

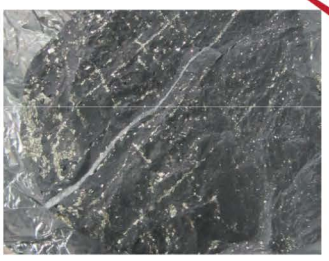



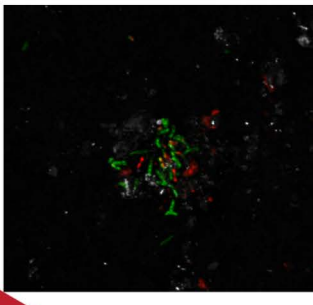
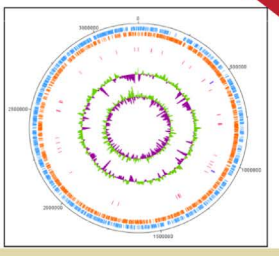

Tânia de Jesus Travassos Leandro

# Microbial diversity and anaerobic metabolisms in the subsurface of the Iberian Pyritic Belt

Tese de doutoramento em Biociências, especialização em Microbiologia,
orientada pelo Professor Doutor Ricardo Amils Pibernat e pelo Professor Doutor Milton Simões da Costa, co-orientada pelo Professor Doutor José Luis Sanz
e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Janeiro 2018

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Universidade de Coimbra

Departamento de Ciências da Vida
Faculdade de Ciências e Tecnologia
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# Microbial diversity and anaerobic metabolisms in the subsurface of the Iberian Pyritic Belt

Tânia de Jesus Travassos Leandro

Tese de Doutoramento apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biociências, especialização em Microbiologia, realizada sob a orientação científica do Professor Doutor Ricardo Amils Pibernat (Centro de Biologia Molecular Severo Ochoa (CSIC-UAM), Departamento de Virologia e Microbiologia, Universidade Autónoma de Madrid) e do Professor Doutor Milton Simões da Costa (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra) e co-orientação do Professor Doutor José Luis Sanz (Departamento de Biologia Molecular, Universidade Autónoma de Madrid)

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**2018**

Front Cover: Photograph images related to the environment area under study and techniques used in this thesis. (From left to right) Peña de Hierro pit lake; Río Tinto headwaters at Peña de Hierro; rock core recovered from the deep subsurface at Peña de Hierro depicting pyrite minerals; anaerobic bottle with enrichment culture; anaerobic chamber; culture plate of bacterial strain T2.26MG-19.2B, isolated from rock samples; CARD-FISH image of bacterial (green) and archaeal (red) cells enriched from a rock core sample (culture T1.2MG); representation of the circular genome of *Tessaracoccus* sp. strain T2.5-30 isolated from a rock core sample.

Este trabalho foi realizado entre o Centro Biologia Molecular Severo Ochoa (CSIC-UAM), Departamento de Virologia e Microbiologia, Universidade Autónoma de Madrid, Espanha e no Centro de Neurociências e Biologia Celular (CNC), Universidade de Coimbra, Portugal, ao abrigo da bolsa de Doutoramento financiada pela Fundação para a Ciência e Tecnologia (SFRH/BD/87076/2012) no âmbito do Programa Operacional Potencial Humano do Quadro de Referência Estratégico Nacional (POPH-QREN) e com o apoio financeiro do projecto ERC Advanced grant IPBSL-250350.

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# Table of Contents

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# Abstract

Río Tinto is an acidic river that originates at Peña de Hierro (Spain) and is characterized by its low pH values, high concentration of ferric iron, sulfate and heavy metals. Peña de Hierro, is localized in the Iberian Pyritic Belt (IPB), a geological formation that hosts one of the largest massive sulfide ore deposits known. Previous studies on the geomicrobiology of the deep subsurface of Río Tinto aquifer at Peña de Hierro have indicated the occurrence of microorganisms inhabiting rocks in the deep subsurface. Recent drilling studies, reaching deeper into the subsurface of the IPB, produced two boreholes. Gases such as hydrogen, carbon dioxide and methane as well as anions such as the nitrate, nitrite, acetate, formate, propionate were detected within both boreholes.

This study focused as a starting point on culture-dependent methods to investigate the metabolic capacity of microbial populations inhabiting rock cores retrieved from several depths along both boreholes. The establishment of enrichment cultures designed to promote methanogenic and nitrate reducing activities resulted in methane production activity and nitrate reduction activity, respectively, from diverse rock samples of both boreholes. Positive enrichment of microorganisms with either metabolic capacity suggested that these pathways may potentially play a role in supporting microbial populations within the subsurface.

Culture-dependent techniques were also applied to isolate and identify the culturable microbial diversity enriched from selected cultures with methane production or nitrate reducing activity. These studies resulted in the isolation of several members of the domain *Bacteria*, belonging to the phyla *Actinobacteria, Proteobacteria, Firmicutes* and *Bacteroidetes*. Facultative anaerobic and strict anaerobic strains were identified as affiliated with bacterial strains known for their metabolic capacity for fermentative metabolism, nitrate reduction, sulfate reduction, iron reduction and acetogenesis. Additionally, an isolation assay with selected methane-producing enrichment cultures, was also performed with focus on the isolation of methanogenic archaea. So far, this study has resulted in the co-culture of an archaeal strain affiliated with the genus *Methanosarcina* in culture with a bacterial strain.

Three enrichment cultures with active methane production were selected to further study the diversity of microbial populations enriched under methanogenic conditions by 16S rRNA gene pyrosequencing. Data retrieved indicated the enrichment of additional bacterial populations not identified by culture-dependent assays. Bacteria affiliated with known sulfate reducing, fermentative, nitrate reducing and acetogenic

bacteria were identified. This study identified the enrichment from rock cores of archaeal populations affiliated with members of the orders *Methanosarcinales* and *Methanocellales*. So far, this is the first report of the presence of members of *Methanocellales* from a deep terrestrial subsurface environment.

Both culture-dependent studies and culture-independent applied methods revealed the occurrence of diverse subsurface rock dwelling microbial populations which may potentially play an important role in the cycling of carbon, nitrogen, sulfur and iron elements through the subsurface ecosystem.

Integrative analysis of data within the IPB drilling project indicated that members of the genus *Tessaracoccus* are widely distributed in the subsurface. To further access metabolic potential of members of this genus isolated from the deep subsurface, *Tessaracoccus* sp. strain T2.5-30, isolated in this study from BH10 at 138.5 meters below surface, was selected for whole genome sequencing. Analysis of the annotated genome suggested potential for heterotrophic growth. A tentative complete pathway for fixation of inorganic carbon, as well as the capacity for nitrate reduction with ammonia formation and fermentative pathways, were found encoded in the genome.

Further studies with enrichment cultures established under methanogenic conditions resulted in the establishment of three subcultures. Preliminary studies based on in situ hybridization, electron microscopy and cloning indicated the co-enrichment in bacterial and archaeal populations. Identification of microbial diversity by 16S rRNA gene cloning for each culture identified so far the presence of bacteria affiliated with either the genus *Paenibacillus, Sphingomonas, Rhodoplanes* and an archaeon affiliated with the genus *Methanobacterium*.

**Keywords:** Continental terrestrial deep subsurface, Iberian Pyritic Belt, rock cores, enrichment cultures, methanogenesis, nitrate reduction, microbial isolation, 16S rRNA gene, microbial diversity, microbial genome.

# Resumo

O Río Tinto tem origem na Peña de Hierro (Espanha), sendo caracterizado pelo seu pH acídico, elevadas concentrações de ferro férrico, sulfato e metais pesados. A área da Peña de Hierro está localizada na Faixa Piritosa Ibérica (FPI) que consiste numa formação geológica que inclui um dos maiores sulfuretos maciços conhecidos. Estudos geomicrobiológicos prévios do subsolo profundo, no aquífero do Río Tinto na Peña de Hierro, indicam colonização microbiana de rochas. A perfuração recente de dois furos resultou no alcançe de uma maior profundidade no subsolo da FPI. Gases como hidrogénio, dióxido de carbono e metano, bem como aniões nitrato, nitrito, acetato, formato e propionato foram detectados em ambos os furos.

O nosso estudo recorreu como ponto de partida à aplicação de métodos dependentes de cultura, para investigar a capacidade metabólica de populações microbianas, que habitam a zona central de rochas de diferentes profundidades em ambos os furos. O estabelecimento de culturas de enriquecimento desenhadas para promover atividades metanogénicas ou de redução de nitrato, resultou na produção de metano ou redução de nitrato, respetivamente, em diversas rochas de ambos os furos. O enriquecimento de microrganismos com a respectiva capacidade metabólica investigada, sugere que essas vias metabólicas podem potencialmente desempenhar um papel no suporte de populações microbianas no subsolo.

Técnicas dependentes de cultura foram também aplicadas para isolar e identificar a diversidade microbiana cultivável, enriquecida a partir de culturas selecionadas com produção de metano ou redução de nitrato. Estes estudos resultaram no isolamento de vários membros do domínio *Bacteria* pertencentes aos filos *Actinobacteria, Proteobacteria, Firmicutes* e *Bacteroidetes*. Foram identificadas estirpes anaeróbias facultativas e anaeróbias estritas que apresentam maior similaridade com estirpes bacterianas com capacidade metabólica descrita para fermentação, redução de nitrato, de sulfato, de ferro férrico ou acetogénese. Adicionalmente foi realizado um ensaio focado no isolamento de arqueas metanogénicas a partir de culturas de enriquecimento com produçao de metano ativa. Até ao momento, este estudo resultou na co-cultura de uma arquea associada filogeneticamente ao género *Methanosarcina* em cultura com uma estirpe bacteriana.

Foram selecionadas três culturas de enriquecimento com produção ativa de metano para uma caracterização adicional da diversidade microbiana enriquecida em condições metanogénicas, recorrendo à pirosequenciação do gene 16S rRNA. Os dados indicam o enriquecimento adicional de populações bacterianas não identificadas por ensaios dependentes de cultura. Foram identificados organismos relacionados

com bactérias descritas como redutoras de sulfato, fermentativas, redutoras de nitrato ou acetogénicas. Este estudo resultou ainda na identificação de arqueas filogeneticamente relacionadas com membros das ordens *Methanosarcinales* e *Methanocellales.* Até ao momento, este é a primeira descrição da presença de membros da ordem *Methanocellales* num ambiente subterrâneo profundo.

Ambos os estudos dependentes e independentes de cultura revelaram a ocorrência de diversas populações microbianas subterrâneas que podem potencialmente desempenhar um papel importante no ciclo do carbono, nitrogénio, enxofre e ferro através do ecossistema subterrâneo.

A análise integrativa de dados no projeto de perfuração da FPI indicou que membros do género *Tessaracoccus* estão amplamente distribuídos no subsolo. Para investigar o potencial metabólico de membros deste género, isolados a partir do subsolo profundo, a estirpe *Tessaracoccus* sp. T2.5-30, isolada a partir do furo BH10 a 138,5 metros abaixo da superfície, foi selecionada para sequenciação integral do genoma. A análise e anotação do genoma sugere um potencial para crescimento heterotrófico. Uma possível via para a fixação de carbono inorgânico, assim como a capacidade de redução de nitrato com formação de amónia e vias fermentativas, foram encontradas codificadas no genoma.

Estudos adicionais com culturas de enriquecimento estabelecidas em condições metanogénicas resultaram em três subculturas. Resultados preliminares baseados em hibridação in situ, microscopia eletrónica e clonagem indicam o co-enriquecimento de populações bacterianas com arqueas. A identificação da diversidade microbiana através da clonagem do gene 16S rRNA para cada cultura, identificou a presença de bactérias afiliadas com os géneros *Paenibacillus, Sphingomonas, Rhodoplanes* e uma arquea relacionada filogeneticamente com o género *Methanobacterium.*

**Palavras-Chave:** Subsolo terrestre continental, Faixa Piritosa Ibérica, rochas, culturas de enriquecimento, metanogénese, redução de nitrato, isolamento microbiano, gene 16S rRNA, diversidade microbiana, genoma microbiano.

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| **ABC-transporter** | ATP-binding cassette transporter |
| **ANI** | Average Nucleotide Identity |
| **BLAST** | Basic Local Alignment Search Tool |
| **CARD-FISH** | Catalyzed Reporter Deposition - Fluorescent in situ Hybridization |
| **CBB cycle** | Calvin-Benson-Bassham cycle |
| **CDS** | Coding DNA sequence |
| **$CH_4$** | Methane |
| **$CO_2$** | Carbon dioxide |
| **COG** | Clusters of orthologous genes |
| **CRISPR** | Clustered Regularly Interspaced Short Palindromic Repeats |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DNRA** | Dissimilatory nitrate reduction to ammonia |
| **EDX** | Energy-dispersive X-ray spectroscopy |
| **EMP** | Embden Meyerhof Parnas |
| **EPS** | Extracellular polymeric substances |
| **FISH** | Fluorescence in situ Hybridization |
| **GEI** | Genomic Islands |
| **GGD** | Genome-to-genome distance |
| **$H_2$** | Hydrogen |
| **IC** | Ion chromatography |
| **IPB** | Iberian Pyritic Belt |
| **IPBSL** | Iberian Pyrite Belt Subsurface Life project |
| **MARTE** | Mars Analogue Research and Technology Experiment |
| **mbs** | Meters below the surface |
| **MCR** | Enzyme methyl-coenzyme M reductase |
| ***mcrA*** | Gene coding for the subunit A of MCR protein |
| **$N_2$** | Nitrogen gas |
| **$N_2O$** | Nitrous oxide |
| **NaBr** | Sodium bromide |
| **NO** | Nitric oxide |
| **$NO_2^-$** | Nitrite |
| **$NO_3^-$** | Nitrate |
| **$O_2$** | Oxygen |

| | |
|---|---|
| **OTU's** | Operational Taxonomic Units |
| **$PP_i$** | Pyrophosphate |
| **PTS-transporter** | Phosphotransferase system transporter |
| **rRNA** | Ribosomal RNA |
| **SEM** | Scanning Electron Microscopy |
| **$SO_4^{2-}$** | Sulfate |
| **TCA cycle** | Tricarboxylic acid cycle |
| **TEM** | Transmission Electron Microscopy |
| **tRNA** | Transfer RNA |
| **VSC** | Volcanic Sedimentary Complex |

# Chapter 1

## General Introduction

## 1.1. The deep subsurface - exploring the deep dark biosphere

The deep subsurface biosphere contemplates the collection of ecosystems and correspondent organisms inhabiting the deep layers of sediment and rock beneath ocean floor and beneath soils on the continental surface (Madsen 2008).

In the last decades there was an increase in publications related with the deep subsurface, which translates the increasing recognition of the importance of microbial life in such environments. It has been suggested that the deep subsurface harbors a significant fraction of the total microbial biomass on Earth, as estimated initially by a first global study of Whitman *et al.* (Whitman *et al.* 1998) and subsequent studies in subseafloor sediments (Kallmeyer *et al.* 2012) and in groundwater (McMahon and Parnell 2014).

Indeed, studies on the deep subseafloor (Bale *et al.* 1997; Schippers *et al.* 2005; Roussel *et al.* 2008; Smith *et al.* 2011; Orsi *et al.* 2013; Ciobanu *et al.* 2014), as well as studies on the continental terrestrial subsurface, such as aquifer groundwater (Kotelnikova and Pedersen 1998; Takai *et al.* 2001b; Haveman and Pedersen 2002; Hallbeck and Pedersen 2012; Pedersen 2013) have demonstrated the existence of metabolic viable and active microorganisms living in the deep. Although investigations are beginning to shed some light on the diversity and metabolic potential in these ecosystems, the deep subsurface still remains one of the least understood environments on Earth.

Studying the deep subsurface is a challenging process due to difficulties with accessing and sampling this type of environment, as well as the expenses involved in the process. Therefore, the great majority of subsurface locations around the planet still remain unexplored (Edwards *et al.* 2012; Orcutt *et al.* 2013).

Several studies on subseafloor sediments and the igneous oceanic crust have been conducted within programs such as the Integrated Ocean Drilling Project (IODP), or its predecessor Ocean Drilling Program (ODP) (Edwards *et al.* 2012). On the continental terrestrial subsurface, microbial ecology studies have been performed around the world on several locations at various depths. Some examples are the case of deep subsurface groundwater studies at Fennoscandian Shield in Europe (Kotelnikova and Pedersen 1998; Haveman *et al.* 1999; Haveman and Pedersen 2002; Hallbeck and Pedersen 2008; Itävaara *et al.* 2011; Hallbeck and Pedersen 2012; Nyyssonen *et al.* 2014; Wu *et al.* 2016a), in South Africa (Takai *et al.* 2001a; Gihring *et al.* 2006; Chivian *et al.* 2008; Borgonie *et al.* 2011; Lau *et al.* 2014; Magnabosco *et al.* 2016), in the Canadian Shield in North America, as well as in deep mines and other locations in The United States (Stevens and McKinley 1995; Lehman *et al.* 2001;

Chapelle *et al.* 2002; Onstott *et al.* 2009; Swanner and Templeton 2011; Osburn *et al.* 2014) or deep subsurface groundwater studies in Japan (Shimizu *et al.* 2006; Fukuda *et al.* 2010; Suzuki *et al.* 2014; Ino *et al.* 2016). Some deep subsurface studies, besides analysis of groundwater, have also investigated microbial diversity in rock core samples or deep mine soil samples (Russell *et al.* 1994*;* Zhang *et al.* 2005*;* Lehman *et al.* 2001; Sahl *et al.* 2008; Cockell *et al.* 2009; Rastogi *et al.* 2009; Rastogi *et al.* 2010). In general, in the majority of all studied subsurface environments, both below sea and below continent, it has been demonstrated that microbial life is ubiquitous (Colwell and D'Hondt 2013).

Drilling studies have many times taken advantage of previous existing deep mines (e.g. gold mines in South Africa), which have allowed reaching lower depths (several kilometers) into the subsurface and reduced the cost of the process (Chivian *et al.* 2008; Borgonie *et al.* 2011; Magnabosco *et al.* 2016).

In the majority of deep continental subsurface studies, sampling involved the collection of groundwater rather than rock sampling. The nature of samples can influence the results obtained when characterizing deep subsurface environments. The free-living planktonic community carried with water can have a distinct composition from the populations inhabiting rocks (Colwell and D'Hondt 2013). Furthermore, the study of microbial communities on rocks may also support a real understanding of the microbial community interaction with the local geology.

Recovering of rock cores is a more troublesome process as additional risk exists of introducing contamination. However, continuous development of several methodologies has improved the sampling and controls over contamination in rock core drilling procedures (Wilkins *et al.* 2014).

Understanding the geomicrobiological composition, metabolic capabilities and strategies to face the challenges of live in the deep, dark and anaerobic biosphere, as well as uncovering interactions between populations in subsurface habitats has an undeniable value. Furthermore, it is also expected that microbial activities operating in the deep subsurface might have an important impact on the biogeochemical cycles of various elements (e.g. carbon, nitrogen, sulfur...), yet little is known about their role. Exploration of subsurface environments also offers the opportunity to reveal life diversity that might not have yet been reported from other environments.

Thus, uncovering the microbial diversity in the deep, the physiological and metabolic processes exploited by microbial populations will elucidate how versatile life can be to inhabit, thrive or survive in such harsh environments. It also poses a chance for better understanding processes that could have supported life on early Earth (Bomberg and Ahonen 2017). Deep subsurface studies, could in turn provide clues as

how life might possibly exist not only in other planets or moons in our solar system but elsewhere in the universe. Within, our solar system, good candidates such as the planet Mars, Jupiter's moon Europa or Saturn's moon Enceladus, are being investigated to search for conditions and clues that could support life (Moissl-Eichinger *et al.* 2016; Deamer and Damer 2017; Fox and Strasdeit 2017).

Within our own planet we have seen that microbial life is very robust, having been found in several extreme environments which previously we thought incompatible with life (Pikuta *et al.* 2007). Therefore, it is conceivable that life might also have found a foothold on other worlds, and the deep surface environment might have provided a place for its development. In our own planet, during early Earth formation the surface was heavily bombarded with asteroids and comets that would frequently sterilize the surface. It has been hypothesized that deep subsurface environments might have provided important shelter for life in that period (Cockell 2006). Hence, these ecosystems are also useful models for life on the early Earth and as a model of life as it could occur in other planets.

Understanding the geomicrobiological composition on the deep subsurface environments also has important socioeconomical impact. In many cases studies on the deep terrestrial subsurface have been promoted by interests of anthropogenic activities such as mining (e.g. metals, minerals of economical value), quality of groundwater supplies or disposal of radioactive wastes, just to mention a few (Pedersen 2000; Edwards *et al.* 2012). These activities are influenced by how microbial activity can change the local geophysicochemical proprieties of the subsurface.

## 1.2. Aerobic and anaerobic metabolisms in the dark - energy production and carbon assimilation

In subsurface environments at shallower depths or where water moves through fractures of rocks, oxygen might enter the system and support aerobic metabolisms. Nevertheless, this oxygen will be rapidly depleted by aerobic organisms on the upper layers generating anoxic conditions (see section 1.4). In dark environments, metabolisms such as oxygenic or anoxygenic photosynthetic processes would not occur. So, in anaerobic and dark environments as the deep subsurface, anaerobic respiration and fermentative metabolism constitute possible strategies for energy generation.

In respiratory metabolisms (aerobic or anaerobic), oxidation of an inorganic or organic molecule (electron donor) is associated with the reduction of an oxidized compound that acts as the electron acceptor. This proceeds through a membrane associated electron transport chain where, through a series of redox reactions, electrons are transferred from reduced to oxidized compounds, until a final terminal acceptor. Transfer of electrons is coupled with release of energy which is used to pump protons or sodium ions across the membrane creating an electrochemical gradient. Energy can be conserved from the resulting proton or sodium gradient for ATP formation by proton or sodium ion driven ATP synthases (Hogg 2005).

While in aerobic respiration, oxygen acts as terminal electron acceptor, in the case of anaerobic respiration, in the absence of oxygen ($O_2$), other molecules act as terminal electron acceptors, such as nitrate ($NO_3^-$), manganese ($Mn^{4+}$), ferric iron ($Fe^{3+}$), sulfate ($SO_4^{2-}$) or carbon dioxide ($CO_2$). If the electron donor is an organic molecule, the microorganisms can be designated as chemoorganotrophs, while if the electron donor is inorganic the microorganisms are named chemolithotrophs. Molecules such as hydrogen ($H_2$), hydrogen sulfide ($H_2S$), ferrous iron ($Fe^{2+}$) and ammonia ($NH_3$) are known inorganic donors (Madigan *et al.* 2012).

There are several types of anaerobic respiratory processes such as reduction of ferric iron into ferrous iron, nitrate reduction, reduction of sulfate or other oxidized sulfur compounds, methanogenesis ($CO_2$ reduction) or acetogenesis ($CO_2$ reduction) (Madigan *et al.* 2012).

In the case of fermentation, cells use organic compounds both for generation of energy (chemoorganotrophs) as well as a source of carbon. Nevertheless, in fermentation, ATP is produced directly from energy rich intermediates generated during the catabolism of a fermentable source in a process termed substrate level phosphorylation. In this process, in the majority of cases a substrate acts as both the electron donor and acceptor (oxidation of the substrate with the reduction of a intermediate oxidation product) (Müller 2001; Madigan *et al.* 2012). Some examples of substrates fermented are the case of sugars, organic acids, some amino acids, purines and pyrimidines. Fermentation products vary dependent on the substrate fermented but the most prevalent are organic acids, alcohols, hydrogen or carbon dioxide (Müller 2001; Madigan *et al.* 2012). Byproducts of fermentative metabolism can in turn be used by other populations of microorganisms as a substrate for energy or as carbon source.

Regarding the sources of carbon to fuel biosynthetic pathways in the cell, they could be supplied either by assimilation of organic compounds or through fixation of inorganic carbon. If the carbon source is organic, the organisms are termed heterotrophs. In turn, if the carbon source is inorganic the organisms are termed

autotrophs. Most of the times, chemolithotrophy coincides with autotrophy, thus autotrophic microorganisms using inorganic substrates as energy sources are designated as chemolithoautotrophs (Madigan *et al.* 2012). Some microorganisms while using inorganic compounds as a source of energy (chemolithotrophs) may require an organic compound as a source of carbon (mixotrophs).

## 1.3. Overview of methane metabolism and nitrate reduction metabolisms

### 1.3.1. Methane metabolism

Two key groups of organisms are involved in methane ($CH_4$) metabolism, the methanogens and methanotrophs. Methanogens operate an anaerobic respiratory process named methanogenesis, which results in the production of methane as the end byproduct, while methanotrophs use methane as energy and carbon source (Madigan *et al.* 2012).

All known methanogens belong to the domain *Archaea*. The majority of known methanogens constitute a deep branching within the phylum *Euryarchaeota* and are assigned into seven orders (i.e. *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales* and *Methanomassiliicoccales*) (Sakai *et al.* 2008; Borrel *et al.* 2013; Hedderich and Whitman 2013; Iino *et al.* 2013). Methane production can occur from several substrates including acetate (acetoclastic methanogens), from $H_2$ + $CO_2$ (hydrogenotrophic methanogens, $H_2$ is a major electron donor for $CO_2$ reduction but some microorganisms can also use other substrates as formate or some alcohols) and from other methylated compounds as methanol, methylamines or methyl sulfides among others (methylotrophic methanogens) (Hedderich and Whitman 2013; Costa and Leigh 2014). Interestingly, recent findings are reshaping our current knowledge on methanogenic archaea, demonstrating that methanogenic metabolism might be phylogenetically widespread within the *Archaea* domain, with the detection of genes involved in methane metabolism in members of the recent discovered phyla "*Candidatus* Bathyarchaeota" and "*Candidatus* Verstraetearchaeota" (Evans *et al.* 2015; Vanwonterghem *et al.* 2016).

Methanotrophs do not constitute a defined clade, they are distributed between many groups. Aerobic methanotrophs that couple assimilation of methane with oxygen reduction are widely known and belong to the classes *Gammaproteobacteria,*

*Alphaproteobacteria* and phylum *Verrucomicrobia* (Dunfield *et al.* 2007; Trotsenko and Murrell 2008). Anaerobic known methanotrophs comprise NC10 bacterium *Methylomirabilis oxyfera* (the only recognized anaerobic methanotrophic bacteria with an intracellular aerobic oxidation of methane coupled to nitrite dismutation) and also the anaerobic methane oxidizing archaea (ANME) (Cui *et al.* 2015; Timmers *et al.* 2017). The mechanisms of anaerobic methane oxidation by ANME are not fully understood. While methane oxidation by ANME has been suggested to be coupled to sulfate and nitrate reduction activity of bacterial partners, recently it has also been shown that in some cases ANME can perform solely sulfate or nitrate reduction metabolism (Costa and Leigh 2014; Cui *et al.* 2015; Rotaru and Thamdrup 2016; Scheller *et al.* 2016).

The enzyme methyl-coenzyme M reductase (MCR), encoded by the *mcr* gene, is responsible for the catalysis of the last step of all known methanogenic pathways, and also occurs in methanotrophs (Hedderich and Whitman 2013; Timmers *et al.* 2017). The amplification of *mcrA* gene, which codifies for the subunit A of MCR enzyme, has been used to infer phylogenetic relationships in molecular ecology studies in combination with amplification of 16S ribosomal RNA (rRNA) gene (Springer *et al.* 1995; Hales *et al.* 1996; Luton *et al.* 2002).

### 1.3.2. Nitrate reduction

Nitrate reduction can occur through a pathway designated dissimilatory nitrate reduction to nitrogen (denitrification) or by dissimilatory nitrate reduction to ammonia (DNRA). In the complete denitrification pathway, nitrate ($NO_3^-$) is sequentially reduced, in separate reactions, to nitrite ($NO_2^-$), nitric oxide (NO), nitrous oxide ($N_2O$) up to nitrogen ($N_2$), with each reaction being catalyzed by a specific enzyme (Zumft 1997). Not all denitrifiers present the four enzymes involved in the process. Denitrifiers can thus be classified as complete denitrifiers (reduce both $NO_3^-$ and $NO_2^-$ up to $N_2$), exclusive nitrite reducers (can only reduce $NO_2^-$ and not $NO_3^-$ up to $N_2$), incomplete denitrifiers (reduce $NO_3^-$ incompletely up to $NO_2^-$, NO or $N_2O$) or as nitrite reducers (reduce $NO_2^-$ incompletely up to NO or $N_2O$) (Lu *et al.* 2014). The pathway of dissimilatory nitrate reduction to ammonia differs from denitrification pathway with a sequential reduction of nitrate and/or nitrite into ammonia (Shapleigh 2013).

The majority of denitrifiers are facultative anaerobes, only using oxidized nitrogen compounds when oxygen is not available. The electron donors can be either organic compounds or inorganic (e.g. hydrogen, reduced sulfur compounds). With respect to the carbon source, microorganisms might present a heterotrophic,

autotrophic or mixotrophic metabolism (Shapleigh 2013; Lu *et al.* 2014). Many heterotrophic chemoorganotrophic denitrifiers are recognized (e.g. *Pseudomonas* spp., *Shewanella* spp., *Lactobacillus* spp., among others), which is also a reflection of their ubiquitous habitat distribution (Shapleigh 2013). However, fewer chemolithoautotrophic denitrifiers are known. Examples of chemolithoautotrophic denitrifiers are the case of species such as *Sulfurimonas paralvinellae*, *Sulfurimonas denitrificans*, *Paracoccus denitrificans* or *Thiobacillus denitrificans*, among others (Shao *et al.* 2010; Lu *et al.* 2014).

## 1.4. Life in the deep subsurface - factors conditioning the occurrence of life

The occurrence, diversity and challenges encountered by life in deep subsurface habitats will vary depending on the type of environment. Nevertheless, some of the main factors that can condition life are the presence of water, the availability of energy and carbon sources, the existence of physical space for colonization and temperatures within values compatible with life (Madsen 2008; Akob and Küsel 2011).

### 1.4.1. Water availability and physical space

The presence of water is essential for biochemical reactions to occur. Water availability is dependent on the permeability of the rocks, which in turn is determined by its porosity and if fractures or faults are present (Wicander and Monroe 2006).

Sedimentary rocks are more porous than igneous and metamorphic crystalline rocks, while both of the latter can often present fractures and shears, which increases permeability. On another hand, strongly cemented sedimentary rocks presents little porosity (Wicander and Monroe 2006). Thus, water movement will vary accordingly with the geological features.

Both factors, porosity and fracturing, determine the flow of water through the deep subsurface and in turn, also the transport of dissolved nutrients and energy sources. Microorganisms on the deep subsurface inhabit rock pores and fractures, where microbial life occurs in microniches, as microcolonies or biofilms (Akob and Küsel 2011).

The physical pore space within rocks can thus determine the space for microbial colonization. Higher porosity means more space available for colonization

and higher water availability. In the case of fractures and faults, since water flow might increase in these locations, higher biomass is also expected to be encountered (Akob and Küsel 2011; Edwards *et al.* 2012). Therefore, in rocks where the pore space is minimal and also at lower depths where pore space might be further reduced due to pressure (e.g. compaction) and other geological effects, the presence of fractures would represent important conducts of water into lower depths and thus determinant of microbial colonization (Wicander and Monroe 2006).

The residence time of water in the subsurface is dependent in the topography, on the geologic structures and the depth that water can reach since its path from recharge area to discharge areas. In deep continental basins the residence time of water can be even of thousands of years (Madsen 2008).

### 1.4.2. Energy and carbon sources

The deep subsurface is a dark and essentially anaerobic ecosystem. Oxygen carried by surface water would be essentially consumed at shallow depths by aerobic respiration processes, rendering deeper layers anaerobic. Organic carbon either carried through recharging groundwater into aquifers or buried within sedimentary rock also decreases with depth, becoming more recalcitrant. Therefore, farther from organic rich strata or petroleum reservoirs, the deep subsurface bedrock habitats are generally considered oligotrophic environments (Lovley and Chapelle 1995; Morita 1999; Hoehler and Jorgensen 2013). Thus in the deep subsurface, inorganic compounds seem to have a more critical role in supporting microbial populations (Stevens and McKinley 1995; Pedersen 1997). Indeed, in 1992 Thomas Gold first proposed the existence of a deep, vast chemolithoautotrophic biosphere (Gold 1992).

In deep, dark, oligotrophic and anaerobic environments, microorganisms living on oxidation/reduction of inorganic compounds provided by the local geochemistry and relying on the fixation of inorganic carbon would be expected to represent the base of the trophic chain in these ecosystems. The in situ metabolic activity of these primary producers could in turn sustain other heterotrophic populations (Stevens and McKinley 1995; Pedersen 1997; Pedersen 2000; Edwards *et al.* 2012).

Hydrogen can act as electron donor and has been suggested as a key energy source in the deep subsurface since it can be produced in situ from geochemical processes (Stevens 1997; Chapelle *et al.* 2002). Hydrogen can be produced by abiotic reactions such as mafic and ultramafic rock water interactions at low temperatures (Stevens 1997), diffusion of gases from the deep mantle or magma-water interactions (Stevens 1997), or radiolysis of water in habitats with radiation fluxes (Lin *et al.* 2005).

Methane and carbon dioxide can also be abiotically generated in the interior of our planet as well as from various types of water rock interactions and may also represent sustainable sources of carbon and energy to drive microbial life (Pedersen 2000; Sherwood Lollar *et al.* 2006).

Metabolisms such as hydrogenotrophic methanogenesis and acetogenesis uses as substrates hydrogen and carbon dioxide resulting in the production of biomass and organic byproducts as methane and acetate, respectively. Methanogens and acetogenic bacteria may represent important primary producers in deep subsurface environments (Pedersen 1997).

Sources of organic carbon might be present, as refractory ancient buried organic matter (kerogen) or from other sources as byproducts of chemolithoautotrophic metabolisms or as recycling of dead biomass. Organic carbon could be used either as energy source - through respiratory metabolisms or fermentation processes - or as carbon source for heterotrophic microorganisms. Nevertheless, if present they are considered scarce (Fredrickson *et al.* 1997; Fredrickson and Balkwill 2006; Wilkins and Fredrickson 2015).

Furthermore, other mineral associated electron donors (e.g. metal sulfides) if present in the subsurface can also fuel anaerobic respiratory metabolisms for energy production coupled to reduction of inorganic electron acceptors (e.g. nitrate, ferric iron).

Besides the effect of the local geochemistry on microbial growth, the presence of microorganisms itself also shapes the local environment. The products of metabolic activities and even the physical presence of microbial colonies influences the geophysicochemical proprieties of their habitats (Newman and Banfield 2002; Falkowski *et al.* 2008).

The energy generated by anaerobic metabolisms is lower in comparison to the energy obtained by surface microorganisms from aerobic respiration of organic compounds or photosynthetic processes. Therefore, in deep oligotrophic environments it has been hypothesized that slow turnover rates of nutrients and energy sources could result in highly slow growth rates, with generation times that could be from hundreds to thousands of years (Jørgensen 2011). Besides, the need for efficiently coupling between different biogeochemical cycles is fundamental.

### 1.4.3. Temperature influence

Temperature is a key factor that can limit the extent of life in the deep subsurface, as temperature increases with depth. High temperatures can compromise the integrity of the cellular membranes and other cellular components.

In the continental terrestrial subsurface, temperature is controlled by geothermal gradients, which in turn depend on the type of geological formations (Kashefi and Lovley 2003). The upper limit at which life was found so far to be able to survive is 122ºC, at conditions of both high temperature and high pressure (Takai *et al.* 2008). Considering that on average geothermal gradients increase 25ºC per kilometer, this means a limit for life to occur at around 5 km into the subsurface (Akob and Küsel 2011). This limit would be variable according to the geologic topography and could indeed extend up to 12 km in locations that host lower geothermal gradients (Omar *et al.* 2003).

Increasing depth in the subsurface is also associated with an increase in pressure. Since life has been found that could withstand pressures greater than the ones that exist in the subsurface, one can assume that pressure would not constitute a limit to the existence of life (Kato *et al.* 1998; Sharma *et al.* 2002; Itävaara *et al.* 2011). It is considered that temperature will likely set a limit for the existence of life before extremely high pressures are reached (Fredrickson and Onstott 1996).

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As discussed in this section, life might be subjected to various constrains that condition its presence and metabolic activity. Thus, it is expected that life might exhibit physiological and metabolic capabilities that will help it to cope with its surrounding environment, in order to optimize energy conservation for metabolic efficiency and maintenance of genetic and cellular integrity over time.

## 1.5. Diversity of life in the deep continental subsurface

The deep subsurface is a heterogeneous environment due to its geochemical and mineralogical variability. This also means that subsurface environments can present contrasting physical and chemical proprieties over very short distances. Thus, the occurrence of life and its diversity will ultimately reflect the conditions of each environment.

When exploring environmental diversity distinct methods either culture-dependent or culture-independent are used, having both advantages and disadvantages, as will be addressed in section 1.7, nevertheless, when combined, they provide a further complete picture of how the ecosystem operates.

Subsurface studies are beginning to shed some light on diversity and processes occurring on the deep. As mentioned before, most of the deep subsurface studies have been performed in groundwater ecosystems. Studies on microbial communities hosted in different subsurface environments have shown the presence of diverse bacterial phyla and also that besides the variability of bacterial populations in different environments, it is frequent the detection of microorganisms related to phyla *Proteobacteria* and *Firmicutes* as the dominant microbial communities as reported in studies on groundwater (Gihring *et al.* 2006; Zhang *et al.* 2006; Sahl *et al.* 2008; Itävaara *et al.* 2011; Nyyssonen *et al.* 2014), rock or mine soils (Zhang *et al.* 2005; Sahl *et al.* 2008; Rastogi *et al.* 2009). Archaea have also been detected in groundwater from several deep subsurface environments (Kotelnikova and Pedersen 1998; Takai *et al.* 2001a; Zhang *et al.* 2005; Gihring *et al.* 2006; Ciobanu *et al.* 2014; Nyyssonen *et al.* 2014).

Most of the studies of microbial diversity within deep subsurface environments are dominated by culture-independent techniques. Nevertheless, several microorganisms have been isolated from terrestrial deep subsurface (Boone *et al.* 1995; Kotelnikova *et al.* 1998; Motamedi and Pedersen 1998; Krumholz *et al.* 1999; Takai *et al.* 2001b; Hallbeck and Pedersen 2008). Isolated microorganisms, even if not representative of the dominant populations on the environment from which they were isolated, are still key for demonstrating that life on the deep is viable and represent also an opportunity for both physiological and metabolic studies of features that may enable their presence in the deep subsurface.

Microbial diversity studies by culture-independent techniques also revealed the presence of several bacterial and archaeal novel lineages (Takai *et al.* 2001a; Chivian *et al.* 2008; Osburn *et al.* 2014; Wu *et al.* 2016b). One example is the discovery in fracture waters recovered at 2.8 km in Mponeng gold mine (South Africa) of an

ecosystem constituted almost entirely by the firmicute "*Candidatus* Desulforudis audaxviator" (Chivian *et al.* 2008). This microorganism appears to obtain energy by reduction of sulfate, using hydrogen generated by water radiolysis and sulfate resulting from chemical oxidation of pyrite by oxygen species radiolytically generated. Genome sequencing have identified the presence of a complete pathway of dissimilatory sulfate reduction, the presence of hydrogenases and the pathway for $CO_2$ fixation via Wood–Ljungdahl pathway (Chivian *et al.* 2008). This ecosystem thus represents a case in which both carbon and energy sources could be supplied by the local geology.

Besides the detection of bacteria and archaea, the presence of fungi have also been reported in the deep terrestrial subsurface groundwater (Sohlberg *et al.* 2015). Although their actual role in such environments still remains mainly unknown, they may play an important role in weathering of rocks and in the degradation of refractory and ancient materials (Sohlberg *et al.* 2015). Even the presence of nematodes has also been reported in fracture water at 3.6 km in the deep gold mines of South Africa. They live in deep fractures subsisting through grazing on attached microbes (Borgonie *et al.* 2011; Borgonie *et al.* 2015).

The presence of viruses is widely recognized on subseafloor sediments (Engelhardt *et al.* 2014; Engelhardt *et al.* 2015) and has also been reported in studies on groundwater from deep continental terrestrial subsurface environments (Kyle *et al.* 2008; Eydal *et al.* 2009). The presence of viruses in deep subsurface environments might have an important impact on the fluctuations of microbial populations as a result of predation and may also play a role as vectors for horizontal gene transfer throughout the microbial communities (Anderson *et al.* 2013; Engelhardt *et al.* 2015).

## 1.6. Iberian Pyritic Belt and Río Tinto

The Iberian Pyritic Belt (IPB) is a geological formation that extends between the south of Spain and Portugal (250 x 20-70 km) (Inverno *et al.* 2015). The IPB hosts one of the largest sulfide ore deposits known (Leistel *et al.* 1998). The IPB consists of three main lithostratigraphic units: the Phyllite-Quartzite Group (PQG), dating from the late Devonian; the Volcanic Sedimentary Complex (VSC), dating from late Devonian and early Carboniferous; the Culm group also known as Baixo Alentejo Flysch Group (BAFG), dating from early to late Carboniferous (Gómez-Ortiz *et al.* 2014; Inverno *et al.* 2015).

The formation of the Volcanic Sedimentary Complex was associated with the volcanic activity trigged by tectonics during the Variscan Orogeny. Tectonism was also

in the origin of the hydrothermal activity (on early Carboniferous) that conducted to the formation of stockwork networks of pyrite and quartz and the massive sulfide ore deposits, hosted in the volcanic and sedimentary rocks (Inverno *et al.* 2015). The major minerals in the massive ores are pyrite (an iron sulfide), sphalerite (a zinc and iron sulfide), chalcopyrite (a copper iron sulfide) and galena (a lead sulfide), which also occur in stockwork ore in different proportions with a quartz-chlorite-sericite-carbonate assemblages (Inverno *et al.* 2015).

Río Tinto is an acidic river in the south of Spain and its origin is located at the core of the IPB at Peña de Hierro, an abandoned mine site. The river is characterized by its low pH (mean of 2.3) and high concentration in heavy metals, ferric iron and sulfate (González-Toril *et al.* 2010). Stratigraphy at Peña de Hierro is inverted as a result of tectonism-associated compression during the Variscan Orogeny that resulted in an inverted anticline that propagates along a 110º N thrusting front. This structure is intersected by NNE-SSW normal faults which are associated with the creation of Río Tinto acidic springs (Fernández-Remolar *et al.* 2008a). Geological composition of Peña de Hierro basement structure include the massive sulfide ore intrusions embedded into the older materials of the volcanic sedimentary complex which are emplaced on the younger Culm unit by the thrusting structure (Gómez-Ortiz *et al.* 2014).

It has been demonstrated that the extreme conditions observed in the river have an ancient natural origin, instead of being the result of 5000 years of mining activity in the area (Fernández-Remolar *et al.* 2008b; Gómez-Ortiz *et al.* 2014).

Along the river bed, iron oxides precipitate and deposit seasonally and with time they consolidate as ferric iron terraces. The massive ferric iron deposits in the older terraces date back to 2 million years, presenting evidence that the high concentrations of ferric iron and sulfate in river waters, that contributed to the progressive formation of these geological formations, is much older than the first mining activities in the area (Fernández-Remolar *et al.* 2003; Fernández-Remolar *et al.* 2005). In young terraces the common iron oxides associated with sulfates observed are namely hydronium jarosite, schwertmannite, copiapite, coquimbite, natrojarosite or gypsum; in older terraces predominates goethite and hematite (Fernández-Remolar *et al.* 2005; Amils *et al.* 2011).

Recently it has been demonstrated that the recharge area for Peña de Hierro aquifer is located northwest of Peña de Hierro pit lake. The proposed model describes that meteoric water percolates and flows southwards through normal faults intersecting the massive sulfide ore bodies. The presence of water and the rock matrix supports the existence of an underground reactor (see section 1.7) (Fernández-Remolar *et al.* 2008b). Metabolic activity of chemolithotrophic microorganisms oxidizing the iron

sulfide ore, of which pyrite ($FeS_2$) is the main sulfide, is in the origin of the acidic, sulfate and ferric iron rich waters. The same group of normal faults carries these acidic waters to the surface generating the artesian acidic springs in the area surrounding Peña de Hierro that feed Río Tinto headwaters (Gómez-Ortiz *et al.* 2014).

Intensive mining activity has exposed sulfide bodies to the atmosphere and left tailing deposits which cover many of the river springs. Although weathering of sulfides on rainy seasons produce acidic solutions that contribute to the river acidity upon mixing with subsurface artesian waters, it would not account for the constant acidic flow of the river in the dry season (Fernández-Remolar *et al.* 2008b; Gómez-Ortiz *et al.* 2014).

Therefore, the acidic nature of the water and the characteristic high concentration of ferric iron, sulfate and protons are explained as the byproducts of the oxidation of sulfide minerals (e.g. pyrite) on the subsurface (Amils *et al.* 2014).

## 1.7. Studies on the subsurface of the Iberic Pyritic Belt at Peña de Hierro

The first studies centered on exploration of Río Tinto subsurface were initially performed within the framework of the Mars Analogue Research and Technology Experiment – MARTE project (2003-2006), a collaboration project between NASA and Centro de Astrobiología in Madrid (Fernández-Remolar *et al.* 2008b).

Río Tinto is a known terrestrial analogue for some locations on Mars due to similar geochemical and mineralogical features (e.g. similarities between main sulfide bioleaching products on Río Tinto basin and sulfate and iron oxide deposits on Mars) (Amils *et al.* 2014). Although this project had its major focus on the development of new technology for the study of life on future Mars missions, it also aimed at characterizing subsurface life at Río Tinto, as well as the resources to support it and demonstrate if an underground bioreactor, supported by anaerobic oxidation of iron and sulfur minerals, was operating on the deep subsurface of Río Tinto aquifer (Stoker *et al.* 2005; Fernández-Remolar *et al.* 2008b).

Two deep boreholes, BH4 and BH8, each with 165 meters below surface (mbs) were drilled upslope of the Peña de Hierro pit lake intersecting the sulfide ore body and a third borehole BH1 (59 mbs) was drilled downslope (Fernández-Remolar *et al.* 2008b).

Analysis of rock leachates and analysis of water and gas samples recovered from MLDS (multi-lever diffusion sampler - placed in situ and analyzed at different time periods after drilling to allow equilibration of borehole fluids with the ones from

surrounding formations) indicated the presence of diverse compounds in the system that could support microbial metabolisms. Electron acceptors available include oxygen, nitrate, nitrite, ferric iron, sulfate and inorganic carbon, while possible electron donors detected were hydrogen, ferrous iron, methane and reduced sulfur (Amils *et al.* 2008; Fernández-Remolar *et al.* 2008b). The presence of high concentrations of sulfate indicates oxidation of sulfides and the presence of both ferric iron and ferrous iron suggested the occurrence of an active iron cycle. Oxygen was detected primarily above the water table. Consumption of oxygen in this area was suggested to induce anoxia in the deeper regions (Fernández-Remolar *et al.* 2008b; Puente-Sánchez *et al.* 2014a).

Enrichment cultures showed positive results for activity of iron and pyrite oxidation, anaerobic thiosulfate oxidation (using nitrite as electron acceptor), sulfate reduction and methanogenesis (Amils *et al.* 2008). Scanning Electron Microscopy with Energy-dispersive X-ray spectroscopy (SEM-EDX) analysis also indicated evidence for microorganisms attached, occurring in microniches on the mineral surface. Besides, fluorescence in situ hybridization studies also demonstrated the presence of viable and active bacteria on the rock cores at different depths along the veins and fractures that supply water to the system (Fernández-Remolar *et al.* 2008b; Puente-Sánchez *et al.* 2014a). Studies also, demonstrated prevalence of iron oxyhydroxides and sulfates, the byproduct of the alteration of sulfides (Fernández-Remolar *et al.* 2008b). Moreover, biological analysis of samples with multiple culture-independent techniques (e.g. antibody microarray, DNA microarray and cloning) demonstrated the presence of Gram positive bacteria from the phyla *Actinobacteria* and *Firmicutes* and indication of the presence of sulfate reducers, denitrifying and hydrogenotrophic bacteria; methane, metal and sulfur oxidizers and methanogenic archaea (Puente-Sánchez *et al.* 2014a). Additionally, aqueous sample extraction from BH4 and BH8 gave pH values between 5 and 7, while the pH values from water samples from BH1 were acidic with pH values lower than 3 (Fernández-Remolar *et al.* 2008b). This data supported the model for a natural origin of Río Tinto acidic waters as discussed in section 1.6, in which as water interacts with the massive sulfide ore bodies, the activity of oxidizing chemolithotrophic microorganisms results in biooxidation of sulfides and acidification of water that sources the acidic springs of Río Tinto (Fernández-Remolar *et al.* 2008b; Gómez-Ortiz *et al.* 2014).

Between 2011-2012 a second drilling campaign as part of the project Iberian Pyrite Belt Subsurface Life (IPBSL) (2011-2015) was performed to continue the research started with the previous MARTE project, in order to further characterize the microbial diversity on the deep subsurface of the IPB at Peña de Hierro and elucidate the interactions between microbial activities and the local geology. This project

attempted to recover more samples, reach lower depths into the aquifer and answer questions raised during the previous MARTE project.

An extensive geophysical study was previously conducted between 2010-2011 by a team of geologists to profile the Peña de Hierro area, identifying the occurrence of groundwater and sulfide bodies (Gómez-Ortiz *et al.* 2014).

Based on the data retrieved, two locations were chosen for the drilling of two boreholes, BH11 and BH10, one with 320 mbs and the other with 620 mbs, respectively (Figure 1.1) (Amils *et al.* 2014).

Borehole BH10 is located upslope of the pit lake at an elevation of 525 meters above sea level and settled on the volcano sedimentary complex, intersecting the massive sulfide ore. Mineralogy of B10 has been thoroughly characterized and comprise volcanoclastic breccias, zones of intense quartz-sericite-pyrite alteration, aphyric rhyolites, felsic volcaniclastic sandstone and siltstones, dacite and rhyodacite dome complexes, purple shale with lenses of jasper, dark shale with intercalations of volcaniclastic sandstone and lenses of pyrite. At 500 mbs occurs a transition from the volcano sedimentary complex to the Culm Group, where stratigraphy is dominated by tectonic and hydrothermal breccias, shale and graywacke (Amils *et al.* in preparation).

BH11 borehole is located at an elevation of 428 meters above sea level and is settled on the Culm younger deposits downslope of the pit lake, consisting of shale and sandstone with pyrite inclusions. The water table in BH10 was located at 90 mbs and in BH11 was located at 4 mbs (Amils *et al.* in preparation).

The IPBSL project in the footsteps of MARTE project also counted with the contribution of a multidisciplinary team in order to accomplish a more detailed overview of how life occurs in the subsurface and interacts with the geology. The work presented in this thesis was developed in the framework of the IPBSL project.

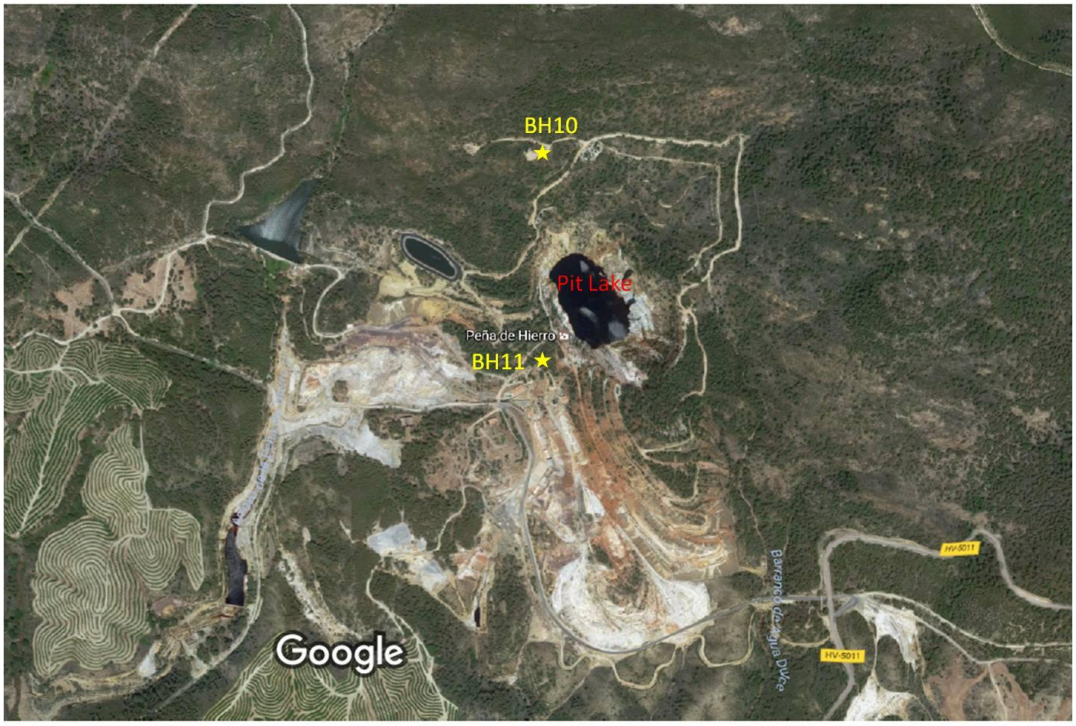


**Figure 1.1.** Satellite image of the Peña de Hierro area depicting the sites of boreholes BH10 and BH11 drilled in IPBSL project (yellow stars). Adapted from Goggle Maps 2017.

Following the knowledge obtained with drilling and rock sampling from the MARTE project, a chemical tracer, sodium bromide (NaBr) was used in drilling water and latter rock leachates were analyzed by ion chromatography to control if contamination occurred. In addition, in order to reduce and avoid contamination, core sections were recovered encased in plastic liners, flushed with nitrogen upon retrieval from the borehole. At the field laboratory, rock sampling for biological analysis was executed in anaerobic and aseptic conditions (Amils *et al.* 2014) by recovering the unexposed center of the cores.

Mineralogical analysis using X-ray diffractometer (XRD) revealed the presence of pyrite and its alteration products (e.g. hematite, magnesite) in both boreholes; elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS) of leachates shows the presence of iron and other metals (Amils *et al.* 2014). Analysis of rock leachates by ion chromatography (IC), besides control of contamination, were also used to estimate the resources available from the rock matrix. Ion chromatography showed the presence of inorganic anions such as nitrate, nitrite and sulfate, and also the presence of organic anions such as acetate, formate and propionate. Gas analysis

on core samples also indicated the presence of hydrogen, carbon dioxide and methane in both boreholes (Amils *et al.* 2014).

In the IPBSL project a diverse set of techniques, both culture-dependent and culture-independent, were applied into the study of the microbial diversity and its interaction with the local geology. Similar to what was performed in MARTE project, in order to demonstrate the metabolic capacity of microorganisms inhabiting the rock cores for selected metabolisms, seven activities were chosen for preparation of enrichment cultures: sulfate reduction, denitrification, methanogenesis, methanotrophy, acetogenesis and also iron reduction/oxidation. Positive activity was detected at several depths in both boreholes for all the tested metabolisms (Amils *et al.* 2013; Amils *et al.* 2014). The work developed on this thesis focused on the analysis of enrichment cultures that presented methane production - methanogenic enrichments - and the ones that presented nitrate reduction - denitrification enrichments. The following sub-sections addresses the general techniques applied in the development of this work.

### 1.7.1. Culture-dependent techniques

It is recognized that only around 1% of total diversity in an environment is possible to cultivate in laboratory conditions (Amann *et al.* 1995). Nevertheless, isolation in pure culture of microorganisms is an essential step for further biochemical, genetic and physiological studies for elucidation of the potential role of the microorganisms in the deep subsurface. Preparation of enrichment cultures is also an important tool to demonstrate the presence of microorganisms with capacity for a particular metabolic activity as well as a tool to increase biomass from low biomass samples.

### 1.7.2. Culture-independent techniques

Techniques independent on culture can be based on the analysis of nucleic acids and are essential tools in microbial ecology studies. They assist in the study of diversity, interactions and the metabolic potential of the populations without the necessity of having the organisms in pure culture on the laboratory.

Sequencing based techniques can target a specific molecule (e.g. massive sequencing of an amplified gene), study the genetic makeup of the population as a whole (metagenomic analysis and reconstruction of genomes from metagenomic data) or whole genome sequencing of isolated strains. In the case of the massive

sequencing of amplified genes, these studies may target the 16S rRNA gene as is the case in phylogenetic studies or target genes encoding key enzymes within a metabolic pathway as is the case in functional studies. Nevertheless, one should keep in mind that the identification of certain pathways encoded in the genome only indicates potential for a given metabolic pathway and not a proof that it is actually active in situ.

Other techniques, such as Fluorescence in situ Hybridization (FISH) and Catalyzed Reporter Deposition - Fluorescent in situ Hybridization (CARD-FISH) have found a broad application in environmental microbial studies (Kubota 2013). FISH uses fluorescently labeled oligonucleotide probes targeting ribosomal RNA (rRNA), in the analysis of the composition of microbial communities. Oligonucleotide probes can thus be designed targeting several taxonomic levels (Amann *et al.* 1990b; Amann and Fuchs 2008). The basic principle involves fixation and permeabilization of cells, hybridization of the probes with their intracellular target rRNA molecules, following observation of fluorescent signal by fluorescence microscopy (Amann and Fuchs 2008). The CARD-FISH, on the other hand, introduce higher sensitivity to the FISH technique with amplification of fluorescence signal (Pernthaler *et al.* 2004). Here, horseradish-labeled probes are used with catalyzed deposition of fluorescent labeled tyramines (Amann and Fuchs 2008). This improvement to FISH technique is especially important to study microorganisms inhabiting oligotrophic environments, which frequently present a lower ribosome content and therefore lower rRNA targets (Amann and Fuchs 2008; Kubota 2013). Moreover, this technique is very important for microbial ecology study of microniches in solid surfaces such as sediments and rocks (Amils 2015).

## 1.8. Research objectives

Investigations of microbial diversity in the deep subsurface of the IPB at Peña de Hierro on the area of Río Tinto aquifer presents great interest since little is known about the geomicrobiology of this environment.

Primary studies began to take the first steps into this exploration and the opportunity to analyze rock samples from deeper depths on BH10 and BH11 offered a unique window into this ecosystem. The work developed on this thesis aimed at the following objectives:

1. Establishment of enrichment cultures with methanogenic and nitrate reducing activities, as well as the isolation of the microbial diversity enriched.
2. Development of culturing assays aiming at isolation of methanogenic archaea.
3. Study by culture-independent techniques, namely pyrosequencing analysis of 16S rRNA gene sequence, to determine the microbial diversity present in active methanogenic enrichment cultures.
4. Explore of the genomic potential of *Tessaracoccus* sp. strain T2.5-30 isolated from the borehole BH10 in this study.
5. Investigate the microbial diversity in bacterial and archaeal co-cultures established from methanogenic enrichment cultures of rock cores.

## 1.9. Outline of the thesis

The work described in this thesis explored the microbial biodiversity and metabolic potential of microorganisms on the deep subsurface with focus on enrichment cultures, as well as on the study of axenic strains isolated during this study. **Chapter 2** describes the establishment and isolation of microorganisms from enrichment cultures with positive activity for methane and nitrate reduction. **Chapter 3** describes the microbial diversity enriched on cultures with active methane production, using culture-independent techniques. **Chapter 4** reports the complete sequencing, annotation and analysis of the genome from *Tessaracoccus* sp. strain T2.5-30, isolated from a core sample at 139.5 mbs. **Chapter 5** describes the establishment of bacterial and archaeal co-cultures, as well as the preliminary results on characterization of the microbial diversity enriched. **Chapter 6** summarizes the major findings across this work and further discusses important questions to address in future investigations.

# CHAPTER 2

# Isolation and identification of microorganisms from the deep subsurface of the Iberian Pyritic Belt

**This work is partially presented in:**

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## 2.1. Introduction

As discussed in Chapter 1, in deep subsurface oligotrophic ecosystems, metabolisms fueled by oxidation/reduction of inorganic compounds are suggested to play a key role in supporting life. As a result of limited nutrient availability, life in the deep subsurface is suggested to operate with lower metabolic rates. This translates into slower growth and consequently lower cell numbers, which becomes more pronounced with increasing depth. In fact, studies performed by colleagues analyzing rock cores from BH10 borehole by CARD-FISH with probes targeting different taxa have demonstrated that microbial colonies are sparsely distributed (Escudero *et al.* in preparation).

Rock sampling preparation for techniques such as the case of culture-based techniques or DNA extraction based techniques, requires a substantial amount of rock sample for the analysis. These samples represent a homogenization of several microniches within the rocks, where distinct physicochemical proprieties may support diverse populations with distinct metabolisms and physiologies. Thus, these techniques provide only global information of microbial populations living within rocks.

Enrichment cultures, although they may modify the original abundances of microbial diversity as they occur in situ, they comprise a valuable tool for study metabolism, namely in our case to demonstrate if populations with capability for methanogenic and nitrate reduction were present within the rocks. Besides, due to the mentioned low biomass, enrichment cultures proved an asset to enhance microbial growth and thus increase biomass of indigenous microbial populations from rock samples, for studies such as isolation assays. Moreover, the opportunity to isolate subsurface microorganisms in pure culture represents a crucial source of biological material for further studies towards a better understanding of their physiological and metabolic capabilities. In fact, only recently a novel species isolated from the deep subsurface of IPB was described (Puente-Sánchez *et al.* 2014b).

This chapter describes the enrichment, isolation and identification of several bacterial species enriched from rock cores from the deep subsurface of the IPB, as well as the enrichment of a methanogenic strain affiliated with the genus *Methanosarcina*.

## 2.2. Materials and methods

### 2.2.1. Site description, borehole drilling and sampling

The IPBSL campaign at Peña de Hierro resulted in the drilling of two boreholes, BH10 and BH11, each with a depth of 620 and 320 mbs, respectively. A chemical tracer, NaBr, was introduced in the drilling fluid to evaluate possible contamination of samples during drilling. Cores were retrieved encased in plastic liners and were flushed with nitrogen to maintain anaerobic conditions, followed by transport to a nearby laboratory at the Museo Minero at Minas de Riotinto for rock sampling in an anaerobic chamber. Core samples of selected sections were retrieved in sterile and anaerobic conditions using a hydraulic core splitter and a rotary hammer to remove the central part of the cores (Appendix I- Figure I.1). Temperature was measured with an infra-red thermometer to control temperature of the hammer bit. A total of 20 depths for BH11 and 39 depths for BH10 were sampled and used for preparation of enrichment cultures.

### 2.2.2. Methanogenic and denitrification enrichment cultures

Rock samples (~6 g) corresponding to different depths along both boreholes were used as inoculum. All procedures were performed in an anaerobic chamber. For enrichment of methanogenic microorganisms, serum bottles with three different anoxic media were prepared, composed of the same basal mineral solution and differing in added energy sources. The basal media had the following composition (per liter of distilled water): 0.3 g $NH_4Cl$, 0.3 g $K_2HPO_4.3H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 1 ml trace element solution (Sanz *et al.* 1997) at an initial pH of 7. Cysteine (0.5 g/L) was used as reducing agent and resazurin (0.2 mg/L) was used as redox indicator. Medium designated MG1 was supplemented with 20 mM acetate and a headspace composed of $N_2$:$CO_2$ (80:20, v/v), medium MG2 with the gas mixture $H_2$:$CO_2$ (80:20, v/v) and medium MG3 was supplemented with a solution composed of propionate:butyrate:methanol (5 mM each). All supplements were added from anaerobic and sterile stock solutions. The anaerobic culture media for enrichment of chemolithoautotrophic denitrifying microorganisms consisted in the same basal media as described above to which 40 mM of nitrate was added as the electron acceptor and 20 mM thiosulfate with $H_2$:$CO_2$ (80:20, v/v) were added as possible electron donors. Enrichments were incubated at 30ºC without shaking for up to one year. Activity of the different cultures was measured by generation of methane for methanogens and disappearance of nitrate for nitrate reducers at different intervals.

After one year incubation, some of the bottles with positive activity for methanogenesis or for nitrate reduction were chosen for isolation of enriched microorganisms.

### 2.2.3. Culturing techniques and media for isolation

Isolation of anaerobic microorganisms was performed by the Hungate roll-tube method (Hungate 1969; Balch *et al.* 1979). Culture media for isolation of anaerobes had the same composition as the enrichment culture medium with the addition of Agar Noble (15 g/L) (Difco) as solidifying agent. Hungate roll tubes were inoculated with serial dilutions of each enrichment culture, respectively. Incubation was at 30ºC in the dark until development of isolated colonies. Colonies were picked in anaerobic conditions with a bent Pasteur pipette and transferred into culture plates with the same cultivation conditions. Plates were incubated in anaerobic jars with AnaeroGen sachets (Oxoid) to generate anaerobic conditions and in the case of cultures incubated with $H_2$:$CO_2$ (80:20, v/v), the gas mixture was injected inside the jar. Subculturing was done for at least two to three transfers. Colonies were screened for unique morphologies and representatives of each morphotype were selected for further identification.

A set of eleven methane-producing enrichment cultures were chosen for further assays to isolate methanogens by serial dilution method in liquid media. The basal media (final pH 7) was prepared in anoxic conditions having the following composition (per liter of distilled water): 0.3 g $NH_4Cl$, 0.3 g $K_2HPO_4$, 0.3 g $KH_2PO_4$, 0.1 g $MgSO_4.7H_2O$, 2.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 10 ml trace element solution (DSMZ medium 141, https://www.dsmz.de/?id=441), 10 ml vitamin solution (DSMZ medium 141, https://www.dsmz.de/?id=441). Cysteine (0.5 g/L) was used as reducing agent and resazurin (0.1 mg/L) was used as redox indicator. After autoclave sterilization, bottles were supplied with the appropriate substrates and antibiotics according to the conditions tested. The following substrates were used: 20 mM acetate or $H_2$:$CO_2$ (80:20, v/v) or a mix of acetate:methanol:formate:$H_2CO_2$ (10:10:10 mM, 80:20 (v/v)). The following combinations of antibiotics were tested to inhibit growth of bacteria: 100 µg/ml vancomycin; mix of 100 µg/ml each vancomycin:penicillin:ampicillin; 100 µg/ml erythromycin; mix of 50 µg/ml each erythromycin:streptomycin:kanamycin; 100 µg/ml kanamycin; mix of 25 µg/ml each rifampicin:kanamycin; mix of 25 µg/ml clindamycin and 50 µg/ml kanamycin; mix of 50 µg/ml each kanamycin:erythromycin; mix of 25 µg/ml each rifampicin:erythromycin. Methanogenic activity was followed by measurement of methane production by gas chromatography (see 2.2.4 section). The purity of the subcultures that develop methane production was checked by CARD-FISH with 16S rRNA-targeted

oligonucleotide general probes for domain *Archaea* and *Bacteria*, respectively (see 2.2.7 section). Subcultures with active methane production were maintained by transfer to fresh liquid media with the same previous composition without addition of antibiotics. One of these subculture designated as T1.2MG-K100 was enriched from the original enrichment culture using a mix of acetate:methanol:formate:$H_2CO_2$ (10:10:10 mM, 80:20 v/v) and treatment with the antibiotics vancomycin and kanamycin (100 µg/ml each). This subculture was selected for further molecular biology studies.

### 2.2.4. Analytical Measurement

Methanogenic activity was monitored by measurement of headspace methane concentration by gas chromatography using a Varian Star 3400CX gas chromatographer equipped with a split/splitless injector and a flame ionization detector. Development of methane production over time was considered a positive indication for methanogenic activity. Nitrate reducing activity was monitored by measurement of nitrate levels through ion chromatography using an 861 Advanced Compact IC instrument (Methrom). Decreasing levels of nitrate was used as a proxy for nitrate utilization.

### 2.2.5. DNA extraction, amplification of 16S rRNA gene and phylogenetic identification of isolates

DNA was extracted using a cetyltrimethylammonium bromide (CTAB) based extraction method as described before (Wilson 2001). Briefly, cell pellets were harvested by centrifugation at 4ºC (10000 g, 10-15 minutes). Cell pellets were resuspended in 567 µl of Tris-EDTA buffer (TE, 10 mM Tris-HCl and 0.1 mM EDTA, pH 8) and lysed by incubation over one hour at 37ºC with 30 µl sodium dodecyl sulfate (SDS, 10%) and 3 µl proteinase K (20 mg/ml). Then, 100 µl of a 5 M NaCl solution was added, mixed by inversion and 80 µl of a CTAB/NaCl solution (10% w/v hexadecyltrimethylammonium bromide in 0.7 M NaCl) was added. After mixing by inversion, tubes were incubated for 10 min at 65ºC. Successive extractions were followed, first with equal volume of a chloroform/isoamyl alcohol (24:1) mix, and then with equal volume of a phenol/chloroform/isoamyl alcohol (25:24:1) mix. Within each extraction, the suspension was centrifuged for 5 minutes at 10000 g for recovering of aqueous phase. Nucleic acids dissolved in the aqueous phase were precipitated with 0.6 volumes of isopropanol and washed with 70% ethanol. Dried DNA pellets were resuspended in 100 µl of sterile deionized water.

The 16S rRNA gene was amplified by PCR using the primers 27F-1492R (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, (5'- TACGGYTACCTTGTTACGACTT-3')) (Lane 1991). PCR amplicons were generated with AmpliTaq DNA polymerase (Applied Biosystems) according to manufacturer instructions. The PCR conditions were the following: initial denaturation at 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing for 1 min at 56ºC and elongation at 72ºC for 2 min. The final elongation was at 72ºC for 7 min. PCR products were checked for the correct length by electrophoresis on a 1% agarose gel stained with ethidium bromide and purified with a DNA purification JetQuick kit (Genomed). Purified DNA products were quantified using a Nanodrop ND-1000 device (NanoDrop) and sent for sequencing at the Center of Astrobiology (Madrid, Spain). Taxonomic identification of amplified sequences was assessed using EzTaxon-e (Kim *et al.* 2012).

### 2.2.6. Physiological tests

For some selected isolated strains, growth was tested in oligotrophic conditions under both anaerobic and aerobic conditions in basal media, and in richer media under aerobic conditions. The oligotrophic media had the following composition (per liter of distilled water): 0.3 g $NH_4Cl$, 0.3 g $K_2HPO_4.3H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 10 ml trace element solution (DSMZ 141 medium, https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf) and 15 g Agar Noble (Difco). For the anaerobic media cysteine (0.5 g/L) was used to reduce the media and resazurin (0.1 mg/L) was added as a redox indicator. Anaerobic oligotrophic plates were incubated in anaerobic jars as previously described. Growth under aerobic conditions was also examined in the following media: R2A (Difco), Nutrient Agar (NA) (Difco), Tryptic Soy Agar (TSA) (Difco). All plates were incubated at 30ºC for up to 10 days.

### 2.2.7. Catalyzed Reporter Deposition - Fluorescent in situ Hybridization (CARD-FISH)

Culture samples were fixed with formaldehyde (4% (v/v), final concentration). Fixed samples were filtered using black polycarbonate filters (0.2 µm pore size, Millipore). In situ hybridizations with horse-radish peroxidase (HRP)-labeled probes followed by the catalyzed deposition of fluorescently labeled tyramide were carried out as described before (Pernthaler *et al.* 2004).

Briefly, after filtration of samples, filters were embedded in sterile 0.2% (w/v) agarose to avoid the detachment of cells. Then filters were treated with a solution of hydrogen peroxide in methanol for inactivation of endogenous peroxidases, and with lysozyme and proteinase K, for permeabilization of cells. The filters were washed with absolute ethanol and sections of each filter were cut to use and the remaining filter stored at -20ºC. Filters sections were incubated with the hybridization buffer, washed, incubated with the CARD solution and washed again. Probe mix EUB338 I-III (Amann *et al.* 1990a; Daims *et al.* 1999) was used to target members of the domain *Bacteria* (formamide concentration of 35% (v/v) in hybridization buffer) and the general probe ARCH915 (Stahl and Amann 1991) was used for targeting members of *Archaea* (formamide concentration of 20% (v/v) in hybridization buffer). For a double CARD-FISH, these steps were first performed for hybridization with bacteria-targeting probe mix and then repeated for a second hybridization with the archaea-targeting probe, including a inactivation step of peroxidases between each hybridization, to inactivate the first peroxidase probe. Probe NON338 (Wallner *et al.* 1993), a nonsense probe was used as negative control probe to evaluate non-specific probe binding (formamide concentration of 0% (v/v)). Tyramide-Alexa Fluor 488 and tyramide-Alexa Fluor 594 were used in the CARD solution. Probes and fluorophores were purchased from Biomers (Ulm, Germany).

Finally, the filter sections were mounted on slides with a 4:1 mix of Citifluor: Vectashield and with DAPI (4',6-diamidino-2-phenylindole) (Thermo-Fisher) which stains DNA molecules. The mounting medium Citifluor (Citifluor Ltd, UK) and Vectashield (Vector Laboratories) were used to decrease the rate of fluorescence fading from the preparations. Filters stained with DAPI and dually hybridized with bacteria and archaea probes were examined under an epifluorescence microscope (Axioplan 2, Zeiss).

### 2.2.8. DNA extraction and sequencing (partial archaeal and bacterial 16S rRNA genes; partial *mcrA* gene) from T1.2MG-K100 culture

Total DNA of culture T1.2MG-K100 was extracted using the protocol described in section 2.2.5. Partial amplification of bacteria 16S rRNA gene was performed using the primer set 27F-1492R (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). Archaeal 16S rRNA gene was partially amplified with the primer set 21F-958R (21F: 5'-TTCCGGTTGATCCYGCCGGA-3'; 958R: 5'- YCCGGCGTTGAMTCCAATT-3') (DeLong 1992). The *mcrA* gene encoding the alpha subunit of the methyl-coenzyme M

reductase was partially amplified with a nested PCR as follows: first with primer set ME1-ME2 (ME1: 5'- GCMATGCARATHGGWATGTC-3'; ME2: 5'- TCATKGCRTAGTTDGGRTAGT-3') and then with MLf-MLr (MLf: 5'- GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'; MLr: 5'- TTCATTGCRTAGTTWGGRTAGTT-3') (Hales *et al.* 1996; Luton *et al.* 2002). PCR amplicons were generated with AmpliTaq DNA polymerase (Applied Biosystems) according to manufacturer instructions. The PCR conditions for each PCR are described in Table 2.1. Purification and sequencing of PCR products was performed as described in section 2.2.5.

The 16S rRNA bacterial gene sequences and the partial *mcrA* gene sequence obtained in this study have been deposited at the DDBJ/ENA/GenBank under the accession numbers MG720000, MG720001 and MG736925.

**Table 2.1.** PCR conditions for amplification of partial 16S rRNA gene using general bacterial and archaeal targeting primers and partial amplification of *mcrA* gene fragment from subculture T1.2MG-K100.

| Primer pair | Target | PCR Thermal Profile |
|---|---|---|
| **21F-958R** | 16S rRNA gene (*Archaea*) | 94ºC for 5 min, 30 cycles at 94ºC for 1 min, 56ºC for 1 min, 72ºC for 2 min; and finally 72ºC for 10 min |
| **ME1-ME2** | *mcrA* gene | 94ºC for 5 min, 30 cycles at 94ºC for 1 min, 50ºC for 1 min, 72ºC for 2 min; and finally 72ºC for 10 min |
| **MLf-MLr** | *mcrA* gene | 95ºC for 5 min followed by 5 cycles consisting of 95ºC for 30 sec, 55ºC for 30 s and 72ºC for 30 s, and a temperature ramp of 0.1ºC/s between the annealing and extension step. Then 30 cycles at 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec; and finally a step of 72ºC for 10 min |
| **27F-1492R** | 16S rRNA gene (*Bacteria*) | 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing for 1 min at 56ºC and elongation at 72ºC for 2 min. The final elongation was at 72ºC for 7 min |

### 2.2.9. Phylogenetic analysis of archaeal population on culture T1.2MG-K100

The partial 16S rRNA gene sequence and partial *mcrA* gene sequence were compared with the closest known strains using the NCBI Basic Local Alignment Search Tool (BLAST) sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ez-BioCloud (Yoon *et al.* 2016). Phylogenetic analysis was performed with MEGA 6 (Tamura *et al.* 2013) software. Closely related gene sequences were retrieved from NCBI GenBank and aligned with ClustalW (Thompson *et al.* 1994). A phylogenetic tree based on 16S rRNA gene sequence was reconstructed using the neighbor-joining (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) methods based on the Jukes and Cantor model (Jukes and Cantor 1969) with a bootstrap analysis of 1000 replicates for evaluation of tree topology robustness.

## 2.3. Results

Enrichment cultures showed positive activity at several depths, throughout the borehole. Enrichments with methane production of at least 10 times higher than that of the control without added substrate were selected for isolation studies (Table 2.2; Appendix I- Figure I.1). Likewise, enrichments that presented at least more than 500 ppm of nitrate consumed over one year were also selected (Table 2.2; Appendix I- Figure I.1).

**Table 2.2.** Summary of the enrichment cultures selected, respective depths of rock cores (meters in bold), substrate used to promote growth and the positive activity detected.

| Borehole | Enrichment | Substrate added / Activity detected |
|---|---|---|
| **BH11** | T1.2MG_**63.55** | Acetate + $N_2$:$CO_2$ / methanogenesis |
| | T1.2D_**63.55** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T1.17D_**336.75** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| **BH10** | T2.1D_**89.6** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.2D_**90** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.3D_**121.8** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.4D_**130.8** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.5_**139.5** | $H_2$:$CO_2$ / methanogenesis |
| | T2.6_**206.6** | $H_2$:$CO_2$ / methanogenesis |
| | T2.7_**228** | $H_2$:$CO_2$ / methanogenesis |
| | T2.8_**249.8** | $H_2$:$CO_2$ / methanogenesis |
| | T2.9_**266.3** | $H_2$:$CO_2$ / methanogenesis |
| | T2.10_**284** | $H_2$:$CO_2$ / methanogenesis |
| | T2.11_**284** | $H_2$:$CO_2$ / methanogenesis |
| | T.20D_**415.3** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.21D_**420** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.22D_**450** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.22MG_**450** | Mix propionate:methanol:butyrate + $N_2$:$CO_2$ / methanogenesis |
| | T2.26MG_**492** | $H_2$:$CO_2$ / methanogenesis |
| | T2.26D_**492** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.30D_**538.5** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |
| | T2.31MG_**414** | Acetate + $N_2$:$CO_2$ / methanogenesis |
| | T2.31D_**414** | Nitrate+thiosulfate+$H_2CO_2$ / nitrate reduction |

Screening of colonies for unique morphologies led to the selection of representative isolates of each morphotype for further identification based on 16S rRNA gene. All isolates belonged to the domain *Bacteria* and grouped within the phyla *Proteobacteria, Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Table 2.3). The majority of the bacterial isolates belonged to the *Proteobacteria* and the *Actinobacteria* (Table 2.3). Within the phylum *Actinobacteria* strains belonging to the following genera were isolated: *Tessaracoccus, Microbacterium, Cellulomonas, Aestuariimicrobium* and *Nocardioides;* in the phylum *Proteobacteria* strains affiliated with the genera *Citrobacter, Pleomorphomonas, Pseudomonas, Shewanella, Xanthobacter, Rhodoplanes, Desulfovibrio, Brevundimonas, Rhizobium* and *Lelliottia;* in the phylum *Firmicutes* isolates belonging to the genera *Paenibacillus, Bacillus* and

*Acetoanaerobium;* and in the phylum *Bacteroidetes* strains affiliated to the genera *Macellibacteroides* and *Propionicimonas.*

*Archaea* isolates were not obtained in pure culture under the culture conditions used. Enrichment cultures T2.2D, T2.7, T2.8, T.20D, T2.21 and T2.22D failed to produce any isolates. Furthermore, isolates related to *Macellibacteroides* sp. and *Xanthobacter* sp. failed to grow after a few transfers.

For some strains, growth under anaerobic and oligotrophic conditions was also tested to evaluate the effect of the agar matrix on growth. Although weak, the majority of the tested strains could grow under these conditions (Table 2.4). The ability for growth in aerobic conditions in oligotrophic media as well as in richer media was also verified (Table 2.4).

**Table 2.3.** Summary of depth of rock cores where microorganisms were isolated from, isolation conditions and taxonomic affiliation of each isolate based on the sequencing of the 16S rRNA gene.

| Borehole | Depth (m) | Strains | Isolation media | Phylum | Genera | Closest strain in EzTaxon-e database (% 16S rRNA similarity) | Putative designation of the isolated representative strain(s) |
|---|---|---|---|---|---|---|---|
| BH11 | 63.55 | T1.2MG-59 | Basal media + acetate + $N_2$:$CO_2$ | *Bacteriodetes* | *Macellibacteroides* | *Macellibacteroides fermentans* LIND7H$^T$ (99.56) | *Macellibacteroides* sp. T1.2MG-59 |
| | | T1.2D-1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Citrobacter* | *Citrobacter amalonaticus* CECT 863$^T$ (98.70) | *Citrobacter* sp. T1.2D-1 |
| | | T1.2MG-36 | Basal media + acetate + $N_2$:$CO_2$ | | *Pleomorphomonas* | *Pleomorphomonas oryzae* DSM 16300$^T$ (99.70) | *Pleomorphomonas* sp. T1.2MG-36 |
| | 336.75 | T1.17D-1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Actinobacteria* | *Tessaracoccus* | *Tessaracoccus lapidicaptus* IPBSL-7$^T$ (100) | *Tessaracoccus* sp. T1.17D-1 |
| BH10 | 89.5 | T2.1D-1.1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Pseudomonas* | *Pseudomonas aeruginosa* JCM 5962$^T$ (100) | *Pseudomonas* sp. T2.1D-1.1 |
| | 121.8 | T2.3D-1.1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Shewanella* | *Shewanella hafniensis* P010(T) (99.43) | *Shewanella* sp. T2.3D-1.1 |
| | 130.8 | T2.4D-O1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Xanthobacter* | *Xanthobacter flavus* 301$^T$ (99.85) | *Xanthobacter* sp. T2.4D-O1 |
| | 139.4 | T2.5-30 | Basal media + $H_2$:$CO_2$ | *Actinobacteria* | *Tessaracoccus* | *Tessaracoccus lapidicaptus* IPBSL-7$^T$ (100) | *Tessaracoccus* sp. T2.5-30 |
| | | T2.5-50 | | | *Tessaracoccus* | *Tessaracoccus oleiagri* SL014B-20A1(T) (99.85) | *Tessaracoccus* sp. T2.5-50 |
| | | T2.5-46A | | *Firmicutes* | *Paenibacillus* | *Paenibacillus odorifer* DSM 15391$^T$ (98.84) | *Paenibacillus* sp. T2.5-46A |
| | | T2.5-7.3 | | *Proteobacteria* | *Pseudomonas* | *Pseudomonas songnenensis* NEAU-ST5-5$^T$ (99.22) | *Pseudomonas* sp. T2.5-7.3 |
| | 206.6 | T2.6-4D2 | Basal media + $H_2$:$CO_2$ | *Actinobacteria* | *Tessaracoccus* | *Tessaracoccus lapidicaptus* IPBSL-7$^T$ (100) | *Tessaracoccus* sp. T2.6-4D2 |
| | | T2.6-12 | | *Proteobacteria* | *Pseudomonas* | *Pseudomonas songnenensis* NEAU-ST5-5$^T$ (99.85) | *Pseudomonas* sp. T2.6-12 |
| | 266.3 | T2.9-1 | Basal media + $H_2$:$CO_2$ | *Firmicutes* | *Bacillus* | *Bacillus circulans* ATCC 4513$^T$ (98.98) | *Bacillus* sp. T2.9-1 |
| | 284 | T2.10-17 | Basal media + $H_2$:$CO_2$ | *Actinobacteria* | *Tessaracoccus* | *Tessaracoccus lapidicaptus* IPBSL-7$^T$ (100) | *Tessaracoccus* sp. T2.10-17 |
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|---|---|---|---|---|---|---|---|
| | | T2.11-28 | | | *Microbacterium* | *Microbacterium saccharophilum* K-1$^T$ (99.42) | *Microbacterium* sp. T2.11-28 |
| | | T2.11-4.1 | | *Proteobacteria* | *Rhodoplanes* | *Rhodoplanes piscinae* JA266$^T$ (99.85) | *Rhodoplanes* sp. T2.11-4.1 |
| | **414** | T2.31MG-40 | Basal media + acetate + $N_2$:$CO_2$ | *Actinobacteria* | *Cellulomonas* | *Cellulomonas hominis* DMMZ CE40$^T$ (98.88) | *Cellulomonas* sp. T2.31MG-40 |
| | | T2.31MG-18 | Basal media + acetate + $N_2$:$CO_2$ | | | *Cellulomonas pakistanensis* NCCP-11$^T$ (97.66) | *Cellulomonas* sp. T2.31MG-18 |
| | | T2.31MG-1 | Basal media + acetate + $N_2$:$CO_2$ | *Bacteriodetes* | *Propionicimonas* | *Propionicimonas paludicola* DSM 15597$^T$ (98.53) | *Propionicimonas* sp. T2.31MG-1 |
| | | T2.31D-1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Pseudomonas* | *Pseudomonas stutzeri* ATCC 17588$^T$ (99.93) | *Pseudomonas* sp. T2.31D-1 |
| | | T2.31MG-Q8 | Basal media + acetate + $N_2$:$CO_2$ | | *Rhodoplanes* | *Rhodoplanes piscinae* JA266$^T$ (99.85) | *Rhodoplanes* sp. T2.31MG-Q8 |
| | **450.3** | T2.22MG-43 | Basal media + propionate:methanol:butyrate+$N_2$:$CO_2$ | *Actinobacteria* | *Cellulomonas* | *Cellulomonas fimi* ATCC 484$^T$ (97.65) | *Cellulomonas* sp. T2.22MG-43 |
| | | T2.22MG-48A | | *Bacteriodetes* | *Macellibacteroides* | *Macellibacteroides fermentans* LIND7H$^T$ (99.56) | *Macellibacteroides* sp. T2.22MG-48A |
| | | T2.22MG-11 | | *Firmicutes* | *Acetoanaerobium* | *Acetoanaerobium noterae* ATCC 35199$^T$ (98.41) | *Acetoanaerobium* sp. T2.22MG-11 |
| | | T2.22MG-35 | | *Proteobacteria* | *Desulfovibrio* | *Desulfovibrio oxamicus* DSM 1925$^T$ (99.14) | *Desulfovibrio* sp. T2.22MG-35 |
| | **492.6** | T2.26MG-19.2B | Basal media + $H_2$:$CO_2$ | *Actinobacteria* | *Aestuariimicrobium* | *Aestuariimicrobium kwangyangense* R27$^T$ (99.93) | *Aestuariimicrobium* sp. T2.26MG-19.2B |
| | | T2.26MG-1 | | | *Nocardiodes* | *Nocardioides pyridinolyticus* OS4(T) (97.90) | *Nocardiodes* sp. T2.26MG-1 |
| | | T2.26MG-97 | | *Proteobacteria* | *Brevundimonas* | *Brevundimonas mediterranea* V4.BO.10$^T$ (99.77) | *Brevundimonas* sp. T2.26MG-97 |
| | | T2.26MG-48.2 | | | *Pseudomonas* | *Pseudomonas stutzeri* ATCC 17588$^T$ (99.93) | *Pseudomonas* sp. T2.26MG-48.2 |
| | | T2.26MG-10 T2.26MG-112.2 | | | *Rhizobium* | *Rhizobium naphthalenivorans* TSY03b$^T$ (98.88) | *Rhizobium* sp. T2.26MG-10 *Rhizobium* sp. T2.26MG-112.2 |
| | | T2.26MG-98 | | | *Rhodoplanes* | *Rhodoplanes piscinae* JA266$^T$ (100) | *Rhodoplanes* sp. T2.26MG-98 |
| | | T2.26D-8 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | | *Lelliottia* | *Lelliottia amnigena* JCM 1237$^T$ (100) | *Lelliottia* sp. T2.26D-8 |
| | **538.5** | T2.30D-1.1 | Basal media + nitrate + thiosulfate + $H_2$:$CO_2$ | *Proteobacteria* | *Rhizobium* | *Rhizobium selenitireducens* ATCC BAA-1503 (98.96%) | *Rhizobium* sp. T2.30D-1.1 |

**Table 2.4.** Growth of isolates on several media. 1- *Aestuariimicrobium* sp. T2.26MG-19.2B; 2- *Brevundimonas* sp. T2.26MG-97; 3- *Microbacterium* sp. T2.11-28; 4- *Nocardioides* sp. T2.26MG-1: 5- *Paenibacillus* sp. T2.5-46A; 6- *Rhizobium* sp. T2.26MG-10; 7- *Rhodoplanes* sp. T2.26MG-98; 8- *Tessaracoccus* sp. T2.5-30: 9- *Tessaracoccus* sp. T2.5-50; 10- *Pseudomonas* sp. T2.26MG-48.2; 11- *Rhizobium* sp. T2.30D-1.1; 12- *Shewanella* sp. T2.3D-1.1; 13- *Lelliottia* sp. T2.26D-8; 14- *Cellulomonas* sp. T2.22MG-43; 15- *Pleomorphomonas* sp. T1.2MG-36. +, positive; -, negative; w, weak; vw, very weak.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R2A (Aerobiosis) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TSA (Aerobiosis) | + | + | + | - | + | + | - | + | + | + | + | + | + | w | + |
| NA (Aerobiosis) | + | + | + | + | + | - | + | + | + | + | - | + | + | + | + |
| Oligotrophic media (Aerobiosis) | w | w | w | + | - | + | w | - | - | w | + | vw | vw | vw | vw |
| Oligotrophic media (anaerobiosis) | w | w | w | + | - | + | w | - | - | w | + | - | w | w | w |

Attempts of isolation of methanogens by applying the serial dilution method resulted only on a few positive subcultures from original enrichment culture T1.2MG (core sample 63.55 mbs, BH11- Table 2.2) with development of methane production. One of these positive subcultures with methane activity, named T1.2B2- K100, was enriched using the culture T1.2MG as inoculum with a mix of acetate:methanol:formate:$H_2CO_2$ (10:10:10 mM, 80:20 v/v) and treatment with the antibiotics vancomycin and kanamycin (100 µg/ml each). CARD-FISH using general probes for domains *Bacteria* and *Archaea* (Figure 2.1) demonstrated a positive signal for both probes, indicating the presence of bacterial and archaeal populations in co-culture. This culture was further selected to determine the phylogenetic affiliation of bacterial and archaeal populations present.

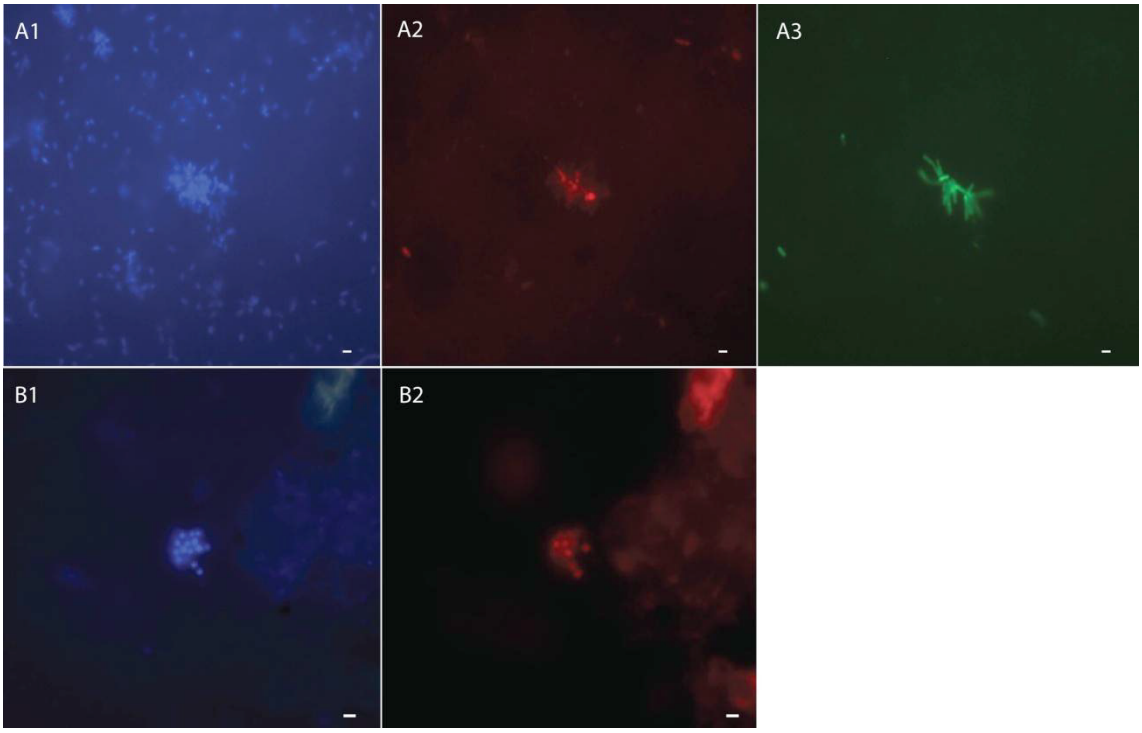


**Figure 2.1.** CARD-FISH of subculture T1.2MG-K100 with DAPI staining of cells **(A1, B1)**, probe ARCH915 targeting *Archaea* **(A2, B2)** and using the probe mix EUB-338 I-III targeting *Bacteria* **(A3)**. Bar, 2 µm.

PCR products obtained with specific primers targeting bacterial 16S rRNA gene, archaeal 16S rRNA gene or *mcrA* gene were amplified without need for cloning, indicating the co-culture in subculture T1.2B2-K100 of a single bacteria and archaea populations, named T1.2MG-B and T1.2MG-A, respectively. Phylogenetic analysis revealed that strain T1.2MG-A is a member of the genus *Methanosarcina* (Figure 2.2). The analysis of 16S rRNA gene sequence of strain T1.2MG-A showed that is closely related to *Methanosarcina horonobensis* strain HB-1 (98.1%). A partial *mcrA* gene sequence was also determined that closely related to *Methanosarcina horonobensis* (94.2%). Identification of the 16S rRNA gene sequence for the bacterial strain T1.2MG-B showed it is closely related to *Rhodoplanes piscinae* JA266$^T$ (100%). By phase contrast microscopy the typical rosette aggregates of *Rhodoplanes* species could be observed as well as coccoid shaped cells correspondent of *Methanosarcina* cells (Appendix I - Fig I.2).

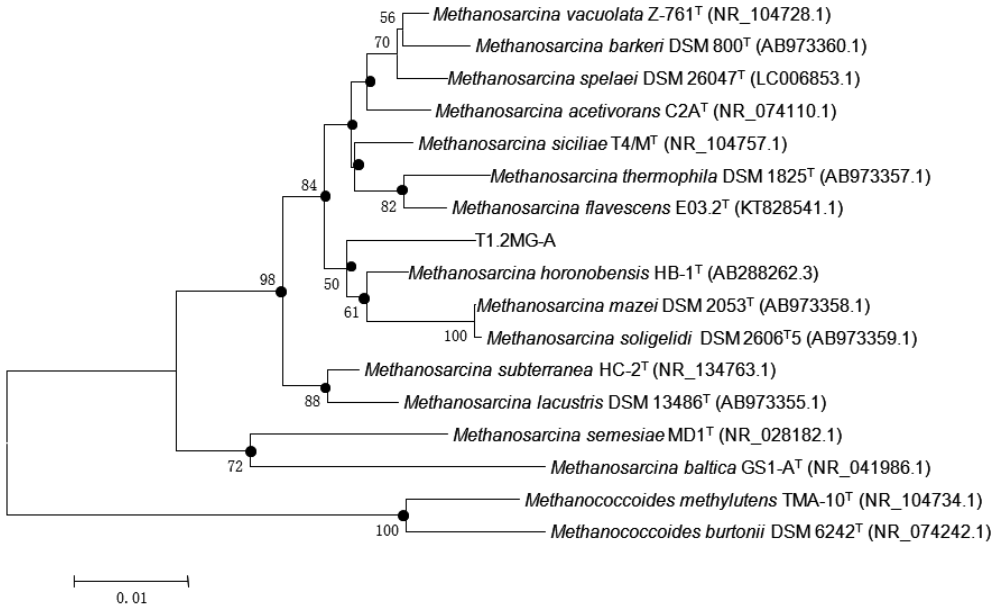


**Figure 2.2.** Phylogenetic tree based on 16S rRNA gene sequence of strain T1.2MG-A and closely related taxa constructed using the neighbor-joining algorithm. The numbers on the nodes indicate bootstrap values (≥ 50%). Filled circles at nodes represent nodes recovered by the maximum-likelihood method. Bar, 0.01 substitutions per nucleotide position.

## 2.4. Discussion

The majority of deep terrestrial subsurface ecological studies have been focused on the analysis of groundwater. While important discoveries were reported on characterization of groundwater geochemistry and the diversity of subsurface planktonic communities, few studies have addressed the study of rock hosted microbial communities as discussed in Chapter 1. Furthermore, within the reported studies based on rock drilling, focus was mainly on sequencing based techniques rather than isolation. On this account, in the present study we applied culture-dependent methods to gain further knowledge into microbial metabolic capabilities and diversity of rock dwelling microbial indigenous populations on the IPB subsurface.

The development of methane production as well as the utilization of nitrate in different enrichment cultures demonstrated the presence of viable and active methanogenic and nitrate reducing populations, respectively. Methane, hydrogen and carbon dioxide were measured in different samples (Amils *et al.* 2014) along the well. Also soluble inorganic anions such as nitrate, nitrite, sulfate and organic acids as acetate, propionate or formate have been extracted from different samples at different depths (Amils *et al.* 2014). Therefore, these results demonstrated that substrates able to sustain methanogenic as well as nitrate reducing activities were present in the system and could indeed be used to support in situ the growth of the microbial populations enriched in these cultures.

Isolation and identification of cultured diversity obtained using traditional techniques showed that all isolates belonged to the phyla *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes* within the domain *Bacteria* (Table 2.3). The majority of the isolates showed high similarity, based on 16S rRNA gene sequence analysis, with cultured strains previously isolated from other habitats, such as marine environment, freshwater, soils, sediments, or wastewater treatment plants.

This study is one of the first massive efforts on the isolation of microorganisms from hard rock samples on a deep subsurface environment. The recovery, of several rock dwelling bacterial strains contributed not only to the identification of viable microbial populations inhabiting the IPB subsurface, but also offered the opportunity for microbial culturing and maintenance, which in turn will open new avenues to future studies aimed at understanding their physiology and metabolic potential, which may held important clues into the processes that support life in this environment.

Several bacterial strains were isolated in basal media supplied with hydrogen and carbon dioxide as substrates (Table 2.3). Nevertheless, growth under these conditions may have been supported by the trace quantities of yeast extract,

incorporated in the media as a source of growth factors, and also by the presence of residual organic molecules in the agar used as solidifying agent. Testing growth in anaerobic conditions in the presence of just the basal solid media (Table 2.4) used for isolation and in the absence of $H_2/CO_2$, indicated that the majority of the strains tested can still grow, although weakly, in these conditions. Additionally, preliminary data on growth with $H_2/CO_2$ in liquid media, suggests that these compounds were not used as substrates (Appendix I - Figure I.3). Therefore, these strains demonstrate oligotrophic capabilities which are in accordance with their presence in an oligotrophic environment as is the deep subsurface.

A number of strains were isolated from enrichment cultures that displayed nitrate reduction activity. These isolates, based on 16S rRNA gene, have high similarity with members of the genera *Citrobacter, Tessaracoccus, Pseudomonas, Shewanella, Xanthobacter, Lelliottia* and *Rhizobium*. Strains belonging to such genera as is the case of *Pseudomonas* (Lalucat *et al.* 2006), *Shewanella* (Brettar *et al.* 2002) or *Citrobacter* (Li *et al.* 2014), have been previously described having capability for denitrification. In turn, some strains affiliated with the genera *Shewanella* (Yoon *et al.* 2015) and *Citrobacter* (Smith 1982; Rehr and Klemme 1989) or *Enterobacter amnigenus* (recently reclassified as *Lelliottia amnigena*) (Fazzolari *et al.* 1990; Brady *et al.* 2013) have also been described with capability for nitrate reduction to ammonia, that is DNRA metabolism. Future studies should evaluate each isolated strain metabolic activity.

The isolates, *Acetoanaerobium* sp. T2.22MG-11, *Desulfovibrio* sp. T2.22MG-35, *Macellibacteroides* sp. T1.2MG-59 and T2.22MG-48A are strict anaerobes, as has been previously described for isolated and taxonomically characterized members of these genera (Jabari *et al.* 2012; Kuever *et al.* 2015; Rainey 2015). All the remaining organisms, isolated under strict anaerobiosis, could also grow under aerobic conditions in R2A medium.

Growth of the some of the facultative anaerobic isolates was also further tested in other richer media than R2A (Table 2.4), i.e. TSA media or NA media. All strains tested demonstrated capability to grow on rich media, with higher biomass production, similarly to what has been described for their closest phylogenetic relatives.

Isolate T2.22MG-11 grouped within the genus *Acetoanaerobium* in the order *Clostridiales*. *Acetoanaerobium* strains are characterized as being able to grow autotrophically on hydrogen and carbon dioxide with the production of acetate. They are also known to ferment carbohydrates (e.g. glucose, maltose), small volatile fatty acids as well as yeast extract (Sleat *et al.* 1985; Bes *et al.* 2015). Strain T2.22MG-11 could also grow on basal media supplemented with hydrogen and carbon dioxide. This

is the first report of an acetogenic rock dwelling bacteria recovered from the IPB subsurface. The identification of the presence of an acetogenic bacteria on rock samples offers an explanation for the acetate measurements obtained from boreholes on both MARTE and IPBSL project. As hydrogen and carbon dioxide are both present in the IPB subsurface (Amils *et al.* 2014), these substrates could support the growth of acetogenic bacteria in situ, and subsequently, in situ production of acetate. The presence of acetate in the subsurface becomes an important source of organic matter that could support growth of heterotrophic populations. In fact, acetate as well as other organic acids have also been detected at various depths from rock leacheates (Amils *et al.* 2014).

Isolate T2.22MG-35 groups within the genus *Desulfovibrio* with *D. oxamicus* strain DSM 1925$^T$ as its closest cultivable relative. The presence of sulfate in rock leachates as well as hydrogen in both boreholes (Amils *et al.* 2014), supports the hypothesis for occurrence of sulfur reducing activities at in situ conditions. *Desulfovibrio* strains have been previously isolated from deep terrestrial groundwater (Motamedi and Pedersen 1998; Sass and Cypionka 2004) and from oceanic subsurface environments (Fichtel *et al.* 2012).

Isolate T2.3D-1.1 closest cultivable relative was *Shewanella hafniensis*. *Shewanella* species are known for their ability to oxidize both organic matter or hydrogen and reduce various compounds of which iron reduction and nitrate reduction has been the subject of various studies (Brettar *et al.* 2002; Yoon *et al.* 2013; Satomi 2014; Yoon *et al.* 2015).

Members of the genera *Bacillus* and *Clostridium* have species with fermentative capacity. Some species of *Bacillus* and the majority of *Clostridium* spp. have been described that can also produce hydrogen as a fermentation product (Logan and Vos 2015; Rainey *et al.* 2015). Therefore, the isolation of *Bacillus* sp. T2.9-1 in this study, as well as the recent isolation by us of a novel *Clostridium* strain (isolated under anaerobic conditions with R2A media) both from the IPB subsurface, supports the hypothesis that if fermentable substrates exist in the deep subsurface, fermentative bacteria present in the rock cores might carry out this type of metabolism and possibly also contribute to generate hydrogen, which in turn could be used by other chemolithotrophic microorganisms. Likewise, strains capable of fermentative metabolism with hydrogen production have also been described within the genera *Paenibacillus* (Lal *et al.* 2012) and *Brevundimonas* (Su *et al.* 2014).

Additionally to the already mentioned genera, several other genera were identified in this study, including species for which metabolic capability for fermentative metabolism has been reported, such as members of *Macellibacteroides* (Jabari *et al.*

2012), *Citrobacter* (Borenshtein and Schauer 2006), *Pleomorphomonas* (Xie and Yokota 2005), *Tessaracoccus* (Finster et al. 2009; Cai et al. 2011), *Cellulomonas* (Christopherson *et al.* 2013; Stackebrandt and Schumann 2014), *Microbacterium* (Evtushenko and Takeuchi 2006; Ohta et al. 2013) or *Propionicimonas* (Akasaka *et al.* 2003)

It is interesting the presence of *Rhodoplanes* from 63.55 mbs (BH11) as well as the isolation of strains related to the genus *Rhodoplanes* from BH10 at several depths (i.e. 284 mbs, 414 mbs, 492.6 mbs), which indicates its ubiquitous distribution in the IPB subsurface. Studies involving massive 16S rRNA gene sequencing from methanogenic enrichment cultures (BH10, 228.67 mbs) and acetogenic enrichment cultures (BH10, 433.32; BH11, 145.47 mbs) also reported sequences affiliated with the genus *Rhodoplanes* (Amils *et al*. in preparation). *Rhodoplanes* species are known purple non-sulfur bacteria, characterized by having diverse metabolic capabilities, being able of both phototrophic and chemotrophic growth (Hiraishi and Imhoff 2015). Strains affiliated with the genus *Rhodoplanes* isolated in this study were recovered with an anaerobic basal media amended with either acetate or under an atmosphere of hydrogen and carbon dioxide in the dark. When grown under such conditions on liquid media or solid media culture/colonies were translucent, while growth under the same conditions upon exposure to light resulted in culture/colonies with a reddish pigmentation. It will be interesting in future studies to explore the physiology of the *Rhodoplanes* strains isolated on this study from dark and anoxic habitats.

Detection of phylotypes affiliated with photosynthetic bacteria from deep subsurface environments has also been previously reported (Miettinen *et al.* 2015; Purkamo *et al.* 2017). Furthermore, sequences affiliated with photosynthetic microorganisms, namely affiliated with the phylum *Cyanobacteria* have also been reported from IPBSL project by massive sequencing of 16S rRNA gene and metagenomic studies with DNA extracted from rocks samples (BH10 borehole) (Puente-Sánchez 2016), by 16S rRNA gene cloning studies (Amils *et al.* in preparation) and by in situ hybridization techniques in rock samples using a probe targeting the phylum *Cyanobacteria* (Escudero *et al.* in preparation).

Within the project IPBSL, diverse applied techniques have recently reported some of the genera identified by the isolation assay from rock samples recovered from different depths. Such are the case of massive 16S rRNA gene sequencing studies of several enrichment cultures (e.g. methanogenic, acetogenic, nitrate reducing and acetogenic enrichment cultures) prepared with rock samples from both BH10 and BH11, from which sequences affiliated with genera *Acetoanaerobium, Bacillus, Cellulomonas, Desulfovibrio, Nocardioides, Propionicimonas, Pseudomonas* and

*Tessaracoccus* were also reported (Amils *et al.* in preparation). Massive 16S rRNA gene sequencing studies and metagenomic studies with DNA extracted from rock cores also indicated the presence at several depths along BH10 borehole of sequences affiliated with several phyla within which, *Actinobacteria* and *Proteobacteria* (dominant at certain depths), as well as *Bacteroidetes a*nd *Firmicutes,* were among the detected phyla (Puente-Sánchez 2016). Immunoprofiling studies using the microarray LDChip300 (with 300 antibodies targeting ecological relevant microbial strains, environmental extracts and functional proteins) also detected a positive identification of *Shewanella* (Amils *et al.* in preparation). Recently, the presence of members of the genus *Tessaracoccus* along several depths and members of the genus *Rhizobium* have also been demonstrated by in situ hybridization studies on rock cores recovered from BH10 borehole by applying probes designed to target members of the genera *Tessaracoccus* and *Rhizobium*, respectively (Escudero *et al.* in preparation). In situ hybridization studies with probes targeting diverse taxa (e.g. *Bacteria, Archaea, Verrucomicrobiales, Plactomycetales, Alphaproteobacteria, Betaproteobacteria*, *Gammaproteobacteria, Cyanobacteria, Firmicutes, Actinobacteria, Bacteroidetes,* among others) have also indicated the presence of microorganisms affiliated with such taxa from different depths on rock samples from BH10 (Escudero *et al.* in preparation).

So far the possible role in the subsurface for many of the genera isolated in this study is still unclear. Further physiological and biochemical characterization on the isolated strains are required to elucidate strategies and metabolic potential that could possibly enable these strains to inhabit subsurface environments. Complementation of these studies by molecular methods, as well as whole genome sequencing will also provide further insights on the genetic potential of the organisms. Recently the complete genome of strain *Tessaracoccus* sp. strain T2.5-30 isolated in this study has been sequenced (Leandro *et al.* 2017). In Chapter 4, the genetic potential inferred from the analysis of the genome of *Tessaracoccus* sp. strain T2.5-30 is discussed.

Isolation on roll tubes from cultures with methanogenic activity did not result in the isolation of methanogenic archaea in pure culture. The resistance to isolation of methanogens could be related with the impossibility in reproducing essential key components present in the enrichment culture, e.g. present in the mineral matrix or a byproduct of the metabolism from other microorganisms that were co-enriched. Furthermore, slow growth might also account for difficulty in isolating. Indeed, difficulty in isolating methanogens have been widely reported in many studies, with slow growth rates and fastidious growth conditions frequently indicated as causes for failure in growth (Lü and Lu 2012).

The experimental approach directed to isolation of methanogenic microorganisms in liquid media was performed by combining addition of antibiotics to the culture media to promote elimination of bacterial populations. Methane activity was only detected in first dilutions ($10^{-1}$ to $10^{-3}$) for a few of the tested growth conditions. CARD-FISH analysis demonstrated that bacteria were still present in those sub-cultures, as well as archaea (Figure 2.1).

For culture T1.2B2-K100 (originating from enrichment culture T1.2MG, rock core 63.55 m deep on BH11), molecular analysis based on amplification of 16S rRNA gene indicated that the bacterial population is related to the genus *Rhodoplanes*, showing highest similarity with the type strain *Rhodoplanes piscinae* JA266$^T$. In fact, observation by phase contrast microscopy (Appendix I- Figure I.2) indicates the presence of cell aggregates and rosette like structures typical of the majority of described species in this genus, occurring in older cultures. The amplification of both 16S rRNA gene and *mcrA* gene revealed the presence of an archaeal strain, designated T1.2MG-A, affiliated with the genus *Methanosarcina* (Figure 2.2). The closest related known strain based on similarity of gene sequence of 16S rRNA gene and partial *mcrA* sequence was *Methanosarcina horonobensis* strain HB-1$^T$. *Methanosarcina horonobensis* has been recently isolated also from groundwater originated from a deep subsurface Miocene formation at Horonobe (Japan) (Shimizu *et al.* 2011). Members of the genus *Methanosarcina* are described as the most metabolic versatile methanogens, capable of using a broader range of substrates (Kendall and Boone 2006). Since few isolates of methanogens have been recovered from deep continental subsurface environments, further characterization of this putative novel methanogenic archaeal strain could be of interest in the future.

The successful enrichment in methanogenic and nitrate reducing activities as well as the isolation of both strict and facultative anaerobic isolates in this study provided another important piece of information towards the global understanding of microbial diversity in the deep subsurface of the Río Tinto aquifer at the Iberian Pyritic Belt. Strains affiliated with microorganisms with metabolic potential for fermentation (e.g. *Bacillus* sp. or *Cellulomonas* sp.), nitrate reduction (e.g. *Pseudomonas* sp.), for chemolithoautotrophic metabolisms such as methanogenesis (e.g. *Methanosarcina* sp.) or acetogenesis (e.g. *Acetoanaerobium* sp.), iron reduction (e.g. *Shewanella* sp.) and sulfate reduction (e.g. *Desulfovibrio* sp.) were either isolated or co-enriched in this study. Moreover, their occurrence suggests a potential important role for these microorganisms on the cycling of elements such as carbon, iron, nitrogen and sulfur through the subsurface ecosystem.

# CHAPTER 3

# Microbial diversity enriched under methanogenic conditions from rock cores of the Iberian Pyritic Belt deep subsurface

**This work is partially presented in:**

Leandro T., Rodriguez N., Rojas P., Sanz J. L., da Costa M. S., Amils R. 2018. Study of methanogenic enrichment cultures of rock cores from the deep subsurface of the Iberian Pyritic Belt. *Under Revision.*

## 3.1. Introduction

As described in Chapter 2, enrichment cultures of rock samples from both BH10 and BH11 boreholes with active methane production were successfully established.

Three methanogenic enrichment cultures representative of each condition used (i.e. acetate, hydrogen/carbon dioxide or propionate/methanol/butyrate as substrates) were selected for total extraction of DNA followed by amplification of 16S rRNA gene and pyrosequencing analysis. This culture-independent strategy provided additional information on both enriched bacterial and archaeal populations which inhabit the IPB deep subsurface at Peña de Hierro aquifer.

## 3.2. Materials and Methods

### 3.2.1. Enrichment cultures, their origin and growth conditions

As described in Chapter 2, two boreholes (BH10 and BH11) were drilled during the IPBSL campaign. The recovered cores were used to establish enrichment cultures designed to promote the enrichment in microorganisms with methanogenic activity.

Three enrichment cultures, with positive methane production were selected for further studies (Table 2.2, Chapter 2). T1.2MG culture (core sample from 63.55 mbs, BH11) enriched with acetate (20 mM) and an atmosphere of $N_2$:$CO_2$ (80:20, v/v); T2.22MG culture (core sample from 450.3 mbs, BH10) enriched with a solution composed of propionate:methanol:butyrate (5 mM each) under an atmosphere of $N_2$:$CO_2$ (80:20, v/v) and T2.26MG culture (core sample from 492.6 mbs, BH10) enriched with $H_2$:$CO_2$ (80:20, v/v).

### 3.2.2. Catalyzed Reporter Deposition - Fluorescent in situ Hybridization (CARD-FISH)

CARD-FISH technique was performed as described in section 2.2.7 (Chapter 2). Filters dually hybridized with bacteria and archaea targeting probes were imaged using a confocal laser scanning microscope LSM710 coupled with an inverted microscope AxioObserver (Carl Zeiss) and equipped with diode (405 nm), argon (458/488/514 nm) and helium and neon (543 and 633 nm) lasers. Images were collected with a 63x/1.4 oil immersion lens.

### 3.2.3. DNA extraction, PCR amplification and 454 pyrosequencing

Total DNA from the enrichment cultures was extracted with PowerSoil DNA extraction kit (MoBio Laboratories Inc., CA). Archaeal and bacterial amplification libraries for high throughput 454 pyrosequencing were prepared by PCR amplification of partial 16S rRNA gene sequence using Invitrogen PlatinumTaq DNA polymerase enzyme. Bacterial 16S rRNA gene fragments were amplified with the primer pair 27F-907R (Lane 1991). Archaeal 16S rRNA gene fragments were amplified with primer pair 21F-915R (Stahl and Amann 1991; DeLong 1992). PCR conditions were the following: initial denaturation at 95ºC for 3 min followed by 28 cycles consisting of denaturation at 95ºC for 30 sec, primer annealing for 45 sec at a 54ºC and elongation at 68ºC for 1.30 min. The final elongation was at 68ºC for 10 min. PCR correct product size was observed in 1% agarose gel stained with ethidium bromide. PCR amplification products were purified with the Invitrogen Purelink kit. Pyrosequencing was performed by Centro de Investigación Tecnología y Innovación (CITIUS, University of Sevilla, Spain) using a 454 FLX + System (Roche).

All sequence processing analysis was done using the software Mothur v.1.36.1 (Schloss *et al.* 2009). Sequences were trimmed to remove primers and barcodes. Sequences with more than eight homopolymers and with ambiguous bases were also excluded. Further quality filtering of the resulting sequences dependent on base quality scores was performed using the script moira.py (https://github.com/fpusan/moira), which contains the python implementation of the Poisson binominal filtering algorithm (Puente-Sánchez *et al.* 2016a). In addition, sequences with a length of less than 400 bp (for bacteria) and 600 bp (for archaea) were excluded. The sequences obtained after the quality filtering were aligned against the SILVA 16S rRNA reference alignment v123 (https://www.mothur.org/wiki/Silva_reference_files). A further pre-clustering step was performed by clustering reads that differed in 1 base. The UCHIME algorithm as implemented in Mothur was used to detect chimeras which were then removed from the dataset. Pairwise distance matrices were built and the sequences were clustered into Operational Taxonomic Units (OTU's) at 97% similarity. Taxonomic classification was performed with the SILVA 16S rRNA gene database v123 (with a 80% confidence threshold). Rarefaction curves were calculated for each sample.

The raw sequences from pyrosequencing obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers SRR6186122 - SRR6186127.

## 3.3. Results

Microorganisms from rock cores at 63.5 mbs (BH11 borehole, culture T1.2MG), 450.3 mbs (BH10 borehole, culture T2.22MG) and 492.6 mbs (BH10 borehole, culture T2.26MG) were enriched under methanogenic conditions. CARD-FISH analysis demonstrated the presence of both bacterial and archaeal microorganisms enriched (Figure 3.1). All cultures showed methane production (see Chapter 2).

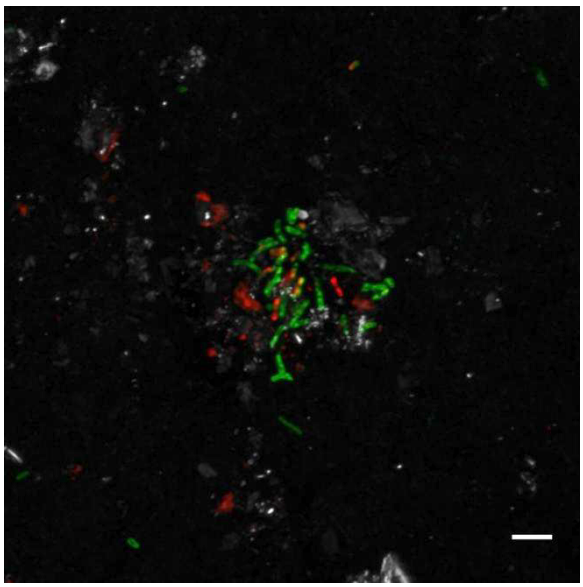

**Figure 3.1.** CARD-FISH of culture T1.2MG with probe EUB-338 I-III targeting *Bacteria* (tyramide-Alexa Fluor 488, green signal) and probe ARCH915 targeting *Archaea* (tyramide-Alexa Fluor 594, red signal). Bar, 10 µm.

Pyrosequencing of bacterial and archaeal 16S rRNA gene sequences from three selected rock core enrichment cultures with active methane production (Table 2.2, Chapter 2), produced a total of 29686 bacterial and 19039 archaeal high quality reads for culture T1.2MG; 28589 bacterial and 3321 archaeal high quality reads for culture T2.22MG; and 12853 bacterial and 24007 archaeal high quality reads for culture T2.26MG. Rarefaction analysis of bacterial community enriched showed no saturation in samples from both cultures T1.2MG and T2.26MG, while it reached saturation for the samples from culture T2.22MG (Appendix II - Fig II.1). In the case of rarefaction analysis of the archaeal community, samples from cultures T1.2MG and T2.26MG reached saturation while saturation was not reached for the sample from T2.22MG culture (Appendix II - Fig II.2). For the cases where saturation was not reached, samples may require further sequencing to ensure the entire diversity present was captured.

The majority of the identified OTU's in the three cultures at the phylum level were affiliated with *Proteobacteria* and *Firmicutes* (Figure 3.2), with minor abundant OTU's (≤ 0.3%) affiliated with the phyla *Bacteroidetes*, *Actinobacteria*, *Chloroflexi* or remained unclassified.

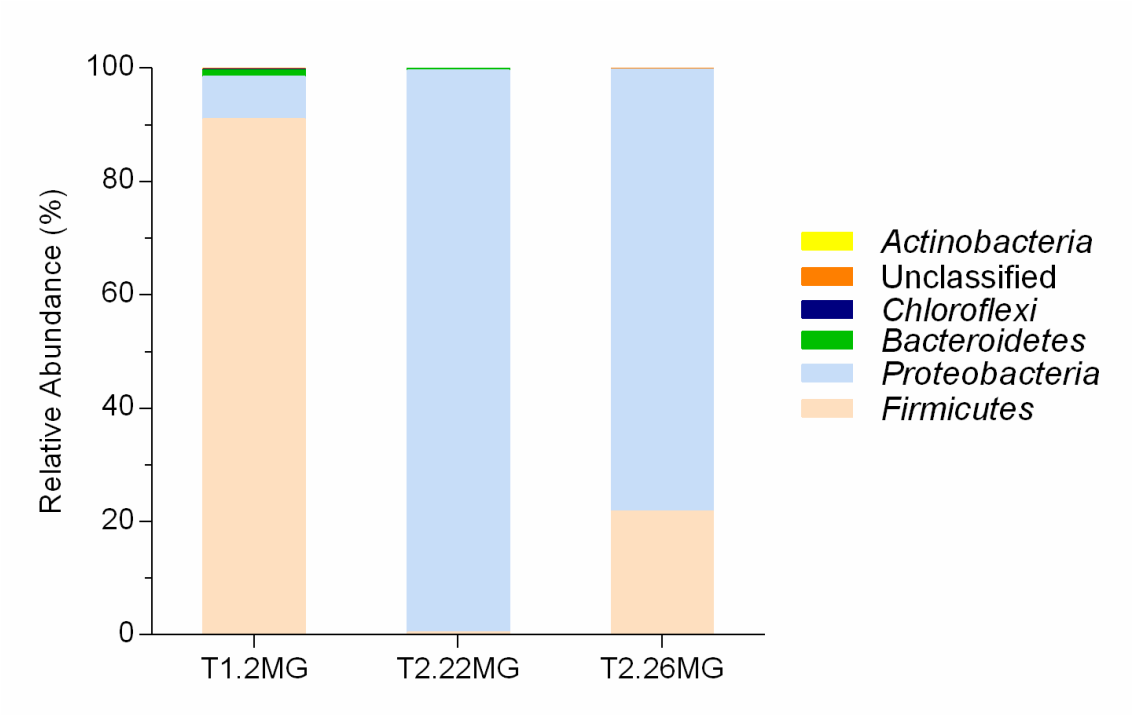


**Figure 3.2.** Relative abundance of bacterial sequences at the phylum level for cultures T1.2MG, T2.22MG and T2.26MG. OTU's could not be taxonomically assigned at the phylum level are reported as "Unclassified".

Culture T1.2MG presented the most diversified bacterial populations enriched. At the genus level (Figure 3.3) the major bacterial OTU's enriched were affiliated with the genera *Sedimentibacter* (43.50%), *Erysipelothrix* (42.58%), and *Rhodoplanes* (5.50%). Other less abundant OTU's at the genus level were classified as *Desulfosporosinus* (2.22%) and *Sporacetigenium* (1.30%) or either assigned as unclassified (4.11%). In culture T2.22MG, pyrosequencing analysis indicated the most abundant bacterial group is closely related to the genus *Azospira* (98.85%) (Figure 3.3). Less abundant OTU's enriched in this culture were classified as affiliated with *Syntrophomonas* (0.33%) or assigned as unclassified (0.45%). For culture T2.26MG the major OTU's enriched were closely related to the genus *Pseudomonas* (76.74%) and to the genus *Sporomusa* (20.28%) (Figure 3.3). Other less abundant OTU's at the genus level were classified as related to the genera *Acetobacterium* (1.06%) or assigned as unclassified (1.22%).

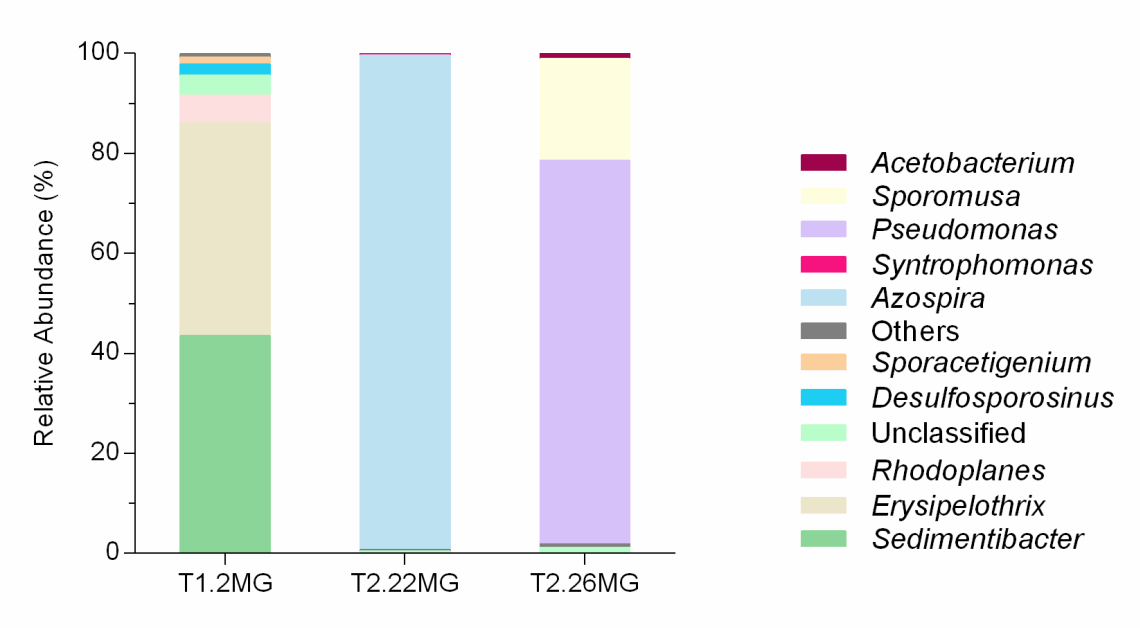


**Figure 3.3.** Relative abundance of bacterial sequences at the genus level for cultures T1.2MG, T2.22MG and T2.26MG**.** Classified genera with relative abundances above the cutoff value of 0.3% are indicated. OTU's could not be taxonomically assigned at the genus level are reported as "Unclassified". The category "Others" comprise all the genera with a relative abundance of < 0.3%.

In both T1.2MG and T2.26MG cultures the major archaeal populations enriched were affiliated at the genus level with *Methanosarcina* which groups within the order *Methanosarcinales* of the phylum *Euryarchaeota* (Table 3.1). In culture T2.22MG, while one of less abundant OTU's was classified at the genus level as *Methanosarcina* (12.89%), the major OTU's enriched were assigned as affiliated with the Rice Cluster I (46.55%) or affiliated with the genus *Methanocella* (40.14%) within the order *Methanocellales* on the phylum *Euryarchaeota* (Table 3.1).

**Table 3.1.** Relative abundance of archaeal sequences for samples T1.2MG, T2.22MG and T2.26MG at the phylum, class, order, family and genus levels.

| Sample | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|
| **T1.2MG** | *Euyarchaeota* (100%) | *Methanomicrobia* (100%) | *Methanosarcinales* (99.85%)<br><br>Others (0.15%) | *Methanosarcinaceae* (99.85%)<br><br>Others (0.15%) | *Methanosarcina* (99.84%)<br><br>Others (0.16%) |
| **T2.22MG** | *Euyarchaeota* (100%) | *Methanomicrobia* (100%) | *Methanocellales* (86.72%)<br><br>*Methanosarcinales* (12.89%)<br><br>Unclassified (0.39%) | *Methanocellaceae* (86.72%)<br><br>*Methanosarcinaceae* (12.89%)<br><br>Unclassified (0.39%) | Rice Cluster I (46.55%)<br><br>*Methanocella* (40.14%)<br><br>*Methanosarcina* (12.89%)<br><br>Unclassified (0.42%) |
| **T2.26MG** | *Euyarchaeota* (100%) | *Methanomicrobia* (100%) | *Methanosarcinales* (100%) | *Methanosarcinaceae* (100%) | *Methanosarcina* (100%) |

Confidence levels higher than 80% have been considered. Percentages have been calculated considering the total of high quality reads for each samples, T1.2MG (19039), T2.22MG (3321) and T2.26MG (24007).

## 3.4. Discussion

As discussed in Chapter 2, enrichment cultures of samples from rock cores were successfully established with active methane production. Pyrosequencing analysis of both bacterial and archaeal 16S rRNA gene amplified from DNA extracted from three selected enrichment cultures, identified archaeal populations enriched as well as bacterial populations, which were not recovered by isolation/culture based assays as described in Chapter 2.

The major bacterial genus enriched among the three cultures were either facultative anaerobic bacteria (e.g. *Erysipelothrix*, *Pseudomonas*, *Rhodoplanes*) or strict anaerobic bacteria (e.g. *Sedimentibacter*, *Sporacetigenium*, *Acetobacterium*, *Sporomusa*, *Desulfosporosinus*).

In culture T2.22MG (inoculated with rock core sample from 450.3 mbs, BH10), the most abundant OTU was assigned to the genus *Azospira* (98.85%) (Figure 3.3), which belong to the class *Betaproteobacteria*. Members of this genus are known for their ability for nitrogen fixation (Oren 2014). *Azospira* spp. are characterized by having a chemoorganoheterotrophic and strictly respiratory metabolism, with some species capable of using nitrate or perchlorate as an electron acceptor (Oren 2014).

With respect to culture T1.2MG (inoculated with rock core sample from 63.55 mbs, BH11), one of the major phylotypes enriched was related to *Sedimentibacter* (43.5%) at the genus level. Members of this genus are fermentative bacteria and have frequently been isolated from sediments. The genus *Erysipelothrix* (42.58%) was also predominantly enriched in this culture. *Erysipelothrix* are chemoorganotrophic facultative anaerobes, with a weakly fermentative and mainly respiratory metabolism (Stackebrandt 2015). *Sporacetigenium* was also enriched although less abundant (1.30%). Members of this genus have a chemoorganotrophic and fermentative metabolism (Chen *et al.* 2006). On the other hand, *Desulfosporosinus* (2.22%), also enriched, are well known sulfate reducing bacteria, able to grow autotrophically (on hydrogen and sulfate) as well as having a fermentative metabolism (Hippe and Stackebrandt 2015).

Pyrosequencing data indicated that members of *Rhodoplanes* (5.5%) were enriched in culture T1.2MG. While strains affiliated with this genus were not isolated from this particular culture, their presence was identified by amplification of 16S rRNA gene in subcultures from this enrichment culture during isolation studies directed at isolation of methanogenic archaea (Chapter 2). Additionally, several other isolates affiliated to *Rhodoplanes* were recovered from various depths along BH10 borehole (Chapter 2, Table 2.3). In culture T2.26MG (inoculated with rock core sample from

492.6 mbs, BH10) at the genus level the most abundant OTU was assigned to *Pseudomonas* (76.74%). Strain T2.26MG-48.2 affiliated with the genus *Pseudomonas* was recovered by isolation from this culture (Chapter 2, Table 2.3).

*Sporomusa* (20.28%) and *Acetobacterium* (1.06%) were enriched in culture T2.26MG. Members of these genera are acetogenic bacteria, with ability to grow on chemolithoautotrophic conditions (with hydrogen and carbon dioxide) but also chemoorganotrophically (Drake and Gössner 2015; Simankova and Kotsyurbenko 2015). Identification of sequences affiliated with acetogenic bacteria as well as the isolation of a bacterial strain affiliated with the genus *Acetoanaerobium* (Chapter 2), further supports that diverse populations of acetogenic bacteria inhabit the deep subsurface and may play a role in acetate production in situ.

Bacterial strains isolated from cultures T1.2MG, T2.22MG and T2.26MG (Chapter 2, Table 2.2), which were affiliated based on 16S rRNA gene with genera such as *Pleomorphomonas, Macellibacteroides, Desulfovibrio, Acetoanaerobium, Rhizobium, Nocardioides or Brevundimonas* were also detected by pyrosequencing analysis of amplified bacterial 16S rRNA gene. OTU's classified as affiliated with these genera were grouped within "Others" (Figure 3.3), since their respective relative abundance was ≤0.3%.

The bacterial diversity enriched differed amid the three studied cultures. Different depth of rock sampled, as well the distinct substrates applied to promote enrichment in each culture, can account for the diversity. Moreover, these may not be the only factors in play which could have influenced the enrichment of the detected populations. Possibly, byproducts from the growth of some populations may also have played a role in promoting growth of other populations.

Pyrosequencing analysis of the archaeal 16S rRNA gene sequences, indicated the enrichment in archaea belonging to the phyla *Euryarchaeota* and related to the genus *Methanosarcina* in all three cultures, i.e. in cultures enriched using as substrate either acetate, a mix of propionate/butyrate/methanol or an atmosphere of hydrogen/carbon dioxide (Table 3.1). As discussed in Chapter 2, isolation assays using as inoculum the culture T1.2MG resulted in a subculture named T1.2MG-K100, comprising an enrichment in an archaeal strain affiliated with the genus *Methanosarcina* and a bacterial strain affiliated with the genus *Rhodoplanes*.

Members of the genus *Methanosarcina* are known to have a more versatile carbon metabolism than other methanogenic taxa, being able to use a wider range of substrates (i.e. acetate, $H_2$:$CO_2$ or methyl compounds) (Kendall and Boone 2006). To date there are fourteen species of *Methanosarcina* described with validly published names (http://www.bacterio.net/methanosarcina.html).

The detection of a positive signal by microarray analysis to members of the family *Methanosarcinaceae* has been previously reported in samples from the deep subsurface at Peña de Hierro (Puente-Sánchez *et al.* 2014a). Archaea affiliated with the genus *Methanosarcina* has also been described in other deep subsurface environments, although from groundwater samples, such as the case of two novel species *M. horonobensis* and *M. subterranea* (Shimizu *et al.* 2011; Shimizu *et al.* 2015).

Besides the enrichment in *Methanosarcina*, in culture T2.22MG, the two major archaeal OTU's enriched were affiliated with the family *Methanocellaceae* (Table 3.1). At the genus level the major phylotype was related to the genus *Methanocella* and the secondly predominant phylotype was classified as Rice Cluster I (Table 3.1).

*Methanocella* is the sole genus so far characterized in the family *Methanocellaceae* of the order *Methanocellales*. The order *Methanocellales* was previously recognized as the group Rice Cluster I (Sakai *et al.* 2007). The Rice Cluster I represented a lineage of uncultured archaea which are dominant in rice paddy soils. Members of the Rice Cluster I lineage remained uncultured for several years until in 2007 Sakai and colleagues developed a co-culture approach that allowed isolation and subsequent characterization of the first cultured member of this group, *Methanocella paludicola* (Sakai *et al.* 2007; Sakai *et al.* 2008). The key to enrichment and isolation of *M. paludicola* strain SANAE$^T$ was the utilization of a co-culture approach with a syntrophic bacterium, which grew on oxidation of propionate and produced hydrogen as a byproduct. The bacterium was a slow grower, therefore it provided low concentration of hydrogen over time, similar to the concentrations encountered in natural habitats, which allowed the enrichment of *M. paludicola* SANAE$^T$ (Sakai *et al.* 2007).

To date, only three strains of *Methanocellales* have been obtained in pure culture, namely *M. paludicola* (Sakai *et al.* 2007)*, M. arvoryzae* (Sakai *et al.* 2010) and *M. conradii* (Lü and Lu 2012). The three isolated strains are hydrogenotrophs. Addition of acetate has also been reported necessary as a carbon source for growth (Sakai *et al.* 2008; Sakai *et al.* 2010; Lü and Lu 2012).

In this work, in culture T2.22MG, rock samples were enriched in a basal media supplemented with a mixture of propionate/methanol/butyrate to promote the simultaneously culture of bacteria growing on the oxidation of small fatty acids and the enrichment of methanogens, either growing on methanol or hydrogen resulting from bacterial fermentation of the supplemented fatty acids. The enrichment in populations affiliated with *Methanocella* and Rice Cluster I, possibly correlated with the co-enrichment with a hydrogen producing syntrophic bacteria. In fact, it was identified in

this culture a minor enriched bacterial phylotype that was affiliated with the genus *Syntrophomonas* (Figure 3.3).

Members of *Syntrophomonas* are characterized by their ability for syntrophic anaerobic oxidization of fatty acids (e.g. butyrate and other longer saturated fatty acids up to $C_{18}$) (Sekiguchi 2015). *Syntrophomonas* can´t oxidize fatty acids, a reaction which is thermodynamically unfavorable, unless hydrogen (a byproduct) is removed. Therefore these bacteria occur in nature on syntrophy with hydrogen-using microorganisms such as hydrogenotrophic methanogens (Müller *et al.* 2010).

To the best of our knowledge this is the first report of the presence of members of the *Methanocellales* in rock cores from subsurface environments. Much is still necessary to be learned about this intriguing group and the identification of members enriched from rock samples retrieved from 450.3 m deep into the subsurface of the Iberian Pyritic Belt provides further information about the ecological distribution of these archaea.

Studies on drilling and analysis of rock cores from BH10 and BH11 revealed the presence of hydrogen, carbon dioxide and methane gases on both boreholes (Amils *et al.* 2014). In addition, soluble inorganic anions such as nitrate, nitrite or sulfate and organic acids such as acetate, propionate or formate have been extracted from different samples at various depths (Amils *et al.* 2014). Therefore, not only methane is present in the deep subsurface of the IPB at Peña de Hierro, but also the substrates (e.g. hydrogen and carbon dioxide) that could support growth of methanogenic populations in situ.

Recently, colleagues analyzing rock core samples by CARD-FISH with order and family specific probes, have demonstrated the presence of live methanogens in situ, affiliated with the orders *Methanosarcinales* and *Methanomicrobiales,* and the family *Methanococcales* (Escudero *et al.* in preparation). Furthermore, methane is a gas, that can diffuse and migrate easily through pores and fractures on rocks and may also comprise an important substrate to support in situ communities of methanotrophic microorganisms, whose presence in rock cores on both BH10 and BH11 boreholes has been inferred by the positive activity in enrichment cultures for methanotrophic metabolism (Amils *et al.* 2014).

The identified microbial diversity enriched by culture-independent techniques, as well as the identification of strains isolated in pure culture as discussed in Chapter 2 indicates that diversified microbial populations occur within the subsurface of Río Tinto aquifer. Moreover, the presence of bacterial and archaeal populations on rock core samples, affiliated with known microorganisms with diverse metabolic potential (e.g. fermentative metabolism, sulfate reduction, nitrate reduction, iron reduction,

acetogenesis or methanogenesis) suggests that these metabolisms may potentially play a role in supporting microbial populations in the subsurface.

# CHAPTER 4

## Whole genome sequencing of *Tessaracoccus lapidicaptus* strain T2.5-30

**This work is partially published in:**

Leandro T, da Costa M. S., Sanz J. L., Amils R. 2017. Complete genome sequence of *Tessaracoccus* sp. strain T2.5-30 isolated from 139.5 meters deep on the subsurface of the Iberian Pyritic Belt. Genome Announcements 5: e00238-17.

## 4.1. Introduction

Isolation studies from enrichment cultures of rock cores retrieved from the IPB deep subsurface (see Chapter 2) resulted in the isolation of several strains affiliated with the genus *Tessaracoccus*.

The genus *Tessaracoccus* was described in 1999 and is classified within the family *Propionibacteriaceae*, phylum *Actinobacteria* (Maszenan *et al.* 1999). The genus contains nine validly published species, namely, *T. bendigoensis* (Maszenan *et al.* 1999), *T. flavescens* (Lee and Lee 2008), *T. lubricantis* (Kämpfer *et al.* 2009), *T. lapidicaptus* (Puente-Sánchez *et al.* 2014b), *T. oleiagri (Cai et al. 2011)*, *T. rhinocerotis* (Li *et al.* 2016), *T. flavus* (Kumari *et al.* 2016), *T. massiliensis* (Seck *et al.* 2016), *T. defluvii* (Srinivasan *et al.* 2017), and one species, *T. profundi* (Finster *et al.* 2009), that has not been validly published. Recently, a novel species *T. arenae* has been published (Thongphrom *et al.* 2017). *Tessaracoccus* species have been isolated from diverse environments and are characterized as Gram-positive non-spore-forming facultative anaerobic bacteria (Maszenan *et al.* 1999). The type strains of *T. profundi* and *T. lapidicaptus* have been isolated from drill cores recovered from deep terrestrial subsurface environments (Finster *et al.* 2009; Puente-Sánchez *et al.* 2014b).

The isolation of several *Tessaracoccus* strains from enrichment cultures of rock cores from both BH10 and BH11 (see Chapter 2), the isolation by colleagues of a novel *Tessaracoccus* species from BH11 (Puente-Sánchez *et al.* 2014b), the identification from methanogenic, acetogenic, nitrate reducing and sulfate reducing enrichment cultures from both boreholes of sequences affiliated with the genus *Tessaracoccus* by massive sequencing of 16S rRNA gene (Amils *et al*. in preparation), as well as the demonstration of the presence of *Tessaracoccus* spp. throughout BH10 borehole by in situ hybridization with a genus specific probe (Escudero *et al.* in preparation), suggested an ubiquitous presence of *Tessaracoccus* strains in the deep subsurface of Peña de Hierro aquifer.

To further investigate the genetic potential of *Tessaracoccus* strains, T2.5-30 strain, isolated from a rock core at 139.5 mbs from BH10 borehole (see Chapter 2), was selected for whole genome sequencing. Simultaneously to our work, a draft genome sequence of the type strain of *Tessaracoccus lapidicaptus* (strain IPBSL-7$^T$), the closest strain based on 16S rRNA gene similarity to our isolate (Chapter 2), has also been reported (Puente-Sánchez *et al.* 2016b).

Here we report strain T2.5-30 genome annotation and analysis as well as its genomic relatedness comparison with strain IPBSL-7$^T$, previously isolated from a rock core at 297 mbs from BH11 borehole (Puente-Sánchez *et al.* 2014b).

## 4.2. Material and Methods

### 4.2.1. Isolation conditions and growth conditions

*Tessaracoccus* sp. strain T2.5-30 was isolated in an anaerobic basal media supplemented with $H_2$:$CO_2$ (80:20, v/v) (see Chapter 2). Inoculation on R2A (Difco) media and incubation at 30-37ºC was latter used for maintenance.

### 4.2.2. DNA extraction

For whole genome sequencing, DNA was extracted using a centyltrimethylammonium bromide (CTAB)-based extraction method (Wilson 2001) as described in section 2.2.5 (Chapter 2), including an additional step of incubation with RNAse A (0.1mg/ml) for 40 min at 37ºC, for RNA degradation. The quantity of extracted genomic DNA was determined with the Qubit version 2.0 fluorometer (Invitrogen), and quality was analyzed by electrophoresis on an agarose gel, as well as on a NanoDrop 2000 (Thermo Scientific) for measurement of $A_{260}/A_{280}$ ratio.

### 4.2.3. Genome sequencing and assembly

Genomic DNA was submitted to the Norwegian Sequencing Centre (University of Oslo, Norway) for PacBio single-molecule real time (SMRT) sequencing (Eid *et al.* 2009). One SMRT cell was used for sequencing on a Pacific Biosciences RSII instrument using P6-C4 chemistry, with 360-min movie time. The generated reads were filtered and then assembled using the Hierarchical Genome Assembly Process (HGAP) (SMRT Analysis Software version 2.3.0; Pacific Biosciences) (Chin *et al.* 2013). The final assembly resulted in four contigs. The Minimus2 software (Amos package) was used to circularize the contigs (Sommer *et al.* 2007). Circularization of contig 0 by joining and trimming of the overlapping 3' and 5' ends resulted in a circular closed chromosome. Contigs 1, 2 and 4 correspond to direct repeats of sequences contained in contig 0 and were excluded from our analysis.

The complete genome of strain T2.5-30 was annotated with the automated pipelines of NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova *et al.* 2016) and within the PATRIC server using RASTtk annotation pipeline, a modular version of RAST (Rapid Annotation using Subsystem Technology) (Wattam *et al.* 2013; Brettin *et al.* 2015). Further functional information was predicted by BLAST searches against GenBank non-redundant (NR) database and using InterPro (Jones *et al.* 2014).

Metabolic pathway prediction was based on mapping of predicted protein coding sequences onto the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata *et al.* 1999). Assignment of predicted protein coding genes to clusters of orthologous genes (COG) was performed with the Clusters of Orthologous Genes database (Tatusov *et al.* 2000) using the WebMGA server (Wu *et al.* 2011). The tools RNAmmer (Lagesen *et al.* 2007) and tRNAscan-SE (Lowe and Eddy 1997) were used for prediction of ribosomal RNA (rRNA) and transfer RNAs (tRNA), respectively. A circular view of the genome was constructed using DNAPlotter (Carver *et al.* 2009). Genomic Islands (GEI) were identified by IslandViewer v 4.0 (Bertelli *et al.* 2017). Further analysis of genes within GEIs and GC content was performed with Artemis (Rutherford *et al.* 2000) using the output from IslandViewer.

The Average Nucleotide Identity (ANI) between genome of strain T2.5-30 and the type strain of *T. lapidicaptus*, strain IPBSL-7 was determined using the ANI calculator from the Kostas' lab (http://enve-omics.ce.gatech.edu/ani/). In addition, genome-to-genome distance (GGD) was also calculated using the Genome-to-Genome Distance calculator 2.1 (Meier-Kolthoff *et al.* 2013).

The genome files for strain IPBSL-7 were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/).

## 4.3. Results and Discussion

### 4.3.1. Strain T2.5-30 general genome features

The genome of strain T2.5-30 has a circular chromosome of approximately 3.2 Mbp and an average G+C content of 70.4% (Figure 4.1, Table 4.1). PacBio SMRT sequencing resulted in approximately 175% coverage. The genome contains 2988 predicted coding DNA sequences (CDSs). Of the total protein-coding genes, 1953 were assigned a putative function while the remaining were annotated as hypothetical proteins. Two rRNA genes operons are present in the genome and 45 tRNA genes (Figure 4.1, Table 4.1).

**Table 4.1.** *Tessaracoccus* sp. strain T2.5-30 genome statistics

| Features | Genome strain T2.5-30 |
|---|---|
| Genome size (bp) | 3212699 |
| DNA G+C content (%) | 70.4 |
| Extrachromosomal elements | 0 |
| Total genes | 3042 |
| Protein coding genes | 2988 |
| Proteins with function prediction | 1953 |
| Hypothetical proteins | 1035 |
| tRNA genes | 45 |
| rRNA genes | 6 (two operons - 5S, 23S,16S) |
| Non-coding RNA's | 3 |
| Genes assigned to COGs | 2463 |
| Genomic islands | 17 |
| CRISPRs repeats | 0 |

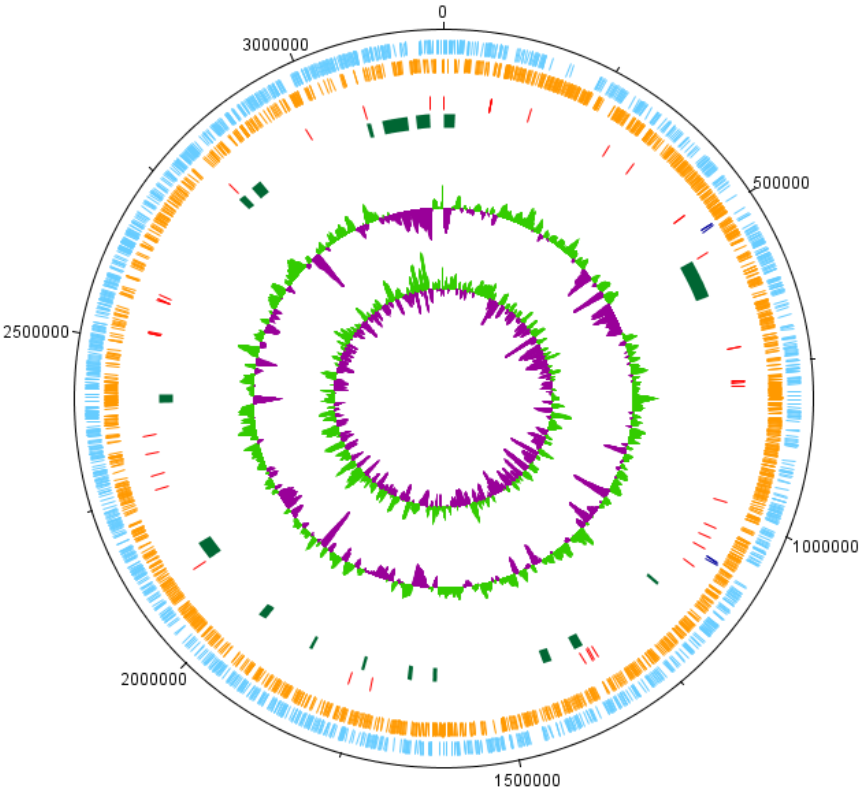


**Figure 4.1**. Representation of the circular genome of *Tessaracoccus* sp. strain T2.5-30. The circles represent from the outside to the center: circle 1, DNA base position (bp); circle 2 and 3, predicted coding sequences on the forward strand (blue) and in the reverse strand (orange); circle 4 and 5 shows the rRNA genes (dark blue) and tRNA genes (red), respectively; circle 6, genomic islands predicted by IslandViewer (integrative view of all the islands predicted with both IslandPath-DIMOB and SIGI-HMM methods); circle 7, G + C content, green (positive deviation from the average), purple (negative deviation from the average); circle 8, GC skew [(G-C)/(G+C)], green positive skew, purple negative skew.

A total of 17 putative genomic islands (Figure 4.1) were identified in the genome of strain T2.5-30 by IslandViewer (Bertelli *et al.* 2017). Bacterial genomic islands evolve from mobile genetic elements such as lysogenic bacteriophages and plasmids (Dobrindt *et al.* 2004). The size of the genomic islands varied from 4785 bp to 69636 bp. Sixteen of the putative genomic islands had a lower GC content (63.1% to 69.9%) while one genomic island had a higher a GC content (72.7%) than the average GC content of the genome (70.4%). Analysis of genes encoded within the predicted islands revealed that a majority were assigned as hypothetical proteins. Within genomic islands, were also encoded putative genes involved in resistance to heavy metals, restriction-modification system, carbohydrate metabolism, sugar transporters or toxin-antitoxin systems. In addition, genes involved in mobilization of genetic elements such as transposases and integrases were present as well, suggesting potential for mobility.

The presence of genomic islands suggests that several episodes of horizontal gene transfer occurred within the genome. As seen in Figure 4.1 the presence of constant shifts in GC skew values along the genome also suggests the occurrence of horizontal gene transfer events (Mira *et al.* 2004; Mann and Chen 2010).

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas is an adaptive immune system present in prokaryotes that provides sequence directed defense against invasion from foreign nucleic acids (Rath *et al.* 2015). Search for CRISPRs elements through both annotation pipelines indicated that these elements were not present within the genome.

### 4.3.2. Comparative genomics

#### 4.3.2.1. Calculation of relatedness between strain T2.5-30 and strain IPBSL-7

Comparison between the genome of both strains resulted in an ANI value of 99.56%. The cut off ANI value for species is set for 95-96% (Richter and Rosselló-Móra 2009). In addition, determination of genome to genome distance resulted in a GGD value of 95%, which is above the 70% cut-off value for species boundaries as well. Therefore both are strains of the species *Tessaracoccus lapidicaptus*.

### 4.3.3. Strain T2.5-30 genomic functional annotation

A total of 82.4% of the predicted CDSs could be assigned to the COG database. From these, 8.1% were assigned to more than one GOG category. The distribution of genes into COG functional categories (Table 4.2) shows that 376 predicted CDSs were involved with carbohydrate transport/metabolism, whereas 131 predicted CDSs were related to inorganic ion transport/metabolism and 214 predicted CDSs were involved in amino acid transport/metabolism.

In the following subsections, pathway prediction based on genes encoded in the genome and putative functions are further discussed. A schematic representation of central metabolic pathways (involving carbon, nitrogen and sulfur), putative transporters among other encoded metabolic features is presented in Figure 4.2.

**Table 4.2.** Distribution of genes into COG functional categories for *Tessaracoccus* sp. strain T2.5-30.

| Functional Categories | | | |
|---|---|---|---|
| **Code** | **Description** | **No. genes** | **%age*** |
| | *Cellular processes and signaling* | | |
| D | Cell cycle control, cell division, chromosome partitioning | 25 | 0.84 |
| M | Cell wall/membrane/envelope biogenesis | 114 | 3.82 |
| N | Cell motility | 5 | 0.17 |
| O | Posttranslational modification, protein turnover, chaperones | 80 | 2.68 |
| T | Signal transduction mechanisms | 87 | 2.91 |
| U | Intracellular trafficking, secretion, and vesicular transport | 38 | 1.27 |
| V | Defense mechanisms | 48 | 1.61 |
| | *Information storage and processing* | | |
| A | RNA processing and modification | 1 | 0.03 |
| J | Translation, ribosomal structure and biogenesis | 154 | 5.15 |
| K | Transcription | 210 | 7.03 |
| L | Replication, recombination and repair | 170 | 5.69 |
| | *Metabolism* | | |
| C | Energy production and conversion | 192 | 6.43 |
| E | Amino acid transport and metabolism | 214 | 7.16 |
| F | Nucleotide transport and metabolism | 78 | 2.61 |
| G | Carbohydrate transport and metabolism | 376 | 12.58 |
| H | Coenzyme transport and metabolism | 144 | 4.82 |
| I | Lipid transport and metabolism | 73 | 2.44 |
| P | Inorganic ion transport and metabolism | 131 | 4.38 |
| Q | Secondary metabolites biosynthesis, transport and catabolism | 37 | 1.24 |
| | *Poorly Characterized* | | |
| R | General function prediction only | 309 | 10.34 |
| S | Function unknown | 209 | 6.99 |
| - | Not in COG's | 525 | 17.6 |

*The total was based on the total number of predicted protein coding genes in the genome.

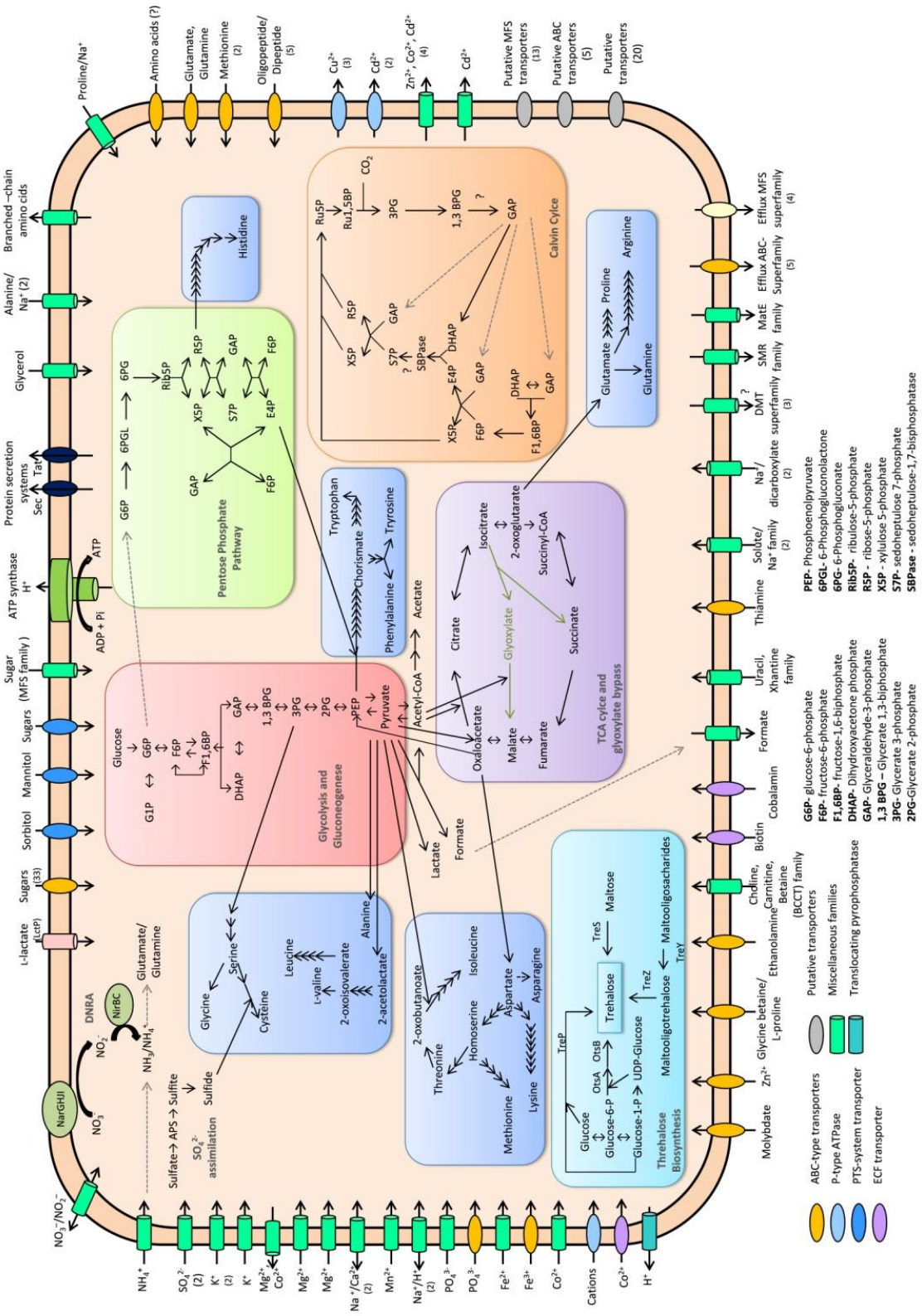


**Figure 4.2.** Schematic representation of predicted central metabolic pathways (involving carbon, nitrogen and sulfur), amino acid biosynthesis, trehalose biosynthesis, and putative transporters in the genome of *Tessaracoccus* sp. strain T2.5-30. Full black arrows represent reactions catalyzed by predicted proteins encoded in the genome. Full green arrows represent glyoxylate pathway specific reactions. Dotted grey lines point to reactions where the respective compound intervenes.

#### 4.3.3.1. Central Carbon Metabolic Pathways

Glucose is a typical substrate used by many microorganisms. Glucose is converted through the Embden Meyerhof Parnas (EMP) pathway (Madigan *et al.* 2012) into pyruvate (Figure 4.2). Genes coding the enzymes which catalyze each step of this pathway are present in the genome of strain T2.5-30. The Entner-Doudoroff pathway, an alternative pathway involved in glucose metabolism, is incomplete, since the genes coding for two key enzymes (phosphogluconate dehydratase and phospho-2-keto-3-deoxygluconate aldolase) are not present in the genome. The genome of strain T2.5-30 also encodes the gluconeogenic metabolic pathway (Figure 4.2). The genome also carries the genes coding for the enzymes involved in the pentose phosphate pathway (Figure 4.2).

In the EMP pathway, the third step consists in the phosphorylation of fructose-6-phospate (using phosphate from ATP) into fructose-1,6-biphosphate, an irreversible reaction that is catalyzed by the enzyme phosphofructokinase (Pfk). This enzyme is encoded in the genome of strain T2.5-30. Additionally, the genome also has a gene coding for a pyrophosphate-dependent phosphofructokinase ($PP_i$-Pfk), which adds a phosphate from pyrophosphate ($PP_i$) to produce fructose-1,6-biphosphate. This enzyme is reversible and may function also in gluconeogenesis (Mertens *et al.* 1998). The utilization of enzymes dependent on $PP_i$ in the EMP pathway has been previously suggested to be related with decrease in ATP consumption, therefore making EMP pathway more efficient, which translates in more ATP available to fuel cellular processes. Among prokaryotes, this enzyme has also mainly been found in anaerobic bacteria (Mertens *et al.* 1998; Moore *et al.* 2002; Reshetnikov *et al.* 2008).

The genes coding for the enzymes involved in the functioning of the tricarboxylic acid (TCA) cycle are present (Figure 4.2). Furthermore, the key enzymes involved in glyoxylate cycle (isocitrate lyase and malate synthase) are encoded in the genome (Figure 4.2). This cycle allows assimilation of acetyl-CoA which could, for example, be derived from beta-oxidation of fatty acids. Functioning of glyoxylate cycle produces malate and succinate, both intermediates of the TCA. The glyoxylate cycle function as an anaplerotic reaction for replenishment of oxaloacetate (Shimizu 2013). Anaplerotic reactions, replenish the intermediates of the main metabolic pathways which are used as precursors in biosynthetic pathways. Oxaloacetate is one of such cases, as is involved in several anabolic processes such as in the biosynthesis of amino acids, pyrimidines and is also a substrate for the phosphoenolpyruvate carboxykinase, the first enzyme in the gluconeogenic pathway (Shimizu 2013). Other anaplerotic routes for replenishment of oxaloacetate are also encoded in the genome

such as conversion of phosphoenolpyruvate into oxaloacetate (catalyzed by a phosphoenolpyruvate carboxylase) or the conversion of pyruvate into oxaloacetate (catalyzed by a pyruvate carboxylase) (Shimizu 2013).

From the analysis of the genome, this strain potentially is unable to use acetate as substrate, since the gene encoding the enzyme acetyl-CoA synthetase, the enzyme that catalyzes synthesis of acetyl-CoA from acetate is missing. Furthermore, genes homologous to the *lutABC* operon are present in the genome. This operon encodes a iron-sulfur enzyme complex that has been shown to be involved in oxidation of lactate into pyruvate (Hwang *et al.* 2013).

As depicted in Figure 4.2, the genome contains genes coding for several ATP-binding cassette (ABC) sugar transporters, phosphotransferase system (PTS) sugar transporters, a lactate permease, ABC-type amino acid transporters and other amino acid transporters (Figure 4.2). Besides, also present are genes coding for glycoside hydrolases, peptidases and proteases.

Within, the encoded glycoside hydrolases, are four gene copies coding for endo-1,4-β-xylanases and one gene coding for a xylan 1,4-β-xylosidase. Both these enzymes are involved in the hydrolysis of xylan, a polysaccharide with a backbone of β -1,4 liked xylose units with diverse side chain residues, e.g. arabinose, glucuronic acid or acetyl (Collins *et al.* 2005; Jordan and Wagschal 2010). Xylan is the second most abundant polysaccharide in nature and enzymes involved in their degradation are of great interest for biotechnological applications (Chakdar *et al.* 2016). Besides, strain T2-5-30 genome also codes for acetylxylan esterases and α-L-arabinofuranosidases, enzymes which are also involved in xylan degradation process (Juturu and Wu 2012).

In anaerobic conditions bacteria may use fermentative pathways for energy production. The genome of strain T2.5-30 encodes a NAD-dependent L-lactate dehydrogenase which may play a role in anaerobic glycolysis by regenerating NADH through conversion of pyruvate into lactate (Figure 4.2) (Wang *et al.* 2014). The enzymes involved in the conversion of acetyl-CoA via acetyl-phosphate to acetate with production of ATP are also encoded in the genome (Figure 4.2). Besides, the enzyme pyruvate formate lyase that catalyzes conversion of pyruvate to formate is also present in the genome. Both acetyl-CoA and formate are produced by this reaction (Figure 4.2). The acetyl-CoA produced would enter the TCA cycle. However, since the enzyme coding a formate hydrogen lyase complex is not encoded in the genome (which would convert formate into carbon dioxide and hydrogen) (Shimizu 2013), the formate produced may be exported by the cell through a formate efflux transporter (Figure 4.2) which is also encoded in the genome.

Some species are known which can use ethanolamine, a common compound found in cellular membranes (e.g. as the phospholipid phosphatidylethanolamine). Ethanolamine can be used as both a carbon and nitrogen source. The process of ethanolamine utilization involves splitting the molecule into ammonia (that can be used as nitrogen source) and into acetaldehyde, which is subsequently converted into acetyl-CoA which can enter the TCA cycle or be used in lipid biosynthesis. Genes homologous to the *eut* operon which encodes proteins involved in the ethanolamine utilization pathway are present in the genome of strain T2.5-30 (*eutH, eutN, eutA, eutBC, eutL, eutE, eutQ, eutT, eutJ*). Additionally, three genes were predicted as part of the operon and were annotated as hypothetical proteins. The gene *eutH* encodes a transmembrane permease which facilitates ethanolamine diffusion (Figure 4.2), *eutBC* encodes the key enzyme responsible for ethanolamine conversion to ammonia and acetaldehyde and *eutE* encodes a acetaldehyde dehydrogenase that converts acetaldehyde into acetyl-CoA. Remaining genes encode auxiliary proteins to this metabolism (Garsin 2010).

The genome of strain T2.5-30 encodes genes involved in the fixation of inorganic carbon through the Calvin-Benson-Bassham cycle (CBB cycle), including genes coding for the key enzymes type II ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) and phosphoribulokinase (Madigan *et al.* 2012). Nevertheless, as has also been previously described in genomes of the chemolithoautotrophs *Nitrosomonas europaea* (Chain *et al.* 2003) and *Pseudonocardia dioxanivorans* (Grostern and Alvarez-Cohen 2013), the genes encoding sedoheptulose bisphosphatase (EC 3.1.3.37) as well as the gene encoding for NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) are not present in the genome of strain T2.5-30. Studies in *Nitrosomonas europaea* and *Pseudonocardia dioxanivorans* have proposed that NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), which functions in glycolysis and gluconeogenesis replace the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In strain T2.5-30, a gene coding for a NADH-dependent glyceraldehyde-3-phosphate dehydrogenase is present. Previous studies have also proposed that the action of a bifunctional fructose biphosphatase can catalyze the dephosphorylation of sedoheptulose biphosphate instead of the enzyme sedoheptulose biphosphatase in the CBB cycle (Amachi and Bowien 1979; van den Bergh *et al.* 1995; Yoo and Bowien 1995; Chain *et al.* 2003; Grostern and Alvarez-Cohen 2013). The genome of T2-5-30 also encodes for a fructose biphosphatase located next to the gene encoding a phosphoribulokinase. The genome also encodes a carbonic anhydrase. Nevertheless,

fixation of carbon dioxide in strain T2.5-30 or in other *Tessaracoccus* strains has yet to be experimentally demonstrated.

The genome of strain T2.5-30 contains genes coding for enzymes that play a role in respiratory chains such as a NADH:ubiquinone oxidoreductase, ubiquinol-cytochrome-c reductase and the two terminal oxidases of aerobic respiration (a cytochrome c oxidase and a cytochrome bd ubiquinol oxidase). Genes coding for an ATPase are also encoded in the genome (Figure 4.2). The presence of genes encoding a terminal nitrate reductase which plays a role in anaerobic respiration of nitrate is addressed in section 4.3.3.2.

Genes encoding hydrogenases are not present in the genome, which supports the observations described in Chapter 2, that regardless of the presence of hydrogen in strain T2.5-30 isolation media, this was not used as substrate for growth. Although several *Tessaracoccus* strains were isolated under an atmosphere of $H_2/CO_2$, possibly growth was supported by the presence of the residual organics in the solid media matrix as well trace quantities of yeast extract, as a result of these strains ability to grow oligotrophically as discussed in Chapter 2.

Metabolic pathways for biosynthesis of all twenty amino acids were encoded in the genome of strain T2.5-30 (Figure 4.2). Pathways for degradation of amino acids such as alanine, proline, glutamine, glutamate, asparagine, aspartate, glycine, serine, threonine were also identified encoded in the genome. In addition, the enzymes involved in the pathway of beta oxidation of straight chain fatty acids are encoded in the genome as well.

#### 4.3.3.2. Nitrogen Metabolism

The pathway for dissimilatory nitrate reduction to ammonia (DNRA) is encoded in the genome of strain T2.5-30. This anaerobic pathway is involved in the reduction of nitrate into ammonia (Mohan and Cole 2007). The genome of strain T2.5-30 carries two genes (*narK*) encoding a putative nitrate/nitrite transporter (Figure 4.2), a membrane bound nitrate reductase (*narGHJI* genes) that catalyzes the reduction of nitrate into nitrite and a soluble cytoplasmic NAD(P)H-dependent nitrite reductase (*nirBD* genes) which reduces nitrite into ammonia. The reduction of nitrate to nitrite may be coupled to energy conservation (proton translocation across the cytoplasmic membrane contributing to generation of a proton motive force), while activity by nitrite reductase may help to detoxify nitrite resulting from nitrate respiring cells and also to regenerate $NAD(P)^+$ (Tiedje 1988). Additionally, ammonia produced may be converted into amino acids (Pengpeng and Tan 2013). The genes encoding glutamine synthase,

glutamate synthase and glutamate dehydrogenase, which are key enzymes involved in assimilation of ammonia into amino acids are encoded in the genome.

Furthermore, a gene coding for an ammonium transporter (*amtB*) (Figure 4.2) is also present in the genome. This transport protein is involved in import of ammonium ions ($NH_4^+$) to be used as a nitrogen source (Wacker *et al.* 2014).

#### 4.3.3.3. Sulfur Metabolism

The genome of strain T2.5-30 contains genes coding for sulfate permeases and an ABC transporter which are involved in uptake of sulfate into the cell (Figure 4.2).

Two mechanisms are recognized to be involved in the assimilation of sulfate into sulfite. In one pathway, after assimilation of sulfate into adenosine phosphosulfate (APS) by an ATP sulfhydrylase, the APS is converted into phosphoadenosine phosphosulfate (PAPS) by the action of an adenylyl-sulfate kinase. Then, PAPS is converted into sulfite by a PAPS reductase (Williams *et al.* 2002). In a second alternative pathway recognized in some microorganisms (e.g. *Mycobacterium tuberculosis*, *Acidithiobacillus ferrooxidans)* after assimilation of sulfate into APS, this compound is directly converted into sulfite by the action of a APS reductase (Williams *et al.* 2002; Valdés *et al.* 2003). The genome of strain T2.5-30 encodes an ATP sulfhydrylase but lack genes coding for adenylyl-sulfate kinase enzymes, suggesting that the APS produced from sulfate may be directly converted into sulfite by the action of a putative APS reductase encoded in the genome. The presence of a putative APS reductase in the genome of strain T2.5-30 genome was inferred as described before (Williams *et al.* 2002; Valdés *et al.* 2003). Briefly, similarity between sequences of APS and PAPS reductase make them difficult to ascertain by bioinformatics annotation programs. In addition both protein sequences present a characteristic signature (KRT)ECG(LS)H. Nevertheless, it has been suggested that APS reductase could be distinguished by the presence in its sequence of two motifs composed of cysteine residues, namely the sequences CCXXRKXXPL and SXGCXXCT, which appear to be present in all known APS reductases but are absent from PAPS reductase sequences.

The sulfite produced from APS may then be converted into sulfide by the action of a sulfite reductase which is encoded in the genome of strain T2.5-30. The assimilation of sulfide into amino acids may then proceed through the biosynthesis of cysteine by the action of cysteine synthase A which is encoded in the genome.

#### 4.3.3.4. Motility

Genes coding for biosynthesis of a flagella apparatus or pilus formation are not present. Lack of motility was also confirmed through observation of cells of strain T2.5-30 by phase contrast microscopy.

#### 4.3.3.5. Secretion systems

Genes coding for the general protein secretion systems (Sec system) and twin-arginine translocation system (Tat system) are present. Sec systems transport unfolded proteins while Tat systems can transport fully folded proteins (Natale *et al.* 2008).

#### 4.3.3.6. Compatible solute production/transport

Trehalose is a sugar disaccharide of glucose which acts as osmoprotectant (Iturriaga *et al.* 2009). Five pathways for trehalose biosynthesis have been described in bacteria: the TreY/TreZ, the TreP, the TreS, the TPS/TPP(OtsA/B) and the TreT pathways (Tournu *et al.* 2013). Four of these biosynthetic pathways (with exception of TreT) are encoded on the genome of strain T2.5-30 (Figure 4.2). A putative ABC-type glycine betaine transporter is also encoded in the genome (Figure 4.2). The accumulation of glycine betaine in the cell cytoplasm is involved in the process of osmoprotection (Kappes *et al.* 1996; Sleator and Hill 2002). In addition, the pathway for the synthesis of the amino acid proline from glutamate is also encoded in the genome. Proline has also been shown to function as osmoprotectant (Sleator and Hill 2002).

Growth under saline conditions has been described for the majority of isolated *Tessaracoccus* spp. Optimum growth values of 0 to 5 % NaCl have been described as well as growth to a upper limit of o 9 - 10% NaCl (Cai *et al.* 2011; Li *et al.* 2016). Strain T2.5-30 was able to growth up to 2% NaCl with optimum value at 0%.

Besides, their role on osmotic stress protection, accumulation of compatible solutes such as trehalose, glycine betaine and proline, have also been implicated in response to other environmental stresses such as desiccation, high temperature, freezing, oxidative stress, as well for their function as energy, carbon and nitrogen sources (Reina-Bueno *et al.* 2012).

#### 4.3.3.7. Genome repair systems

The genome of strain T2.5-30 encodes pathways involved in DNA repair, which protect DNA from errors during replication, oxidative stress, UV light or damage by toxic chemical compounds.

The genome encodes homologous of genes which code for proteins involved in base excision repair pathway (repair of small base lesions), nucleotide excision repair pathway (repair of bulky DNA damage induced by chemical compounds or UV light), non-homologous end-joining repair of double DNA breaks pathway, homologous recombination pathways for repair of single strand breaks as well as double breaks and photolyase activity (repair of UV induced damage).

#### 4.3.3.8. Oxidative stress response

The genome of strain T2.5-30 presents homologous of genes coding for several proteins involved in mechanisms for cellular protection against oxidative stress, which play a critical role for the maintenance of intracellular redox homeostasis required for proper cellular functioning.

Proteins involved in protection against oxidative stress such as superoxide dismutase, thioredoxins, thioredoxin reductase, glutaredoxin-like protein, peroxiredoxins, and proteins involved in the methionine sulfoxide reductase system are encoded in the genome.

Low molecular weight thiols, such as glutathione and mycothiol have a key role in maintenance of a reducing environment for the functioning of various cellular processes, as well as in protecting the cell from oxidative stress and participating on detoxification processes (Rawat and Av-Gay 2007; Johnson *et al.* 2009). Utilization of glutathione is widespread among both eukaryotes and Gram negative bacteria. The majority of Gram positive bacteria also produce glutathione. Nevertheless, the major thiol found in some *Actinobacteria* is mycothiol (Hayward *et al.* 2004; Johnson *et al.* 2009). The genome of strain T2.5-30 presents genes coding for the proteins involved in biosynthesis of both glutathione and mycothiol.

Both *gshA* and *gshB* genes, which are involved with in *de novo* synthesis of glutathione from glutamate, cysteine and glycine, are encoded in the genome. The genome of strain T2.5-30 also presents homologous of genes coding for glutathione reductase (regenerates reduced glutathione), gamma-glutamyl-transpeptidase (recycles glutathione into their component amino acids), glutathione peroxidase (role in

scavenging of oxygen reactive species) and a putative glutathione-S-transferase (role in detoxification) (Pophaly *et al.* 2012).

The genome also encodes the enzymes, glyoxalase I and glyoxalase II, which form the ubiquitous glyoxalase system for detoxification of electrophile compounds, which are highly cytotoxic for the cell, such as methylglyoxal and other reactive aldehydes that are produced as byproducts from typical cell metabolisms. This system converts the metabolic produced α-ketoaldehydes into their correspondent 2-hydroxycarboxylic acid (Suttisansanee and Honek 2011). For the case of methylglyoxal detoxification through the glyoxalase system, thiol group from glutathione is required and D-lactate production as well as glutathione regeneration occur on the last step. It has also been demonstrated that organisms that encode a D-lactate dehydrogenase can convert the D-lactate formed during detoxification of methylglyoxal into pyruvate (Suttisansanee and Honek 2011). The genome of strain T2.5-30 also encodes a putative D-lactate dehydrogenase.

As mentioned above the genome of strain T2.5-30 encodes genes homologous with genes involved in mycothiol biosynthesis (*mshA, mshB, mshC* and *mshD*). Mycothiol, like glutathione also have an important role in protecting the cell against oxidative stress and toxins (Rawat and Av-Gay 2007). The proteins mycothiol conjugate amidase and mycothiol disulfide reductase, which are required for detoxification processes of electrophilic compounds are also encoded in the genome. (Hayward *et al.* 2004).

There are just a few reports of microorganisms that encode genes involved in biosynthesis and utilization of both thiols, such as the case of *Rhodococcus* strain AD45, in which experimental evidence demonstrated the production of both compounds (Johnson *et al.* 2009).

#### 4.3.3.9. Resistance to heavy metals and drugs

The genome of strain T2.5-30 present several putative genes involved in mechanisms which may provide this strain resistance to heavy metals. Among these are: cadmium ($Cd^{2+}$) translocating P-type ATPase; copper ($Cu^{2+}$) translocating P-type ATPase; $Cd^{2+}$ permease (CadD); cation diffusion facilitator (CDF) superfamily which are involved in the efflux e.g. zinc ($Zn^{2+}$), cobalt ($Co^{2+}$) and cadmium ($Cd^{2+}$) (Figure 4.2).

The genome of strain T2.5-30 encodes various putative genes coding drug resistance such as: multidrug resistance transporters of the major facilitator superfamily (MFS); ABC-type multidrug transport system; several genes assigned as permease of

the drug/metabolite transporter (DMT) superfamily which include exporters for a wide range of substrates as toxic compounds or metabolites (Tsuchiya *et al.* 2016); small multidrug resistance family (SMR) proteins (which are members of the much larger DMT superfamily) (Sun *et al.* 2014); glyoxalase/bleomycin resistance protein/dioxygenase superfamily; a putative multidrug and toxic compound extrusion (MatE) family protein (Figure 4.2).

#### 4.3.3.10. Secondary metabolite production

Members of the *Actinobacteria* are known for their production of several terpenoids (or terpenes), which are a group of natural compounds synthesized from two, five carbon isoprene units as building blocks (e.g. C10, C15, C30, C20 or C40 terpenes) (Aldred *et al.* 2009). Biological function of these molecules can be varied. The genome of strain T2.5-30 encodes proteins involved in the pathway for biosynthesis of carotenoids such as zeta-carotene, lycopene, beta-carotene and isorenieratene. *Actinobacteria* are the only lineage apart from green sulfur bacteria where the synthesis of isorenieratene has been shown (Maresca *et al.* 2008), although their role is not completely understood. Additional pathways for synthesis of other terpenoid molecules where not present.

The genome of strain T2.5-30 does not encode enzymes involved in the pathways for biosynthesis of polyketides nor nonribosomal peptides, two classes of bioactive natural compounds (Chen *et al.* 2012).

### 4.3.4. Remarks on genome annotation of *Tessaracoccus* sp. strain T2.5-30 and life on the subsurface

Strain T2.5-30 was isolated at 139.5 mbs from BH10. Additionally, other isolates affiliated with the genus *Tessaracoccus* have also been recovered from rock cores, namely strains T1.17D-1 (BH11), T2.5-50 (BH10), T2.6-4D2 (BH10) and T2.10-17 (BH10) (Chapter 2).

Annotation of predicted protein coding genes for strain T2.5-30 provided evidence of potential for heterotrophic metabolism. Fermentative and respiratory pathways for energy production are encoded in the genome. A metabolic pathway for dissimilatory nitrate reduction to ammonia is also encoded in the genome, which may potentially be used for energy production and support growth in anaerobic conditions. While a putative pathway for carbon fixation by CBB cycle was inferred from the

genome, experimental demonstration is crucial to prove if indeed the reactions catalyzed by the two enzymes whose genes are lacking in this pathway could be bypassed by other encoded enzymes. As such, heterotrophic metabolism may possibly be the main and only route for carbon assimilation.

The genome also encodes several proteins associated with resistance to oxidative stress which is compatible with their ability to grow under aerobic conditions as well. Several pathways for nucleic acid repair are also encoded in the genome, which may be important on ensuring genome stability on slow growing cells over extended periods of time.

One should also consider that genome annotation is ultimately dependent on the information currently available on databases. As such, for around 35% of the predicted genes of strain T2.5-30 annotation was not possible. Genome sequencing studies offers a diverse repertoire of predicted genes and respective encoded proteins which remains to be studied. Proteins encoded by these putative genes may also hold clues to the understanding of both metabolic and physiological mechanisms which help bacteria cope with life on the subsurface.

The deep subsurface of the Río Tinto aquifer is an anaerobic and oligotrophic environment. Most likely, anaerobic chemolithoautotrophic respiratory metabolisms fueled by compounds originated from the rock matrix (e.g. $H_2$, $CO_2$, ferrous iron, sulfides) pave the way to the establishment of a trophic chain by acting as primary producers. In Chapters 2 and 3, we reported the presence on rocks from the IPB subsurface of microorganisms affiliated with known fermenting bacteria, iron reducing, sulfur reducing, nitrogen reducing, methanogenic and acetogenic microorganisms, thus describing that diversified populations inhabit this ecosystem. Moreover, the occurrence of biofilms on rock cores from BH10 borehole has also been recently demonstrated (Escudero *et al.* 2018).

The presence of microorganisms living in biofilms is ubiquitous in natural environments, where multispecies biofilms represent one of the most successful modes of living (Flemming *et al.* 2016). The biofilm matrix imparts both structural, as well as functional advantages which include capacity to retain water, concentrate nutrients, as well as facilitating nutrient recycling (e.g. from dead cells), resistance to grazing, enhance resistance/tolerance to several stresses (e.g. salinity, desiccation, pH, etc), cooperation between populations (e.g. enhance metabolic ability of cells), facilitate genetic exchange and intercellular communication (Flemming *et al.* 2016). Additionally, establishment of a biofilm plays an important role in habitat formation, thus creating a niche with distinct physicochemical proprieties than the surroundings, favoring microbial colonization (Flemming *et al.* 2016). These advantages, as studied for

surface biofilms likely play an analogous role in deep subsurface environments (Wanger *et al.* 2006). Despite the fact that biofilm formation involves a high energy cost to the cell, the presence of biofilms have also been reported before from the deep subsurface (Wanger *et al.* 2006; Jägevall *et al.* 2011; Wu *et al.* 2017), which suggests that growth in biofilms may play a critical beneficial role in colonization of deep subsurface environments.

Therefore, the combined action of bacteria living on biofilms may allow the synergic degradation of even refractory organic matter present on the rocks, as well as concentration of nutrients originating from dead microbial biomass. These potential sources of organic substrates may support heterotrophic growth even at lower depths, providing water is also available. Ultimately, the key for life in subsurface may reside on the interdependent and intricate relationships established between diverse microbial populations inhabiting the same microniche or closely connected microniches within the rocks.

The identification of common genetic determinants involved in biofilm formation across all microorganisms is a difficult process. There are diversified genetic factors which determine biofilm formation and additionally media and growth conditions influence the pattern of gene expression (Beloin and Ghigo 2005). We observed that when *Tessaracoccus* sp. strain T2.5-30 as well as *Tessaracoccus* sp. strain T2.5-50 (both isolated from BH10, see Chapter 2) were grown as standing cultures in R2A media on polystyrene multi-well plates, both strains had the ability to form biofilms (Appendix III- Figure III.1) under the conditions tested. Likewise, identification of production of extracellular polymeric substances (EPS) surrounding *Tessaracoccus lapidicaptus* sp. strain IPBSL-7 cells and their role in precipitation of iron-rich phosphates and carbonates have also been reported before under anoxic growth conditions (Sánchez-Román *et al.* 2015). While we cannot be certain that *Tessaracoccus* strains have the same biofilm ability on rock surfaces in the subsurface as evaluated in this study, recent studies by colleagues on biofilm detection on rock cores recovered from BH10 borehole have indicated the presence of members of the genus *Tessaracoccus* within biofilms as identified using double-labeled oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) targeting members of the genus *Tessaracoccus* (Escudero *et al.* in preparation), which supports the hypothesis for biofilm formation under the environmental conditions within the IPB subsurface.

Regardless of how these microorganisms arrived at the deep subsurface, either being carried by surface waters and migrating over time or deposited during the geological setting of the IPB, bacteria such as *Tessaracoccus* isolates as well and other strains isolated in this work have mechanisms that allowed survival and retaining

of activity. Future studies should attempt to investigate expression of metabolic potential inferred from the genome. Although, undoubtedly a challenge, transcriptomic and proteomic profiling studies of subsurface isolates grown in vitro under conditions designed to emulate in situ conditions, would offer important insights into subsurface microbial activity.

# CHAPTER 5

## Study of bacterial and archaeal co-cultures enriched from 139 and 284 meters below surface

## 5.1. Introduction

In studies of isolation of bacteria and archaeal microorganisms described in Chapter 2, from enrichment cultures with active methane production, three colonies obtained from the roll tube isolation assays were subsequently transferred to liquid culture under the same growth conditions. These conditions consist of a basal medium under an atmosphere of hydrogen and carbon dioxide. Further studies of these cultures were driven by indication of their mixed nature, i.e. presence of both bacterial and archaeal populations.

Here we describe the conditions used for maintenance of these cultures, as well preliminary studies on investigating their microbial diversity using a combination of culture-independent techniques.

## 5.2. Materials and Methods

### 5.2.1. Culturing techniques

As described in Chapter 2, several enrichment cultures that developed methane production were chosen to attempt isolation of the cultivable microbial diversity using a roll tube assay. Three of these enrichment cultures were named T2.5, T2.10 and T2.11 (Table 2.2, Chapter 2). These cultures were enriched in a basal media under an atmosphere of $H_2$:$CO_2$, which comprised the same conditions subsequently used for growth on roll tubes, with the additional addition of Noble agar to create a solid matrix of media lining the tube walls. After colony development on the roll tubes, isolated colonies were transferred into fresh media plates under the same conditions, i.e. basal media and an atmosphere of $H_2$:$CO_2$ as substrate (see section 2.2.3, Chapter 2). Simultaneously to their transfer, microbial biomass was used in PCR screening for the presence of bacteria and archaea, as described below (see section 5.2.2). Three of these samples, due to indication of the presence of archaeal and bacterial cells, were chosen for transfer into a fresh liquid media. The liquid media had the following composition: (per liter of distilled water), 0.3 g $NH_4Cl$, 0.3 g $K_2HPO_4$, 0.3 g $KH_2PO_4$, 0.1 g $MgSO_4.7H_2O$, 2.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 10 ml trace element solution (DSMZ medium 141, https://www.dsmz.de/?id=441). Cysteine (0.5 g/L) was used as reducing agent and resazurin (0.1 mg/L) was used as redox indicator. A mixture of $H_2$:$CO_2$ (80:20, v/v) was used in the headspace. The medium preparation, as well as transfers were performed under strict anaerobic conditions as described in

section 2.2.3 (Chapter 2). These three established cultures were named respectively, T2.5-12, T2.10-10 and T2.11-6. Cultures were incubated in the dark at 30ºC. Maintenance of living cultures was performed by transfer to a fresh medium every six months.

### 5.2.2. Colony PCR screening for amplification of 16S rRNA gene

Colonies were picked with a toothpick and diluted in 25 µl sterile water. Partial amplification of bacteria 16S rRNA gene was performed using the primer set 27F-1492R (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, 5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991). Archaeal 16S rRNA was partially amplified with the primer set 1AF-1100R (1AF, 5'- TCYGKTTGATCCYGSCRGAG-3'; 1100R, 5'- TGGGTCTCGCTCGTTG-5') (Hales *et al.* 1996). PCR reactions (final volume 10 µl) were prepared with 0.5 U of AmpliTaq (Applied Biosystems), 1x AmpliTaq Buffer, 3 mM of $MgCl_2$, 200 µM of each deoxyribonucleotide (dNTP), 0.3 µM of each primer and 3 µl of template (diluted colony). The PCR conditions were the following: initial denaturation at 95ºC for 10 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing for 1 min at (56ºC for bacterial targeting primers and 55ºC for archaeal targeting primers) and elongation at 72ºC for 2 min. The final elongation was at 72ºC for 10 min. PCR products were checked for the correct length by electrophoresis on a 1% agarose gel stained with ethidium bromide. Purification and sequencing of PCR products was performed as described in section 2.2.5 (Chapter 2).

### 5.2.3. Analytical Measurement

Methane, hydrogen and carbon dioxide levels were measured by gas chromatography using a Bruker 450GC gas chromatographer equipped with a flame ionization detector (for methane detection) and a thermal conductivity detector (for hydrogen and carbon dioxide).

### 5.2.4. Catalyzed Reporter Deposition - Fluorescent in situ Hybridization (CARD-FISH)

Double CARD-FISH, using a sequential hybridization with an HRP-labeled oligonucleotide probe targeting *Bacteria* (EUB338 I-III) and an HRP-labeled oligonucleotide probe targeting *Archaea* (ARCH915), was performed for cultures T2.5-

12, T2.10-10 and T2.11-6 as described in section 2.2.7 (Chapter 2) and observed by confocal microscopy as described in section 3.2.2 (Chapter 3).

### 5.2.5. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis

Samples from cultures T2.5-12, T2.10-10 and T2.11-6 were fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for four hours at room temperature. Samples were washed three times with phosphate buffer (0.1 M, pH 7.4). Samples were dehydrated in a graded ethanol series of 50%, 80%, 100%, followed by critical point drying. Sample sections were mounted onto conductive graphite subs and coated with gold to enhance electrical conductivity and preventing sample charging under electron beams. Samples were observed with a JEOL 5600V SEM using an acceleration voltage of 20 kV and a working distance of 20 mm. Analyses were performed at room temperature. The qualitative element composition of samples was determined by Energy Dispersive X-ray (EDX) using an INCAx-sight with a Si-Li Detector (Oxford, England) with a detection limit of 10% of the main element.

### 5.2.6. Transmission Electron Microscopy (TEM)

Samples were prepared at the Centro de Biología Molecular Severo Ochoa Electron Microscopy Unit. Briefly, samples from cultures T2.5-12, T2.10-10 and T2.11-6 were fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for two hours at room temperature. Samples were washed with phosphate buffer (0.1 M, pH 7.4) and post fixed in 1% osmium tetroxide in bidistilled-water. Subsequently to washing steps, samples were incubated with 2% aqueous uranyl acetate followed by additional wash steps. Samples were dehydrated in a graded ethanol series of 30%, 50%, 70%, 95% and 100%. Dehydration was then completed by incubation with a mixture of ethanol/propylene oxide (1:1) for 5 min, followed by incubation with pure propylene oxide twice for 10 min. Samples were infiltrated with propylene oxide/Epon (1:1) for 45 minutes and 100% Epon (TAAB 812 resin; TAAB Laboratories) over night at room temperature. Polymerization of the infiltrated samples was done at 60ºC for two days. Ultrathin sections of each sample were collected on 200 mesh copper grids and stained with 2% uranyl acetate and lead citrate to enhance the contrast of cell structures. Samples were examined in a JEM1010 TEM (JEOL) transmission electron microscope operated at 80 kV.

### 5.2.7. DNA extraction and 16S rRNA gene clone libraries

For cloning assay, total DNA was extracted from cultures T2.5-12, T2.10-10 and T2.11-6 as described before (see section 3.2.3, Chapter 3). The bacterial 16S rRNA genes were amplified with the primer pair 27F-1492R (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, (5'- TACGGYTACCTTGTTACGACTT-3') (Lane 1991). While amplification of archaeal 16S rRNA gene was attempted by testing several primer pairs (e.g. 1AF-1100R (Hales *et al.* 1996), 21F-1492R (Lane 1991; DeLong 1992) or 344F-915R (Stahl and Amann 1991; Casamayor *et al.* 2002), as well as variations in annealing temperature and template concentration, no amplification was obtained.

The PCR reaction mixture for amplification of bacterial 16S rRNA gene (final volume of 50 µl) contained: 1.25 U of AmpliTaq (Applied Biosystems), 1x AmpliTaq Buffer, 3 mM of $MgCl_2$, 200 µM of each deoxyribonucleotide (dNTP), 0.3 µM of each primer and 25 ng template. PCR conditions for amplification of bacterial 16S rRNA gene were as follow: 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing for 1 min at 56ºC and elongation at 72ºC for 2 min. The final elongation was at 72ºC for 7 min. PCR products were checked for the correct length by electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were purified with DNA purification JetQuick kit (Genomed). Subsequently, the purified PCR products were cloned and transformed into *Escherichia coli* DH5α competent cells using pGEM-T Easy Vector System (Promega). Blue/white screening for positive inserts was performed according to the manufacturer's instructions. Randomly selected colonies (resuspended in 25 µl sterile water) were directly screened for inserts by performing colony PCR using the pGEM-T specific primers T7-SP6 (T7, 5'- TAATACGACTCACTATAGGG-3'; SP6, 5'-TATTTAGGTGACACTATAG-3'). The PCR reaction was as described above (section 5.2.2) using 3 µl of the colony suspension. Conditions for amplification were the following: 95ºC for 10 min, 30 cycles at 94ºC for 60 s, 55ºC for 60 s, 72ºC for 2.5 min, and finally 72ºC for 10 min. Inserts with the expected length were sent for sequencing at the Center of Astrobiology (Madrid, Spain), for identification of clones. Sequences were manually curated using the SerialCloner v2.6.1 software (SerialBasics). Sequences were searched for chimeric sequences with the web version of Bellerophon (Huber *et al.* 2004). Sequences were searched by BLAST against the GenBank database and EzBioCloud (Yoon *et al.* 2016) to identify their closest relatives. Representative sequences of each clone group and their respectively closely related gene sequences retrieved from NCBI GenBank were aligned with ClustalW (Thompson et al. 1994). A phylogenetic tree based on 16S rRNA

gene sequence was reconstructed on MEGA 6 software (Tamura *et al.* 2013), using the neighbor-joining (Saitou and Nei 1987) method based on the Kimura 2-parameter model (Kimura 1980), with a bootstrap analysis of 1000 replicates to evaluate inferred tree topology robustness. Nucleotide sequences of amplified 16S rRNA gene have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers MG725257- MG725261.

### 5.2.8. Extraction of DNA by freezing-boiling lysis of cells and amplification of archaeal 16S rRNA gene

DNA extraction using an alternative DNA extraction method, to the one referred in section 5.2.7 used for DNA extraction of the cloning assay, was applied. A culture sample was recovered from T2.11-6 culture and centrifuged for 10 min at 10000 rpm for pellet cells. Cells were resuspended in 500 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8) and incubated for 15 min at -80ºC following incubation at 100ºC for 5 min. To recover the supernatant, samples were centrifuged at 10000 rpm for 2 minutes. DNA was precipitated with 0.6 volumes of isopropanol at room temperature for 15 min and washed with 70% ethanol. DNA pellet was resuspended in 25 µl of sterile water.

Archaeal 16S rRNA gene was partially amplified by nested PCR with the primer set 1AF-1100R (1AF, 5'-TCYGKTTGATCCYGSCRGAG-3'; 1100R, 5'-TGGGTCTCGCTCGTTG-3') (Hales *et al.* 1996) followed by amplification with the primer set 109F-915R (109F, 5'-AMDGCTCAGTAACACGT-3'; 915R, 5'-GTGCTCCCCCGCCAATTCCT-3') (Stahl and Amann 1991; Imachi *et al.* 2006). PCR amplicons were generated with AmpliTaq DNA polymerase (Applied Biosystems) according to manufacturer instructions. The PCR conditions were the following for both PCR's: initial denaturation at 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 1 min, primer annealing for 1 min at 55ºC and elongation at 72ºC for 1.5 min. The final elongation was at 72ºC for 7 min. Purified PCR products were sent for sequencing within the Center of Astrobiology (Madrid, Spain). Taxonomic identification of amplified sequences was assessed using Ez-BioCloud (Yoon *et al.* 2016). Nucleotide sequence of amplified 16S rRNA gene sequence have been deposited in the DDBJ/EMBL/GenBank databases under the accession number MG720002.

## 5.3. Results

Screening of colonies developed on roll tubes by PCR with primers targeting bacterial and archaeal domains, respectively, suggested they were comprised of mixed populations (Appendix IV- Figure IV.1). PCR amplification signal for bacteria was clear on agarose gel, although, the signal for archaea was extremely faint. Three of these colonies were inoculated in liquid cultures composed of a basal media under an atmosphere of hydrogen and carbon dioxide.

Six months following preparation of liquid cultures, measurement of headspace gases indicated that neither hydrogen or carbon dioxide were used. Methane production was also negative (Appendix IV- Figure IV.2).

Double CARD-FISH hybridization with probes targeting, respectively, *Bacteria* and *Archaea*, indicated the presence of a positive signal for both domains in all three cultures (Figure 5.1), indicating that each originally transferred colonies represented mixed colonies. Figure 5.1 represents the dominant features observed on each culture. In both cultures, T2.10-10 and T2.11-6, rod-shaped cells, arranged mainly in aggregates, stained positive independently with bacterial or archaeal domain probes (Figure 5.1 panels a1-5, b1-5). In culture T2.5-12 rod-shaped cells predominated. CARD-FISH hybridization of this culture resulted in the observation of a simultaneous positive archaeal and bacterial signals within the same rod-shaped cells (Figure 5.1 panels c1-5). The absence of nonspecific signals was confirmed in control hybridizations with the nonsense control probe, NON338.

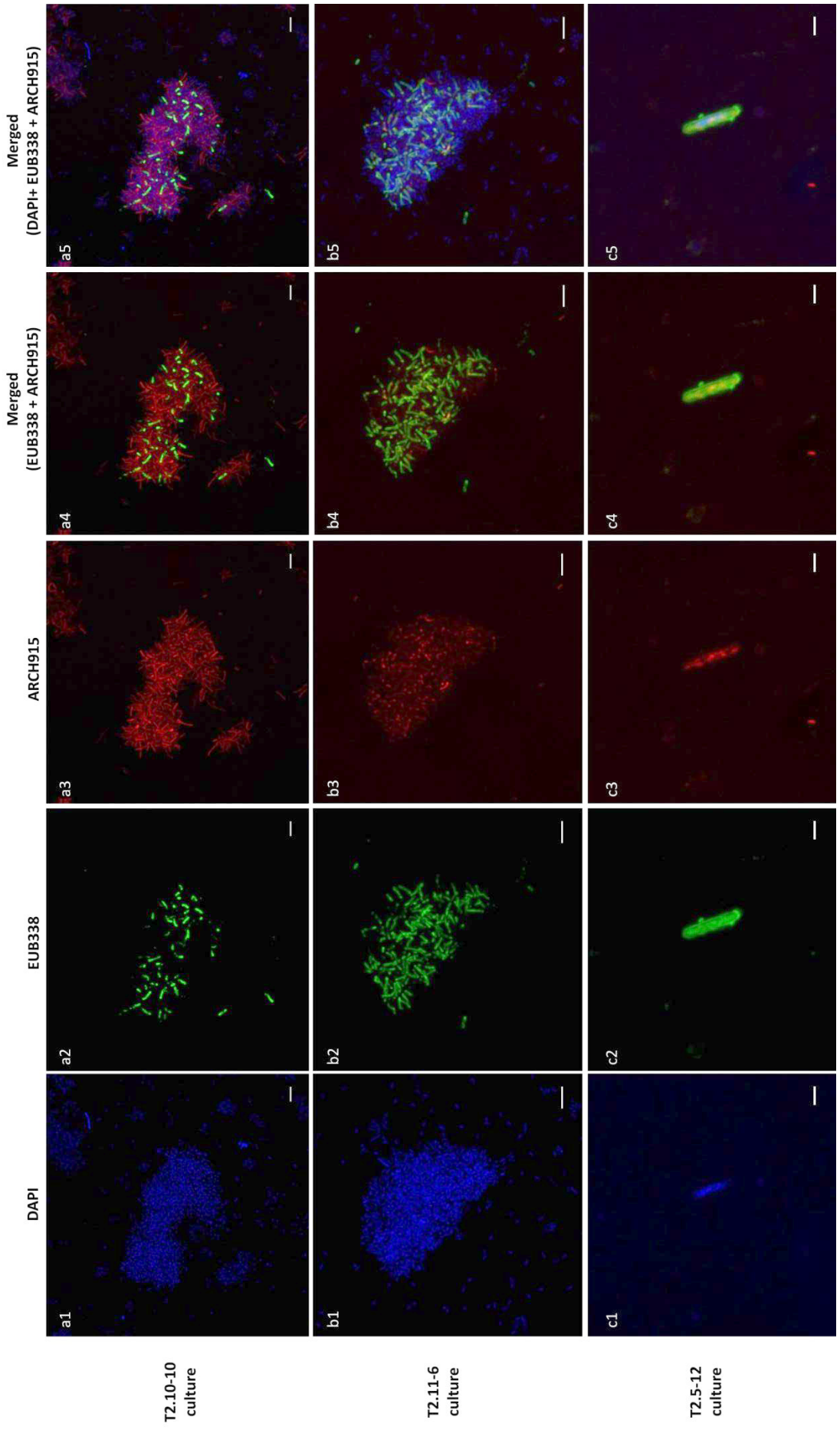
DAPI
EUB338
ARCH915
Merged (EUB338 + ARCH915)
Merged (DAPI+ EUB338 + ARCH915)
T2.10-10 culture
T2.11-6 culture
T2.5-12 culture
a1
a2
a3
a4
a5
b1
b2
b3
b4
b5
c1
c2
c3
c4
c5

**Figure 5.1.** Confocal laser scanner photomicrographs of CARD-FISH hybridization using EUB338 I-III probe mix targeting bacteria (green), ARCH915 probe targeting archaea (red) and DAPI staining. Culture T2.10-10 (panel **a1-5**), T2.11-6 (panel **b1-5**) and T2.5-12 (panels **c1-5**). Scale bar= 5 µm (a1-5, b1-5), 2 µm (c1-5).

Culture T2.5-12 was further investigated by SEM microscopy with EDX spectrometry. Figure 5.2a,d show diverse cell morphologies that vary from long rod-shaped to short rod-shaped cells, as well as sphere-like morphologies. EDX spectrum (Figure 5.2e) indicated an elemental composition of mainly carbon for the matrix observed by SEM where the cells adhere.

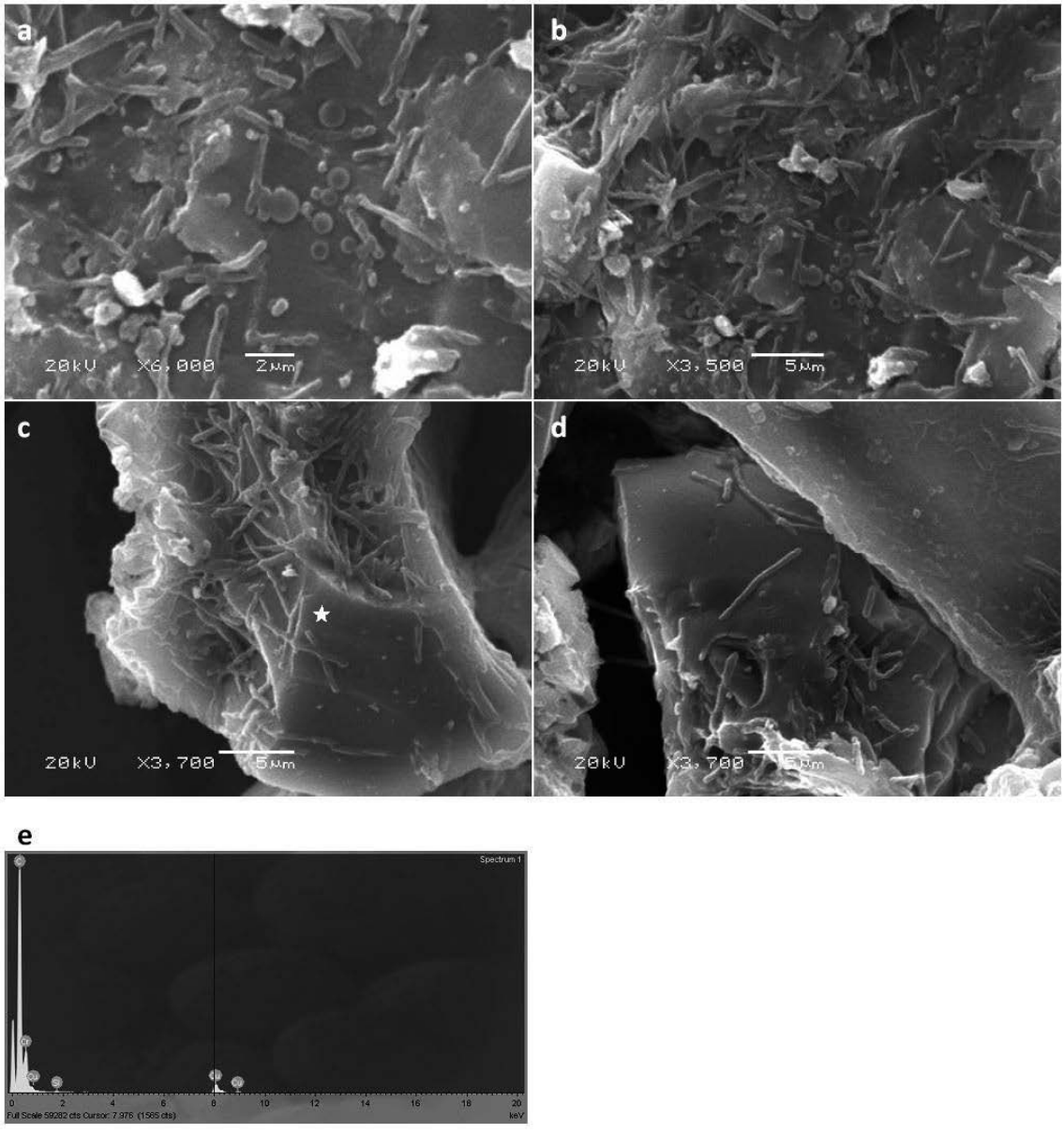


**Figure 5.2.** SEM micrograph of cells from culture T2.5-12 **(a, b, c, d)**. EDX spectrum for the area identified by the star (on panel c) **(e)**. Scale bar= 2 µm (a), 5 µm (b-d).

In order to establish a preliminary insight into the cellular ultrastructure of microorganisms enriched in culture T2.5-12, a culture sample was observed by TEM microscopy. Figure 5.3a-f show diverse ultrastructure planes of cells enriched in this culture. Despite the mixed nature of these cultures, Figure 5.3a and Figure 5.3c, which may either represent a longitudinal or cross-sectional cellular cut, showed the presence of intracytoplasmic membranes. Additionally, Figure 5.3d also suggested the presence of spore forming cells. Figures 5.3e,f depict diverse cellular cuts including longitudinal cuts of rod-shaped cells.

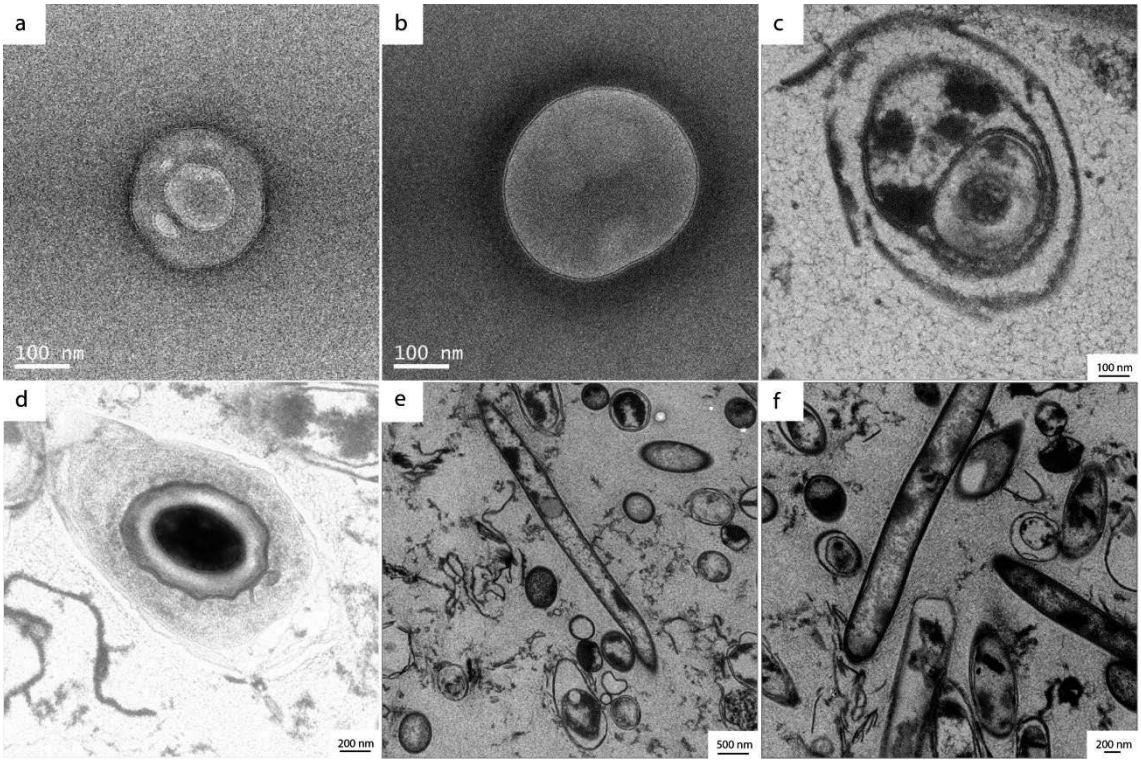


**Figure 5.3.** TEM photomicrographs of cells enriched in culture T2.5-12 **(a-f)**. Scale bar = 100 nm (a, b, c), 200 nm (d, f), 500 nm (e).

Cloning targeting the amplification of 16S rRNA gene sequence identified the presence of bacteria affiliated with both the genera *Paenibacillus* and *Rhodoplanes* in culture T2.10-10 (supplemented with $H_2/CO_2$), bacteria affiliated with *Rhodoplanes* in culture T2.11-6 (supplemented with $H_2/CO_2$), and in culture T2.5-12 (supplemented with $H_2/CO_2$) identified the presence of bacteria affiliated with the genera *Paenibacillus* and *Sphingomonas*. Figure 5.4 represents the phylogenetic position of a representative clone of each clone group identified from each culture.

Furthermore, amplification of 16S rRNA gene using archaea-targeting primers with DNA extracted using an alternative method from culture T2.11-6, resulted in a sequence with 96.98% similarity with the type strain of *Methanobacterium aggregans.*

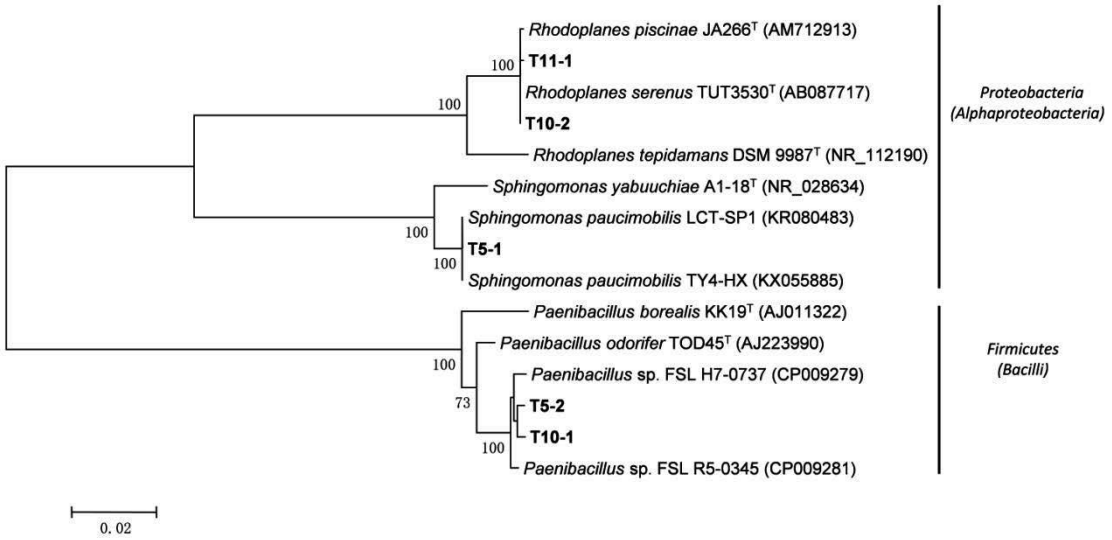


**Figure 5.4.** Phylogenetic tree based on representative 16S rRNA gene sequence of each representative clone sequence identified in culture T2.10-10 (sequences T10-1, T10-2), culture T2.11-6 (sequence T11-1) and culture T2.5-12 (sequence T5-1, T5-2) and closely related taxa constructed using the neighbor-joining algorithm. The numbers on the nodes indicate bootstrap values (≥ 50%). Bar, 0.02 substitutions per nucleotide position.

## 5.4. Discussion

Hydrogen and carbon dioxide represent two important substrates detected in the deep subsurface of the IPB (Amils *et al.* 2014). These substrates can support chemolithoautotrophic microbial metabolisms, representing respectively, important energy and carbon sources in the subsurface.

Cultures T2.5-12, T2.10-10 and T2.11-6 were established with a basal medium under an atmosphere of hydrogen and carbon dioxide. These cultures were prepared with cells previously enriched from cultures T2.5, T2.10 and T2.11, respectively, which were prepared as described in Chapter 2. As such, ultimately, microbial populations grown on culture T2.5-12 originated from a rock core recovered from 139.5 mbs (BH10), while microbial populations grown on cultures T2.10-10 and T2.11-6 originated from a rock cores recovered from 284 mbs (BH10).

Although, the utilization of hydrogen or carbon dioxide in T2.5-12, T2.10-10 and T2.11-6 cultures was not evident over the evaluated incubation time (Appendix IV-Figure IV.2), we cannot discard that utilization of these compounds as substrates may still proceed if microorganisms are operating with extremely slow metabolic rates under the culture conditions applied. After transfer of cultures (10% inoculum) into fresh medium, growth become evident by visual observation of increased turbidity after 2-3 months for T2.10-10 and T2.11-6 cultures and after 4-5 months for T2.5-12 culture.

CARD-FISH studies using domain specific probes has indicated the presence in these three cultures of both bacterial and archaeal cells (Figures 5.1).

In both cultures T2.10-10 and T2.11-6 cellular aggregates dominate, comprising both bacteria and archaeal cells (Figure 5.1 panel a1-5 and panel b1-5). Formation of floating cellular aggregates (floating biofilms) has been described as the result of secretion of a exopolysaccharide (EPS) matrix which encases the cells, similarly to what occurs in biofilms bound to an solid interface (Flemming and Wingender 2010). The close proximity of populations within a biofilm offer unique opportunities for cell cooperation which may translate in synergetic degradation of substrates, exchange of metabolites and nutrients, signaling or exchange of genetic material (Flemming *et al.* 2016). Current studies by Escudero *et al.* (Escudero *et al.* 2018) are beginning to shed some light on the occurrence of biofilms on rock cores and interactions between microbial populations in the IPB subsurface.

A significant fraction of DAPI stained cells (Figure 5.1 panel a1 and panel b1) was not hybridized by either of the probes applied. This may be related with incomplete permeabilization for that population of cells by the protocol applied. Alternatively, the absence of hybridization may have been due to probe coverage issues. Commonly

used probes as the ones applied don´t present full coverage for the respective taxa they are targeting. While probe EUB338-I covers most *Bacteria* (90% coverage), it fails particularly in the detection of members of *Planctomyces* and *Verrucomicrobia,* which can be targeted with the probes EUB338-II (0.8% coverage *Bacteria,* 47% coverage *Planctomyces*) and EUB338-III (1.3% coverage *Bacteria*, 75% *Verrucomicrobia*) (Daims *et al.* 1999). Likewise, probe ARCH915 targeting *Archaea* presents only 85% coverage to members of this domain. Coverage details were inferred with online TestProbe tool at https://www.arb-silva.de/search/testprobe/.

Cloning targeting the amplification of 16S rRNA gene sequence identified the presence of bacteria affiliated with both genera *Paenibacillus* and *Rhodoplanes* in culture T2.10-10, while in culture T2.11-6 detected the presence of bacteria affiliated with the genus *Rhodoplanes* (Figure 5.4). Formation of aggregates has been described before for members of the *Rhodoplanes*, being mainly reported in older cultures (Chakravarthy *et al.* 2012). It should be also taken in consideration that formation of cellular aggregates between diverse populations can also possibly offer an opportunity for cooperation between populations.

Members of the genus *Paenibacillus* are described as facultative anaerobic, spore-forming, rod-shaped bacteria. Members of this genus are characterized by having a respiratory and fermenting metabolism. Particularly, they are known for their ability to hydrolyze many carbohydrates (Priest 2015; Grady *et al.* 2016). Fermentative metabolism with hydrogen production has been reported (Lal *et al.* 2012; Priest 2015). Nitrate respiration, reduction of ferric iron and ability for nitrogen fixation by some species have also been reported (Behrendt *et al.* 2010; Ahmed *et al.* 2012). Autotrophic capability of *Paenibacillus* strains has been reported in a study related to cultivation of anaerobic and facultative anaerobic strains from spacecraft associated clean rooms, which described the isolation of strains affiliated with *Paenibacillus* under an atmosphere of hydrogen and carbon dioxide (Stieglmeier *et al.* 2009).

In turn, members of the genus *Rhodoplanes* are described as purple non-sulfur bacteria, being characterized by their diverse metabolic capabilities, which includes both phototrophic and chemotrophic metabolisms (Hiraishi and Imhoff 2015). In anaerobic conditions growth by photoheterotrophy has been reported as the preferred mode for growth, i.e. with light and organic compounds (Hiraishi and Ueda 1994; Lakshmi *et al.* 2009; Okamura *et al.* 2009; Chakravarthy *et al.* 2012; Srinivas *et al.* 2014; Hiraishi 2017; Hiraishi and Okamura 2017). Photolithotrophic growth has also been reported with thiosulfate (Hiraishi and Ueda 1994; Okamura *et al.* 2009; Chakravarthy *et al.* 2012; Srinivas *et al.* 2014; Hiraishi 2017). For the type strain of *Rhodoplanes tepidamans,* photolithotrophic growth occurs with hydrogen but not with

sulfite, sulfide or thiosulfate (Hiraishi and Okamura 2017). In anaerobic conditions and in the dark, growth by heterotrophic denitrification has also been reported (Hiraishi and Ueda 1994; Lakshmi *et al.* 2009; Okamura *et al.* 2009; Hiraishi and Okamura 2017). For type strains for which fermentative metabolism has been described as negative under the conditions applied (Lakshmi *et al.* 2009; Chakravarthy *et al.* 2012; Hiraishi 2017). Future studies should attempt to elucidate the mechanisms in action supporting growth under the culture conditions applied in this work, oligotrophic media (only 0.01% yeast extract as source of growth factors) under an atmosphere of hydrogen and carbon dioxide, incubated under dark conditions.

As described in Chapter 2, we were able to isolate strain T2.5-46A (see Table 2.3), identified based on 16S rRNA gene similarity as affiliated with the genus *Paenibacillus*, from T2.5 enrichment culture (see Table 2.2). As mentioned above, cellular material originated from culture T2.5 was used in the establishment of subculture T2.5-12 discussed in this chapter from which a clone affiliated with the genus *Paenibacillus* was also identified. Other sequences affiliated with the genus *Paenibacillus* have also been detected by 454 pyrosequencing from acetogenic enrichment cultures (BH10, 311.1 mbs; BH11, 145.4 mbs), from sulfur reducing enrichment cultures (BH11, 93 mbs) or from methanogenic enrichment cultures (BH11, 320.3 mbs) (Amils *et al*. in preparation).

Moreover, as discussed in Chapter 2, isolates/sequences affiliated with the genus *Rhodoplanes* have also been recovered/detected from several depths on the IPB subsurface in this work and by colleagues.

Amplification of archaeal 16S rRNA gene was not possible during bacterial cloning studies. Using an alternative DNA extraction method with culture T2.11-6 and application by a nested PCR protocol it was identified the presence of an archaeal strain affiliated with the genus *Methanobacterium*. Therefore, initial difficulty in amplification of archaeal 16S rRNA gene could have been possibly correlated with low concentration of DNA template resulting from an inefficient DNA extraction from archaeal cells. Future studies should attempt this DNA extraction protocol or alternative DNA extraction protocols with increased starting biomass for extraction of archaeal DNA in order to identify the archaeal populations enriched in T2.5-12 and T2.10-10 cultures, as well.

In culture T2.11-6 the archaeal 16S rRNA gene sequence amplified presented 96.98% similarity with the cultured type strain of *Methanobacterium aggregans,* a hydrogenotrophic methanogen isolated from a biogas plant (Kern *et al.* 2015). The genus *Methanobacterium* comprises 34 validly published species and belongs to the family *Methanobacteriaceae* of the order *Methanobacteriales* within the class

*Methanobacteria* of the phylum *Euryarchaeota*. This sequence belongs to a potentially novel species of the genus *Methanobacterium*. Members of the genus *Methanobacterium* are described as rod-shaped archaea with ability to grow and produce methane from carbon dioxide reduction coupled with hydrogen oxidation, with some strains also being able to use formate, secondary alcohols or carbon monoxide (Boone 2015). Members of *Methanobacterium* have been reported previously in groundwater from continental subsurface environments (Kotelnikova *et al.* 1998; Moser *et al.* 2005; Nyyssonen *et al.* 2014; Purkamo *et al.* 2016). While methane production was not detected in culture T2.11-6 headspace (Appendix IV- Figure IV.2), as discussed, this may be correlated with slow growth rates and the time period of measurement which may have been too short for accumulation of detectable methane levels.

Interestingly, in culture T2.5-12 dominated rod shaped cells within which occurred localization of both bacterial and archaeal probe signal as observed by double CARD-FISH analysis (Figure 5.1 panels c1-5). Archaeal probe signal appeared to be located both inside the rod shaped structure hybridized with bacterial specific probe, as well as outside. Hybridization with control probe NON338 was negative, indicating that non-specific binding does not occur. Besides, testing hybridization of each probe using the opposite fluorophores attached to the respective probe (tyramide-Alexa Fluor 594 for CARD-FISH with bacterial targeting probe and tyramide-Alexa Fluor 488 for CARD-FISH with archaeal targeting probe) indicated the same hybridization pattern (not shown).

The CARD-FISH assay was latter repeated twice with culture samples recovered from subsequent transfer of cultures T2.10-10, T2.11-6 and T2.5-12 into fresh media and the same pattern of hybridization was verified, i.e. predominantly aggregates of bacterial and archaeal cells in cultures T2-10-10 and T2.11-6 and predominantly rod-shaped cells which hybridized with bacterial and archaeal probes in culture T2.5-12.

Cloning by targeting the amplification of 16S rRNA gene, after DNA extraction from T2.5-12 culture identified the presence of bacterial clones affiliated with the genera *Paenibacillus* and *Sphingomonas*. Clones affiliated with the genus *Paenibacillus* were also detected as described above in culture T2.10-10 (Figure 5.4).

Members of the genus *Sphingomonas* have been described as rod-shaped, non-spore-forming chemoheterotrophic bacteria (Balkwill *et al.* 2006). They are characterized by their biodegradative capabilities, particularly recalcitrant aromatic compounds and polysaccharides (Aylward *et al.* 2013). While traditionally described with an aerobic respiratory metabolism, ability to use alternative electron acceptors has

not been extensively tested. Recently, anaerobic growth have been reported for a novel strain described as affiliated with the genus *Sphingomonas* (Ding et al. 2015). The sequence affiliated with members of the genus *Sphingomonas* have also been detected before from deep subsurface environments within fracture waters (Onstott *et al.* 2009; Purkamo *et al.* 2016). Moreover, sequences affiliated with the genus *Sphingomonas* have also been obtained by 16S rRNA gene massive sequencing studies from DNA extracted from rock samples recovered from BH10 borehole (Puente-Sánchez 2016).

Analysis of culture T2.5-12 (after six months incubation) by SEM showed the presence of long and shorter rod-shaped cells as well as spherical-shaped cells of various dimensions (Figure 5.2a-d). Besides, cellular structures, a matrix which EDX spectrometry indicated with an elemental composition of carbon (Figure 5.2e) was also observed. Origin and biological significance of this matrix requires further studies.

Interestingly, TEM analysis of cultures after six months incubation, indicated the presence of several intracytoplasmic membrane delimited structures (Figure 5.3a). In Figure 5.3c the occurrence of intracytoplasmic membrane structure is observed as well as the detachment of an unknown external membrane. Since this is a mixed culture with diverse cellular morphologies, these images may either represent a longitudinal or cross-sectional cellular cuts. While occurrence of intracellular compartmentalization has been described for members of the bacterial phyla *Planctomyces*, CARD-FISH studies with EUB338-II probe targeting members of *Planctomyces* was negative. Nevertheless, in situ hybridization studies with EUB338-II probe on rock samples has identified positive signals at several depths (Escudero *et al.* in preparation), demonstrating the occurrence of member of this phyla in the IPB subsurface. The nature of these observed structures and their role is so far unknown. Notice should also be taken of the small diameter dimensions presented by these cells (0.25-0.3 µm) (Figures 5.3a-b). These may correspond to either ultra-small bacteria, ultra-small archaea or ultramicrocells such as starved cells due to growth under the oligotrophic growth conditions applied.

Moreover, the spore ultrastructure observed by TEM (Figure 5.3d) may correspond to a *Paenibacillus* (as identified by cloning) which are known spore-forming bacteria. Figures 5.3e-f display longitudinal cut of rod-shape cells as well as diverse other cut planes. Nevertheless, observation of intracytoplasmic membranes within rod shaped cells was not apparent. Further studies are necessary to thoroughly identify the phylogeny of the enriched populations, as well as their metabolic and physiological potential in order to clarify preliminary observations made so far. To best of our knowledge, cultivation of strains affiliated with either the genera *Paenibacillus,*

*Rhodoplanes* or *Sphingomonas* in an oligotrophic media under the presence of $H_2$ and $CO_2$ in the dark has not been extensively studied so far.

In this study the detection of members of the genus *Methanobacterium* for the first time in the IPB subsurface at Peña de Hierro, contributed to expand our knowledge on the diversity of methanogenic populations present on rocks within the IPB subsurface. Furthermore, the collected microscopic data, particularly in the case study of culture T2.5-12 also suggested that additional microbial diversity may occur, which was not detected by the cloning assay.

In the recent years, with the development of sequencing technologies (e.g. metagenomic studies and single cell sequencing) and data analysis tools, it is becoming clearer the massive extent of microbial diversity, indicating that it is far more intriguing and unexpected. Studies on diverse environments have reported the occurrence of several candidate phyla, many of which representing deep branching lineages (Colman *et al.* 2016; Hug *et al.* 2016; Kolinko *et al.* 2016; Becraft *et al.* 2017). Such discoveries are conducting to a progressive continuum reassessment of taxonomy and the tree of life. Additionally, inference of metabolic potential from reconstructed genomes is revealing extraordinary discoveries such as detection of genes which were thought to define eukaryotic specific features in members of the novel archaeal phyla *Lokiarchaeota* (Spang *et al.* 2015); detection of a deep branching lineage (Candidate phyla radiation, CPR) whose genomes also appear to encode unusual protein synthesis machinery (Brown *et al.* 2015) or the detection of fermentative or methanogenic pathways in genomes from members of the novel archaeal phylum *Bathyarchaeota* (Lloyd *et al.* 2013; Evans *et al.* 2015), just to mention a few. Therefore, much is still to be discovered and understood.

While members of known phyla such as *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* have been frequently detected and in some cases isolated from both the subseafloor and continental terrestrial subsurface, sequencing based techniques also indicate a far richer diversity in such environments. Lineages that lack cultured representatives, many representing candidate phyla have also been reported from these ecosystems (Takai *et al.* 2001a; Chivian *et al.* 2008; Jungbluth *et al.* 2016; Wu *et al.* 2016a; Jungbluth *et al.* 2017). Considering the geographic extension of subsurface environments, we are just in the process of beginning to understand the diversity, physiology and role of life in these ecosystems.

The preliminary data obtained in this study just offered a glimpse into intriguing features of the microbial populations enriched. Much is still required to clarify the data collected so far. Future studies encompassing both metagenomic analysis and single cell genomics will certainly provide a clearer understanding of phylogenetic diversity,

and metabolic potential of enriched microbial populations. Since growth under the tested conditions has not been investigated so far for the identified bacteria, it would be interesting to combine both transcriptomic and proteomic studies to evaluate the metabolic pathways active under these conditions.

# CHAPTER 6

## Concluding Remarks and Future Perspectives

The Iberian Pyritic Belt is characterized by being one of the largest sulfide ore deposits known in the world, yet little is known about the geomicrobiology of this system.

The work developed in this thesis relied on the combination of both culture-dependent techniques as well as culture-independent techniques to study rock core samples recovered from the subsurface of Río Tinto aquifer, located at the heart of the IPB. The study focused on investigation of the presence and activity of methanogenic microorganisms and nitrate reducers, isolation and study of the biodiversity enriched under such conditions. Results obtained revealed the presence of phylogenetically diverse bacterial and archaea microorganisms inhabiting rocks on the deep subsurface.

The establishment of enrichment cultures with positive activity for both methanogenic and nitrate reduction (Chapter 2) confirmed that viable and active microorganisms with such metabolic potential inhabit these rock cores.

Notwithstanding the important discoveries reported so far by deep subsurface studies much is still to be learned, particularly on the understanding of physiological and metabolic processes that support life in these environments. In comparison to other environments, fewer isolates from deep subsurface environments have been reported. Currently, with reasonable prices and improved methods for sequence analysis, culture-independent techniques based on nucleic acid sequencing are undoubtedly not only attractive but also adopted as the preferred method to explore diversity, since they also provide a further complete picture of the diversity present than the one accessed by culturing techniques alone. Nevertheless, isolation studies offer an invaluable resource. Recovery of microorganisms in pure culture and their continued culturing provide means to design experiments, test and uncover their metabolic and physiological capabilities.

Furthermore, the majority of continental subsurface studies have focused on groundwater sampling. As such, in the current study the recovery in pure culture of several bacterial strains isolated from rock cores provided not only biological information into viable indigenous bacterial populations inhabiting rocks on the IPB subsurface, but also provided an opportunity for future studies aimed at elucidating their physiological and metabolic potential.

While no archaea was isolated in pure culture, we successfully isolated several bacteria affiliated with the phyla *Proteobacteria, Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Chapter 2). Several of these strains are related to strains previously described with potential for fermentation, sulfate reduction, nitrate reduction, iron reduction or acetogenesis. The isolation of these strains from rock samples suggests a

potential role for these microorganisms in the cycling of elements such as carbon, sulfur, nitrogen and iron through the subsurface ecosystem. Furthermore, enrichment cultures of rock cores positive for active sulfate reduction, acetogenesis and iron reduction activities have also been reported from several depths at both BH10 and BH11 boreholes (Amils *et al.* 2013). This demonstrates that microorganisms with potential for these metabolisms appear to be widely distributed throughout the subsurface.

As far as isolation is concerned, this is a challenging process. Bacterial strains isolated resulted from their amiable growth under the set of conditions applied in this study. As discussed before, resistance for growth may be explained by inability to reproduce physiological and nutritional requirements and/or the required timescales for growth in an artificial setting. Besides, it should also be considered that for some instances, due to interdependence on interactions between populations it may actually be restrictive isolation of certain populations in pure culture.

Previous measurements in the MARTE project as well as from BH10 and BH11 boreholes indicated the presence of substrates that could be used to support growth by anaerobic metabolisms, such as hydrogen, methane, carbon dioxide gases; organic acids such as acetate, propionate; inorganic anions such as nitrate, nitrite, sulfate; as well as ferrous and ferric iron (Amils *et al.* 2014). Moreover, organic compounds such as acetate and methane may have a biological origin as byproducts from acetogenic and methanogenic activities, respectively. The presence of refractory organic matter and dead biomass may also represent a source of organic matter which could also play an important role in supporting oligotrophic heterotrophic populations.

Life in such oligotrophic anaerobic environments occurs at a slow pace and microorganisms inhabiting these ecosystems are expected to present strategies for optimization of energy conservation for metabolic efficiency and maintenance of genetic and cellular integrity. The strains obtained in this study were either enriched in methanogenic or nitrate reducing conditions. Further studies are required to test their physiological and metabolic potential as well as establishing their taxonomy at the species level by means of a polyphasic taxonomic characterization. Currently, strains T2.26MG-10 and T2.30D-1.1 isolated in this study and identified as affiliated with the genus *Rhizobium* are under further characterization by colleagues. Particularly interesting would be the study of strains isolated in nitrate reduction conditions in order to effectively test their potential for denitrification or DNRA metabolism.

One should also consider that while the study of microbial activities at in situ conditions is desired in order to infer strategies employed as well as evaluate the impact of microbial activities on element cycling through the subsurface environment,

the low rates of biomass turnover which are described within such ecosystems, will undoubtedly pose a challenge.

The experimental assay focused on isolation of methanogenic archaea, resulted in the establishment of a culture enriched in the archaeal strain named as T1.2MG-A. Identification by sequencing of both 16S rRNA gene and *mcrA* gene indicated that this strain is affiliated with the genus *Methanosarcina* (Chapter 2). This strain was originally enriched from a rock core sample originated from BH11 borehole and amended with acetate. As discussed above, the presence of acetate has also been detected in rock leachates from the IPB (Amils *et al.* 2013) and thus apart from hydrogen, which is also present in the subsurface, it may also support methanogenic archaea growing in the subsurface. Future work should ensue, aiming at isolation of T1.2MG-A strain in pure culture.

In order to further investigate the bacterial and archaeal diversity enriched under methanogenic conditions, three cultures were selected for which 16S rRNA gene pyrosequencing analysis indicated the enrichment in several bacteria affiliated with known fermentative, acetogenic and sulfate reducing bacteria as well as the enrichment of archaea related to the orders *Methanosarcinales* and *Methanocellales* (Chapter 3). To our knowledge, this is so far the first report of detection of members of the *Methanocellales* in deep subsurface environments.

The development of methane in enrichment cultures as well as the phylogenetic identification of methanogenic populations enriched, demonstrated the presence of live and active methanogens inhabiting the rock cores. In turn, within the IPBSL project, CARD-FISH studies on rock samples have also reported the presence of colonies affiliated with methanogenic archaea using order and family level targeting probes (*Methanosarcinales*, *Methanomicrobiales,* *Methanomicrobiales* and *Methanococcaceae*) (Escudero *et al.* in preparation). Nevertheless, metagenomic studies with DNA extracted directly from rock samples recovered from BH10 failed to detect members of the *Archaea*, which could correlate with the presence of low archaeal biomass on the rocks or difficulty in extracting DNA from rock samples (Amils *et al.* in preparation).

The presence of rock dwelling methanogenic microorganisms and the detection of hydrogen or acetate, which could support their growth, suggests that methanogenic metabolisms may be active in the IPB, possibly even contributing to a biological origin for the methane detected in boreholes drilled in the framework of MARTE and IPBSL projects. In turn, this methane itself may be used as substrate to sustain methanotrophic microbial populations in the subsurface. In fact, enrichment cultures

established with rock core samples from BH10 and BH11 with active methanotrophic activity have been reported as well (Amils *et al.* 2014).

The rock subsurface environment is a heterogeneous environment as a result of its geochemical and mineralogical variability. The porous and fractured nature of the rock matrix offers the possibility for life to thrive on microniches which may be connected or independent from each other, even at a short distance apart. This allows diverse microbial metabolisms to occur within the rock matrix. Each microniche, even if just a few millimeters apart can present different proprieties. As such, the metabolisms active within Río Tinto subsurface aquifer will ultimately depend on the physicochemical characteristics established within each microniche on the rock matrix.

Integrative analysis of results from several techniques within the IPBSL project such as 16S rRNA gene analysis of bacterial isolates (Chapter 2), 16S rRNA gene massive sequencing from rock cores (Amils *et al.*, in preparation) and in situ hybridization using a genus-specific probe (Escudero *et al.* in preparation), showed the occurrence of members of the genus *Tessaracoccus* distributed along both BH10 and BH11 boreholes. In Chapter 4 it is discussed the sequencing and annotation of the complete genome of *Tessaracoccu*s sp. strain T2.5-30 isolated from BH10. Genome analysis indicated potential for heterotrophic metabolism, fermentation, as well as nitrate reduction associated with ammonia formation in anaerobic conditions and fixation of inorganic carbon. Further experimental work is required to test the predicted genomic potential.

Moved by our interest in microbial populations enriched under an atmosphere of hydrogen and carbon dioxide, three subcultures amended with these substrates were established. CARD-FISH analysis using domain specific probes demonstrated the presence of both bacterial and archaeal cells (Chapter 5). Preliminary studies indicated the presence of bacterial clones affiliated with either *Paenibacillus* and *Rhodoplanes* in one culture, affiliated with *Rhodoplanes* from a second culture and clones affiliated with *Paenibacillus* and *Sphingomonas* in a third culture. Additionally, testing of alternative DNA extraction methods also allowed the amplification in one culture of an archaeal 16S rRNA gene sequence identified as affiliated with *Methanobacterium*, adding further knowledge into the diversity of archaea inhabiting rock cores. Nevertheless, as addressed in Chapter 5 future studies are undoubtedly required to further investigate diversity, metabolism and physiology of the microbial populations enriched.

This work is an important contribution for the geomicrobiological characterization of the IPB subsurface at Peña de Hierro, not only providing further insight into the indigenous bacterial diversity inhabiting rock samples, but also allowed identification at the genus level of several archaeal genera present in this ecosystem.

The work described in this dissertation fits within the global picture that is emerging from the various studies investigating the geomicrobiology of BH10 and BH11 boreholes, which report a high level of metabolic diversity at Peña de Hierro aquifer. The emerging model predicts that as water enters the VSC, which host the massive sulfides of the IPB, biotic and abiotic processes are activated. Compounds with origin on the rock matrix such as metal sulfides, ferrous iron and hydrogen originated by water/rock interactions may act as electron donors for microbial growth. Also, several electron acceptors such as ferric iron, nitrate, sulfate and carbon dioxide are also available and may support diverse metabolisms (Amils *et al.* 2014).

The deep subsurface of Río Tinto aquifer at the center of the IPB appears to be characterized by a high diversity of microbial life. The heterogeneous nature of the rock matrix accounts for such diversity. Interactions within distinct microbial populations as well as their interaction with the local geology should define the physicochemical characteristics at the microniche scale, within which, conditions conducive to support metabolisms such as methanogenesis and nitrate reduction as well as fermentation, sulfate reduction, acetogenesis, iron oxidation/reduction or methanotrophy may be established.

Prokaryotes have inhabited Earth for at least over 3.8 billion years (date of oldest known fossil) (Gramling 2017), along which they had the opportunity to diversify and adapt to virtually all the conditions existent on the planet. Since microbial life first evolved on Earth, the planet itself has continually been shaped by them. Future studies on life in subsurface environments, will keep on pushing forward novel biodiversity discoveries, as well as unveiling mechanisms and adaptations, providing clues not only on how life first evolved on our planet, as well as how it may currently occur in other planets.

# Appendices

# Appendix I


**Figure I.1.** Drilling campaign at Peña de Hierro (**a**); Core retrieved from the deep subsurface (**b**); Recovering samples from the central area of the core inside an anaerobiose chamber (**c, d**); Bottles with enrichment cultures used in this study (**e**); Colonies on the surface of agar on roll tubes (**f**).

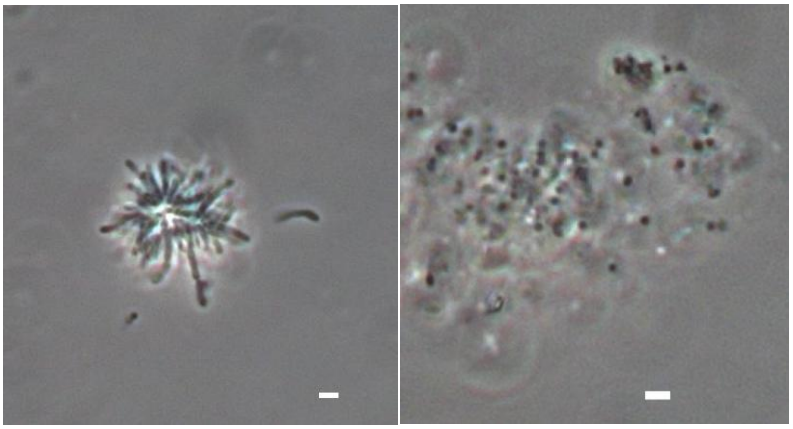

**Figure I.2.** Phase contrast observation of the typical rosette formation of *Rhodoplanes* cells (left panel) and coccus-shaped typical *Methanosarcina* cells (right panel) in T1.2MG-K100 culture (1000x magnification). Bar, 2 µm.

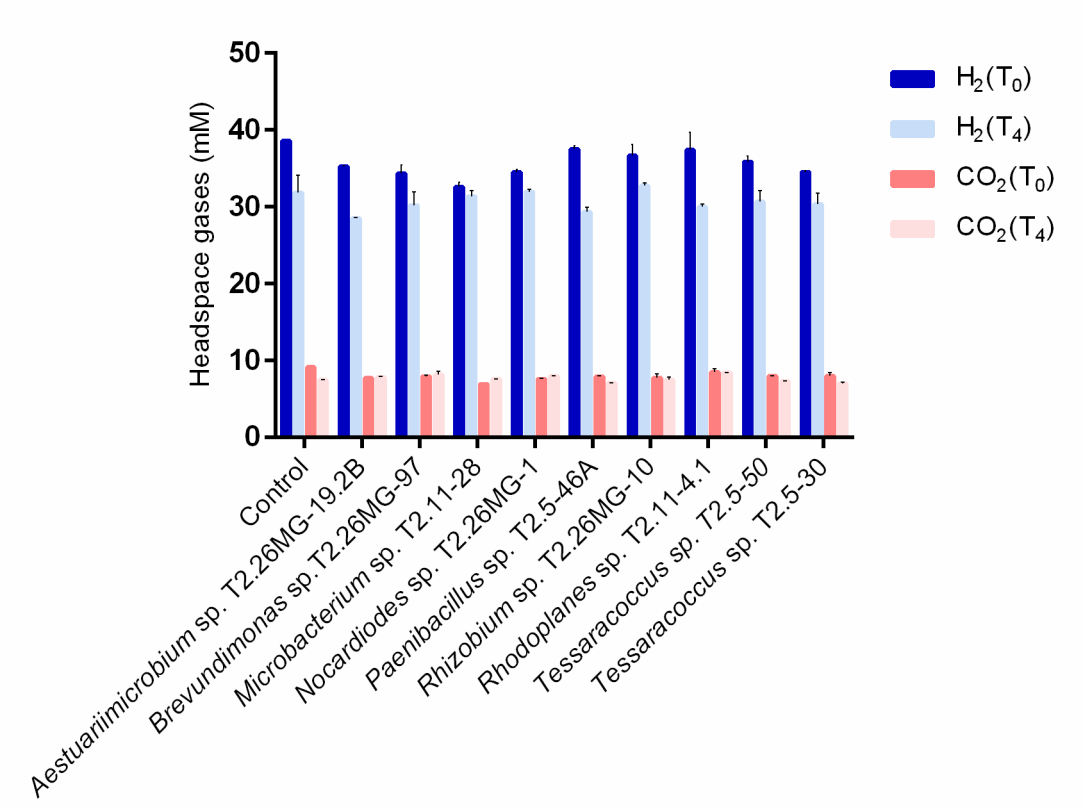


**Figure I.3.** Hydrogen and carbon dioxide levels of selected isolated strains grown in an oligotrophic media with a mix of hydrogen and carbon dioxide as substrates over a period of four months.

# Appendix II

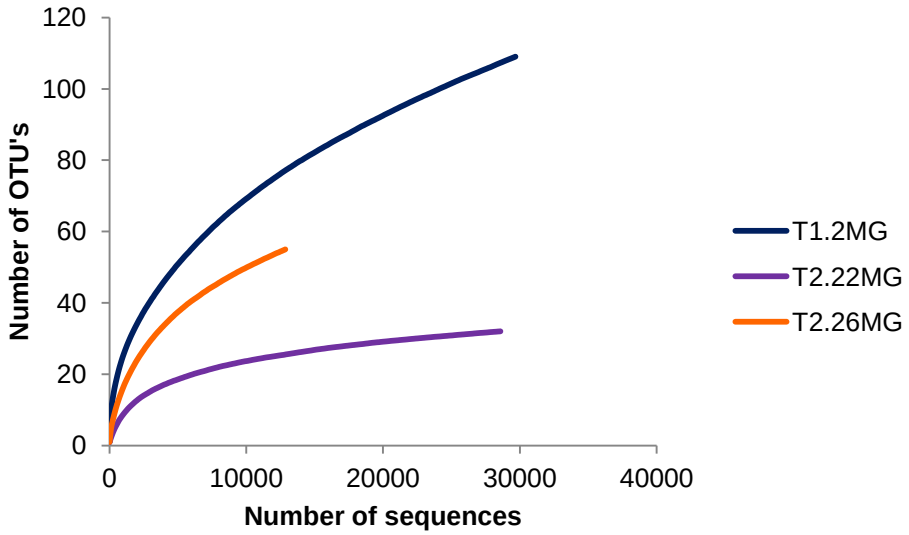


**Figure II.1.** Rarefaction curves for bacterial OTU's (97% cutoff) in enrichment cultures T1.2MG, T2.22MG and T2.26MG.

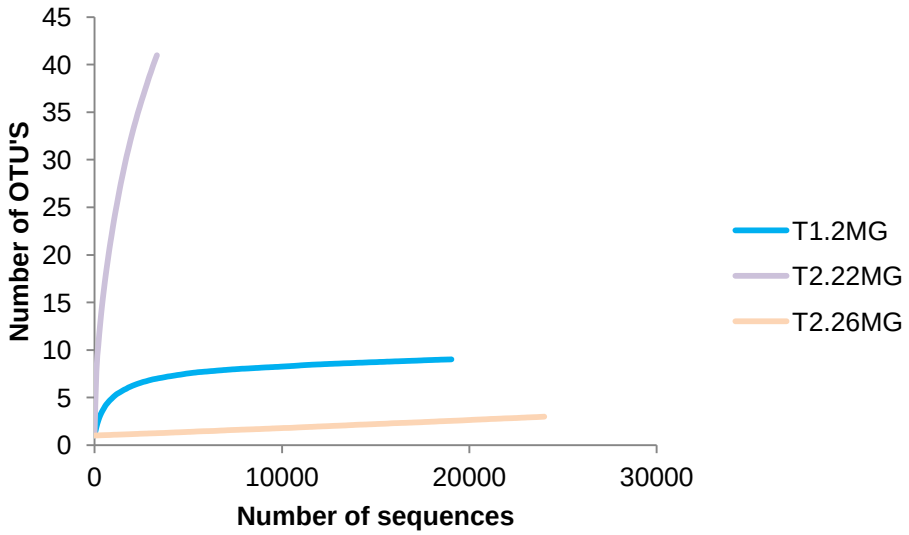


**Figure II.2.** Rarefaction curves for archaeal OTU's (97% cutoff) in enrichment cultures T1.2MG, T2.22MG and T2.26MG.

# Appendix III

### Crystal violet assay for evaluation of biofilm formation ability

To test the ability of bacterial strains for biofilm formation the assay applied was adapted from the method described in Sakurai and Yoshikawa (Sakurai and Yoshikawa 2012). Biofilm assay was performed in multi-well polystyrene plates by growing cells in aerobic R2A media at 30ºC for up to 5 days. Plates were washed with deionized water and stained with 1% (w/v) crystal violet for 15 minutes. Subsequently, plates were washed with deionized water and let to dry at room temperature.

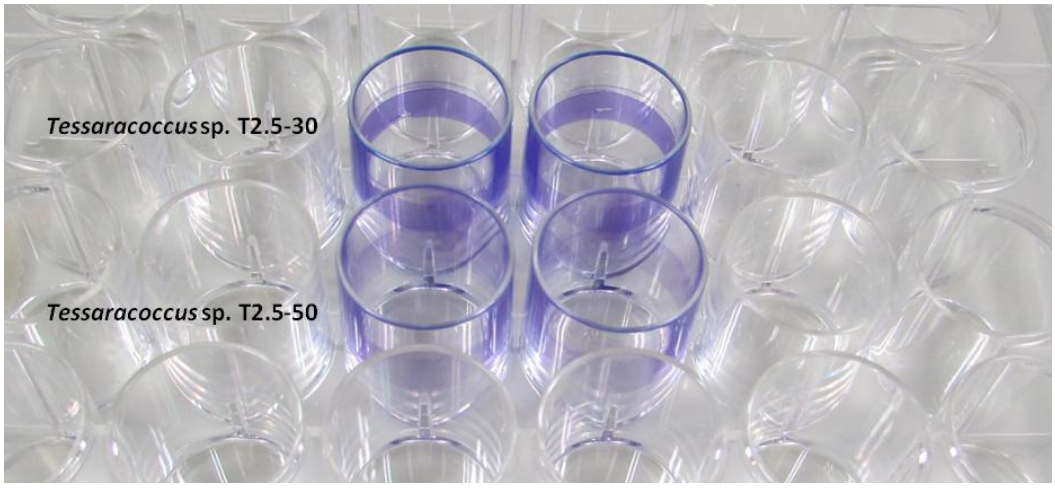


**Figure III.1.** Biofilm formation assay for *Tessaracoccus* strains T2.5-30 and T2.5-50 grown at 30ºC in R2A media on polystyrene multiwell plates. The plates were washed and stained with crystal violet.

# Appendix IV

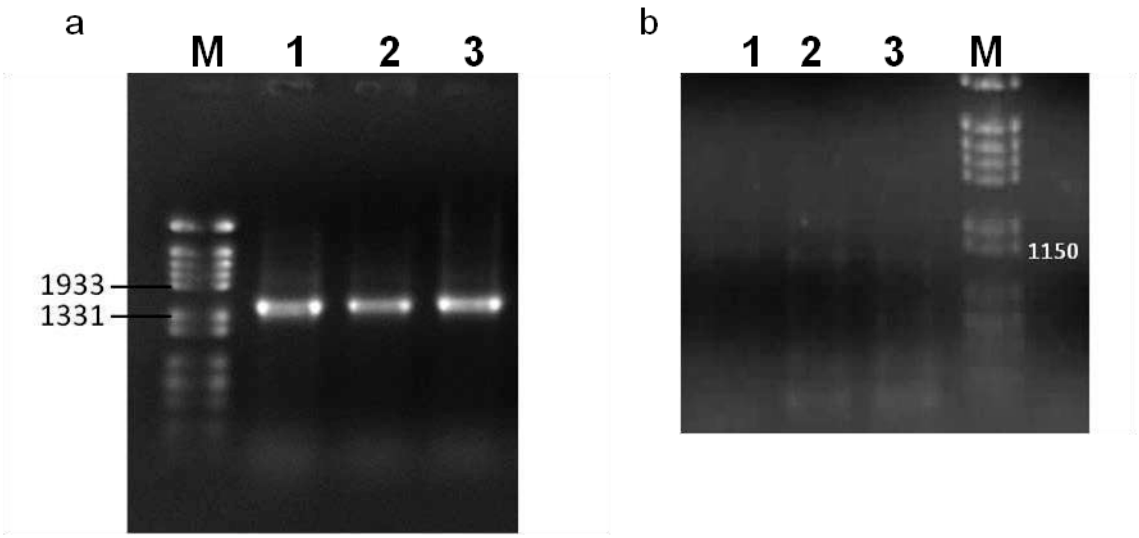


**Figure IV.1.** Agarose gel electrophoresis of PCR products from 16S rRNA gene amplified with primers targeting *Bacteria* **(a)** and *Archaea* **(b)**. M, DNA marker (Φ29 digested with *Hind*III); 1, colony T2.5-12 ; 2, colony T2.10-10 ; 3, colony T2.11-6.

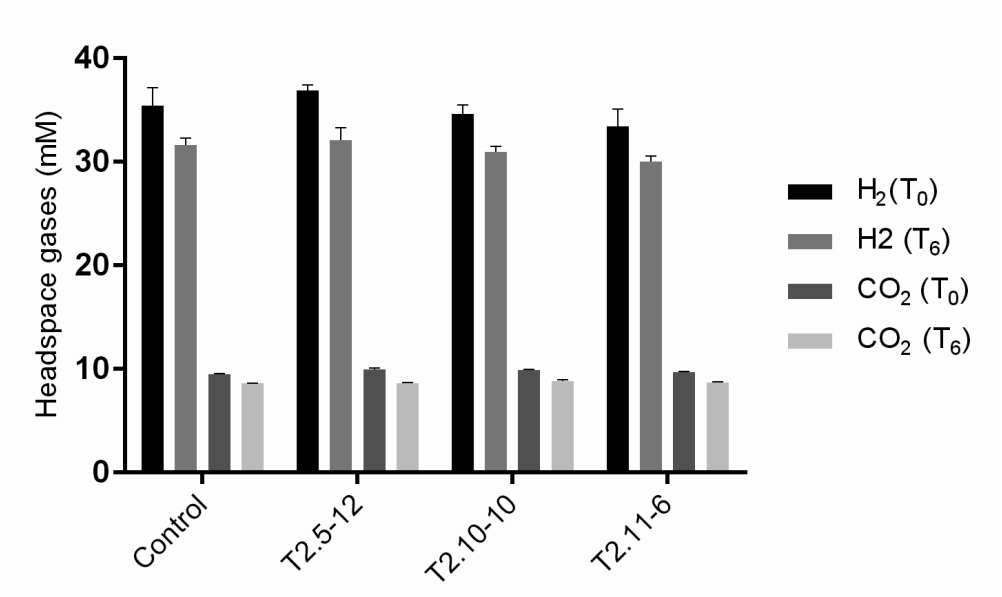


**Figure IV.2.** Measurement of hydrogen and carbon dioxide levels in the headspace of cultures T2.5-12, T2.10-10, T2.11-6 and negative control with uninoculated media, at time zero ($T_0$) and after six ($T_6$) months incubation.

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