkanes metabolism and related pathways

Alkanes are the most easily degraded compounds within aliphatic hydrocarbons. Two types are distinguished, linear and branched-chain alkanes. The latter is considered more recalcitrant due to its structure; however numerous bacteria have been shown to degrade them, *e.g.*, *Alcanivorax* genus (Alvarez *et al.*, 2009; Hara *et al.*, 2003). Typically, linear alkanes are divided into four categories: 1) C1-C7, which include gaseous and

liquid alkanes, 2) C8-C16 aliphatic compounds of low molecular weight, 3) C17-C28 medium molecular weight aliphatic hydrocarbons, 4) >C28 hydrocarbons of high molecular weight. Generally, low molecular weight aliphatic alkanes are more easily degraded than high molecular weight ones, which require multiple metabolic steps to be metabolized (Liu *et al.*, 2011). Alkanes are generally insoluble in water, and the solubility decreases with the increase of molecular weight. The uptake of alkanes by microorganisms can vary widely depending on the species considered and, the physical and chemical characteristics of the environment. Alkanes of low molecular weight can have sufficient solubility allowing a direct uptake from the environment, while medium and high molecular weight alkanes might need surfactants and/or emulsifiers secreted by the microorganisms or of anthropogenic origin which increase the solubility allowing bacteria to uptake them (Beal & Betts, 2000; Noordman & Janssen, 2002). Wang & Shao (2014) recently described the regulation of alkane degradation in *Alcanivorax dieselolei*. The OmpS protein detects alkane molecules and then activates the expression of different proteins and enzymes involved in the chemotaxis and the alkane uptake and hydroxylation. The uptake of alkanes in *Alcanivorax* is operated by the OmpT proteins, which are expressed in the outer membrane (Wang & Shao, 2014). Depending on the length of linear alkanes, different transport proteins are responsible for the uptake (ompT1 for C28–C36, ompT2 for C16–C24 and, ompT3 for C8–C12). The monooxygenase enzymes (AlmA, AlkB) then hydroxylate the alkanes where they overcome the low chemical reactivity of the chemicals by generating reactive oxygen species. The alcohols generated are then oxidized into aldehydes and finally into fatty acids (Figure 1.2) (Rojo,

2009). Branched-chain alkanes are activated the monooxygenases genes *alkB1* and *almA* in *A. dieselolei* B-5 (Liu *et al.*, 2011). A common mechanism for the degradation of branched alkanes is the “activation” through hydroxylation of the terminal methyl group of the branched chain to the corresponding acids or ketones (Wang & Shao, 2013). Other microorganisms able to degrade branched-chain alkanes (*e.g.*, pristane) are *Mycobacterium neoaurum* and *Rhodococcus ruber*, which degrade them into carboxylic acids as final compounds (Cong *et al.*, 2009).

1.4.3 Cycloaliphatic compounds metabolism and related pathways

Crude oil is a complex mixture of different compounds including several cycloaliphatic compounds. For instance, cyclopentane and cyclohexane are commonly found in oil mixtures (Comandini *et al.*, 2014). Generally, the difficulty of the degradation of cycloaliphatic compounds is due to their intrinsic toxicity on biological systems and their insolubility (Hommel, 1994). Bacteria able to degrade these compounds have been identified through an oxidative pathway that transforms cycloaliphatics into the corresponding adipic acids (Iwaki *et al.*, 2008) thanks to a metabolic process that opens the C-C ring with the addition of carboxylic groups. Interestingly, bacterial consortia have shown the ability to degrade cycloaliphatic compounds using them as a source of both carbon and energy. Specifically, Lee and Cho (2008) have investigated a consortium of *Rhodococcus* sp., *Sphingomonas* sp. and *Stenotrophomonas* sp., able to degrade and thrive on cyclohexane. Additionally,

mineralization to CO_2 and H_2O of cyclohexane has been reported for *Rhodococcus* sp. EC1 (Lee & Cho 2008).

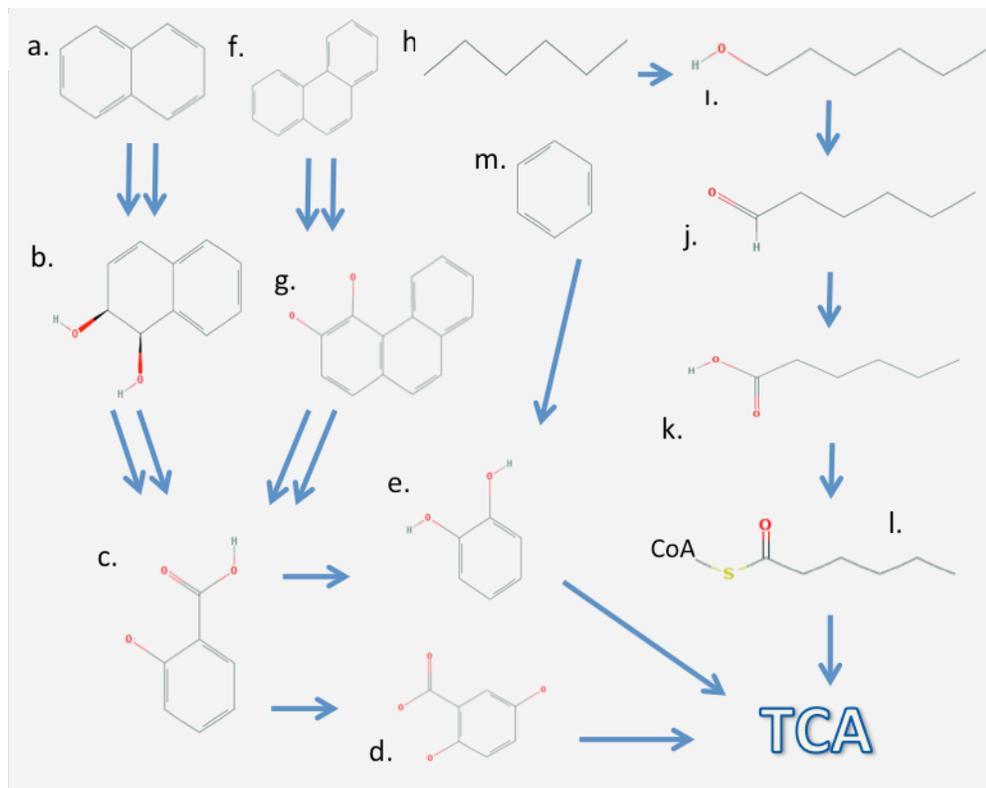


Figure 1.2 Representative schematic of aerobic degradation of Benzene, Naphtalene, Phenantrene and linear alkanes. a. Naphthalene, b. 1,2 – Dihydronaphthalene-1,2-diol, c. Salicylate, e. Cathecol, f. Phenanthrene, g. 3,4 – Phenanthrenediol, h. Alkane, i. Alkanol, j. Alkanal, k. Alkanoic acid, l. Alkanoyl-CoA. TCA, Tricarboxylic acid cycle. Double arrows indicate multi enzymatic steps. Single arrows represent single step reactions.

1.4.4 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a group of hydrocarbons that are particularly recalcitrant in the environment due to their chemical structure composed of multiple aromatic rings. PAHs have attracted a growing interest since their recalcitrance in the environment is a potential threat to ecosystems, *i.e.*, DWH blowout (Allan *et al.*, 2012). Indeed, due to

their genotoxic, mutagenic and carcinogenic potential they represent a significant threat to human health (Franco *et al.*, 2008). Usually, PAHs can form linear, angular or clustered arrangements with bonds between the aromatic rings of different molecules (Figure 1.1). The increase of aromatic ring number corresponds to the higher chemical stability of PAHs resulting in a longer time of persistence in the environment (Liu *et al.*, 2011).

The two main known mechanisms developed for the biotic degradation of PAHs by microorganisms are chemical oxidation or photolysis. Even though the diversity of PAHs is quite important (several hundreds), research has focused on only 16, which are considered by the US Environmental Protection Agency (US EPA) as priority pollutants for remediation (Keith, 2015). Amongst those, the degradation of anthracene, naphthalene and, phenanthrene is the most well described (Pinyakong *et al.*, 2000, Resnick *et al.*, 1996; Annweiler *et al.*, 2000, Menn *et al.*, 1993; Pinyakong *et al.*, 2003a,b). The initial step is the activation of the aromatic ring catalyzed by dioxygenase yielding to cis-dihydroxylated intermediates. Consequently, additional steps involving ring-cleaving dioxygenases produce central intermediates such as catechol and protocatechuates that can be further converted to tricarboxylic acid cycle intermediates (Figure 1.2) (Gibson & Parales, 2000).

The dioxygenases responsible for the initial activation step of the aromatic ring appear to be common in bacteria. These enzymes are components of an enzymatic complex requiring NADH as cofactor (Resnick *et al.*, 1996). Several bacteria have been

described for their ability to degrade PAHs and can generally be categorized depending on the possessed dioxygenase responsible for the initial steps: toluene dioxygenase (TDO), naphthalene dioxygenase (NDO) and biphenyl dioxygenase (BPDO). The specificity substrate range of such enzymes can vary among different bacteria. TDO has been shown to have a broad range of substrates for dihydroxylation, and its activity is limited by the molecular size/weight of PAHs compounds. In contrast, NDO and BPDO are shown to be able to degrade higher molecular weight PAHs. Additionally, for tetracyclic or larger PAHs only BPDO appears to be effective for the initial activation of the aromatic ring through dioxygenase activity (Boyd & Sheldrake, 1998). During the DWH spill, the PAHs degradation was specifically attributed to the genera *Cycloclasticus* and *Colwellia* (Vila *et al.*, 2010; Baelum *et al.*, 2012), indicating that there is a specialization among bacterial taxa towards PAHs.

1.5 Conclusions

Hydrocarbon degradation by microorganisms was largely studied over the past years, especially during important episodes of oil spills (*e.g.*, DWH; Hazen *et al.*, 2010; Baelum *et al.*, 2012; Gutierrez *et al.*, 2013; Camilli *et al.*, 2010; Valentine *et al.*, 2012). During this time, studies have been concentrated on sites that were exposed to either chronic or occasional hydrocarbon pollutions. This allowed for a comprehensive analysis of the different metabolisms and microbial successions that are responsible for hydrocarbon degradation. However, little has been done to investigate the potential of microbial hydrocarbon degradation in a pristine environment before the potential

episode of pollution. In this regard, the Red Sea constitutes an important body of water to study. Due to the diminishing reserves of oil, the rise of its price and despite the difficulties inherent to high depths, there is a strong incentive to exploit the hydrocarbons reserves in the basin (Henni, 2017). This will strongly increase the potential contaminations to the environment. The investigation of the biochemical pathways that are detectable or not at different sites/depths of the basin will shed light on the Red Sea potential for microbial hydrocarbon degradation and could allow for more precise bioremediation solutions. Considering the unique and extreme nature of the environmental conditions of the Red Sea, it is expected that novel microbial taxa have been evolved there, including novel hydrocarbon degraders adapted to the hot, saline and oligotrophic conditions of such a unique ocean.

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Chapter 2:

Alcanivorax marisrubri sp. nov. isolated from the Red Sea

2.1. Introduction

The genus *Alcanivorax* was first proposed to describe a marine bacterium isolated from the North Sea, *Alcanivorax borkumensis* (Yakimov *et al.*, 1998). The description of the genus was further amended in 2003 by the description of *A. venustensis* and the reclassification of *A. jadensis* (Fernández-Martínez *et al.*, 2003). Over twenty years later, the genus *Alkanivorax* included 11 described species, *A. balearicus* (Rivas *et al.* 2007), *A. borkumensis* (Yakimov *et al.* 1998), *A. dieselolei* (Liu & Shao 2005), *A. gelatiniphagus* (Kwon *et al.* 2015), *A. hongdengensis* (Wu *et al.* 2009), *A. jadensis* (Fernández-Martínez *et al.* 2003), *A. marinus* (Lai *et al.* 2013), *A. nanhaiticus* (Lai *et al.* 2016), *A. pacificus* (Lai *et al.* 2011), *A. venustensis* (Bruns & Berthe-Corti 1999) and *A. xenomutans* (Rahul *et al.* 2014). All members of the genus *Alcanivorax* were isolated in marine environment and include ubiquitous alkane-degrading bacteria.

The genus *Alcanivorax* accommodates Gram-stain-negative, aerobic, motile or non-motile rods that are obligate hydrocarbonoclastic bacteria, chemo-organotrophic and, tolerate high salt concentrations (Kwon *et al.* 2015). *Alcanivorax* species have been found to be widespread in the marine environments (North Sea, South China, South Korea, India, Spain etc...), occupying a variety of ecological niches, such as seawater and marine sediment. However, no surveys on the *Alkanivorax* diversity have been performed in the Red Sea, despite such an ocean has unique hydrological, physical and

climatic characteristics including the highest temperature, salinity and oligotrophy among all the sea basins on earth (Thisse *et al.*, 1983; Chase *et al.*, 2006).

In order to further understand their role in marine environments, the current study aimed to classify two marine bacteria, strains ALC70 and ALC75, which were isolated respectively at 1000 m and at 10 m depth in the Red Sea during an effort to describe marine bacteria that could be involved in the degradation of crude oil. Comparative 16S rRNA gene sequence analysis indicated that both strains belonged to the genus *Alcanivorax*. I provide data that support the description of a novel species in the genus.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

Strains ALC70 and ALC75 were isolated at 1000 m and 10 m depth, respectively, at station SR3 (18.97N, 39.54E) during the InDeepSW0416 cruise in April 2016 aboard the R/V Thuwal in the south Red Sea. The water sampling was carried out via with a Niskin Rosette system. Microcosms liquid cultures were setup using 5 ml seawater as inoculum and 45 ml of Filtered Sea Water (FSW). Arabian light crude oil was added as the only carbon source (1% v/v). Urea and potassium phosphate monobasic (KH₂PO₄) were subsequently added as nitrogen and phosphorous source. The liquid cultures were transferred to fresh media every 15 days for a total of four times. Five ml were taken from the liquid culture and transferred to a new microcosm (final volume 50 ml with FSW) with fresh crude oil, KH₂PO₄, and urea. After the 5th transfer, 100 µl of the liquid

culture was used for cultivation screening on petri dish. The screening was conducted using the 10^{-5} to 10^0 serial dilutions of the enriched microcosm. The medium in the plates had the same composition as the liquid microcosms (FSW, Oil, Urea, KH_2PO_4 and agar). The putative isolates were repeatedly restreaked on FSW-Oil petri dishes to ensure the purity of the colony. The strains were then aerobically grown on marine broth (BD, U.S.A.) at 26°C for 2 days. The bacterial cultures were stored at -80°C in marine broth 2216 (BD, USA) supplemented with 20 % (v/v) glycerol.

2.2.2 Genome sequencing and phylogenetic analysis

Genomic DNA was isolated with the QIAGEN Genomic-tip 100/G kit. Overnight cultures grown at 26°C in marine broth (BD) were centrifuged for 10 minutes at 4500 g and the cellular pellet was recovered. DNA extraction was carried out with Qiagen Tip 100/G protocol to ensure the extraction of large size DNA fragments (50-100 Kb). Concentration was measured by Qubit High Sensitivity kit (Thermo-Fischer Scientific) and quality control was performed with Bioanalyzer 2100 (Agilent). The genomes were annotated using an automated annotation pipeline for microbial genomes, INDIGO (INtegrated Data Warehouse of Microbial GenOMes; Alam *et al.* 2013). 16S rRNA gene sequences were extracted from the annotation results. They were subsequently aligned using the SILVA Incremental Aligner (SINA) software (Pruesse *et al.* 2012) along the other described *Alcanivorax* species. The phylogenetic analysis has been performed using the RAxML software, using the GTRCAT substitution model with the autoMRE bootstrapping criteria as suggested by Pattengale *et al.* (2010).

2.2.3 Genome comparison and genomic signature analyses

The genomes of our two strains ALC70 and ALC75 along with the one of *Alcanivorax venustensis* DSM 13974 were compared to *Alcanivorax borkumensis* SK2, *Alcanivorax dieselolei* B5, *Alcanivorax jadensis* T9, *Alcanivorax pacificus* W11-5 (Schneiker *et al.* 2006; Lai *et al.* 2012; Lai and Shao 2012). All sequenced genomes were retrieved from GenBank. COG and KEGG annotations were obtained using the Integrated Microbial Genomes & Microbiomes database (<https://img.jgi.doe.gov/>; Chen *et al.* 2017). Estimates of DNA–DNA hybridization (DDH) were calculated using genome-to-genome distance calculator 2.0 (GGDC) provided by DSMZ (<http://ggdc.dsmz.de>; Meier-Kolthoff *et al.* 2013). The average nucleotide identity (ANI) was calculated using the JSpeciesWS webserver using the default parameters (Richter *et al.* 2016).

2.2.4 Physiological and biochemical analysis

The ability of the strains to grow at different NaCl concentrations (0-15% w/v) and different temperatures (4-50°C) was assessed using Marine Broth. Salt tests were carried out at 26°C, while temperature growth was performed at 4.5% NaCl. Further physiological and biochemical characterizations were performed using different Biolog GN plates (Biolog), including pH, carbon and nitrogen sources. Cellular fatty acids, respiratory quinones and polar lipids composition were analysed by the identification service laboratories of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cell morphology was examined by means of

transmission and scanning electron microscopy. Motility was checked using phase-contrast microscopy.

2.3 Results and discussion

Analysis of the complete 16S rRNA gene sequences showed that strains ALC70 and ALC75 were grouped in a cluster along with *Alcanivorax venustensis* ISO4 and were distinguished from *Alcanivorax gelatiniphagus* MEBiC08158, *Alcanivorax marinus* R8-12, *Alcanivorax* sp. N3-7a and *Alcanivorax pacificus* W11-6, as observed in the phylogenetic tree of the genus *Alcanivorax* (Figure 2.1). The similarity of the 16S rRNA gene sequences of ALC70 and ALC75 was 99.80%. Both strains shared 97.8% 16S rRNA gene sequence similarity with *Alcanivorax venustensis* ISO4 and less than 97% for the other species described.

As the percentage of similarity of the 16S rRNA gene sequences between strains ALC70 and ALC75 and *A. venustensis* ISO4 was above 97%, the usual threshold for species delimitation, I performed phylogenetic analysis of conserved marker genes to assess if the two strains belong to the species *A. venustensis*. As the genome of *A. venustensis* ISO4 was not available, I determined its sequence for further comparisons (Figure 2.2). The results showed that both strains were grouped and separated from the others previously described *Alcanivorax* species, including *A. venustensis*.

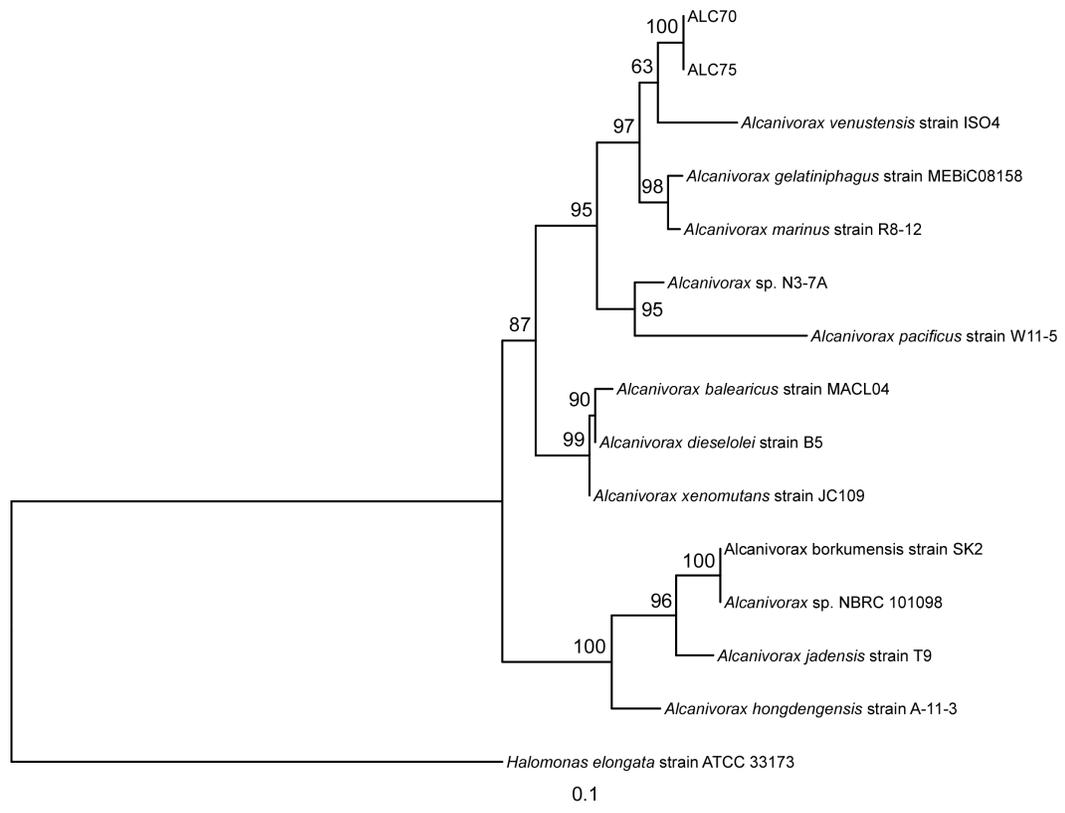


Figure 2.1: Phylogenetic tree highlighting the position of the strains ALC70 and ALC75, relative to the other type species within the *Alcanivorax* genus. The tree has been inferred from 1313 aligned characters of the 16S rRNA gene sequence under the maximum likelihood criterion and rooted with *Halomonas elongata*. The branches are scaled in terms of the expected number of substitutions per site. Numbers above branches are support values from 550 bootstrap replicates.

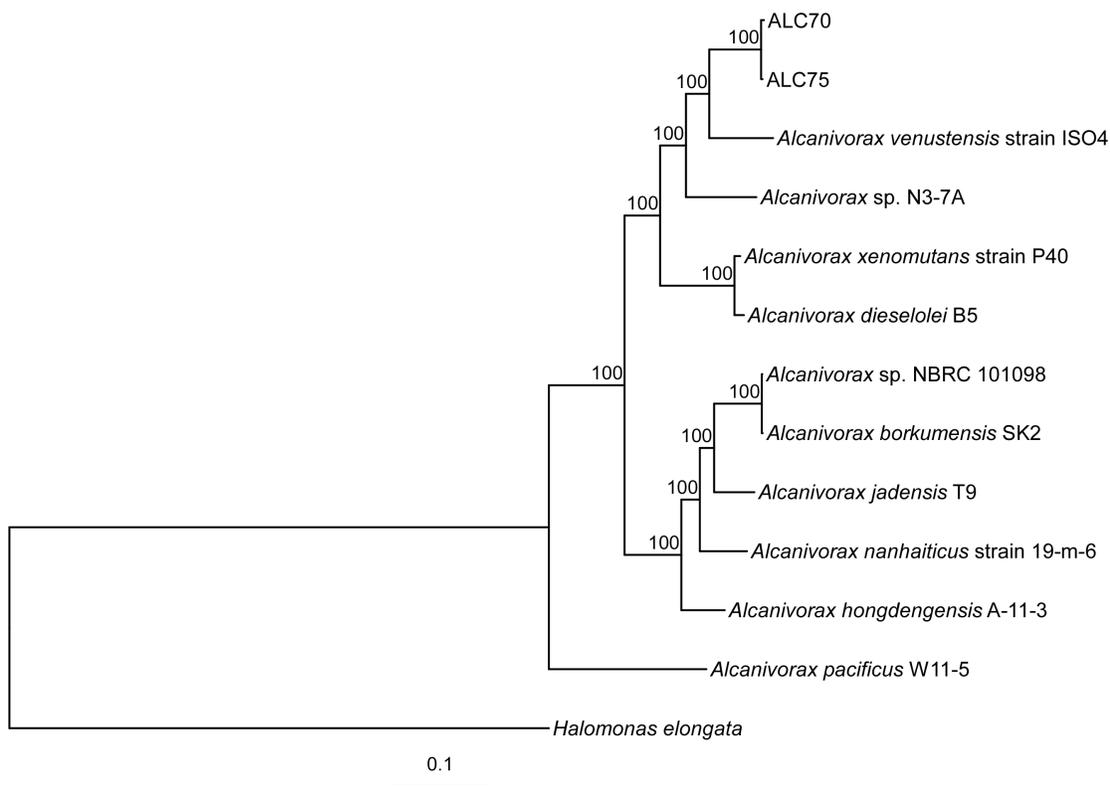


Figure 2.2: Phylogenetic tree using a partitioned maximum likelihood analysis concatenating 107 essential single-copy genes extracted from the genome of *A. marisrubri* ALC70 and ALC75 relative to the other type species within the *Alcanivorax* genus whose genome is determined. The tree was built with the software bcgTree (Ankenbrand and Keller 2016) and rooted with *Halomonas elongata*. Numbers at nodes designate bootstrap support values resulting from 100 bootstrap replicates.

The genomic characteristics of strains ALC70 and ALC75 were similar in term of GC% at ~66 mol% and slightly different in term of size with 4.2 and 4.5 Mb for ALC70 and ALC75, respectively. Compared to the others species described, they have GC percentages (66.17% and 65.95%) higher that those of the other described *Alcanivorax* species (in the range 54.7-64.7%) and their genome size was amongst the largest. The CDS content is proportional to the size of the genome as is the case for the other species ($\cong 1$ CDS/Mb) (Table 2.1).