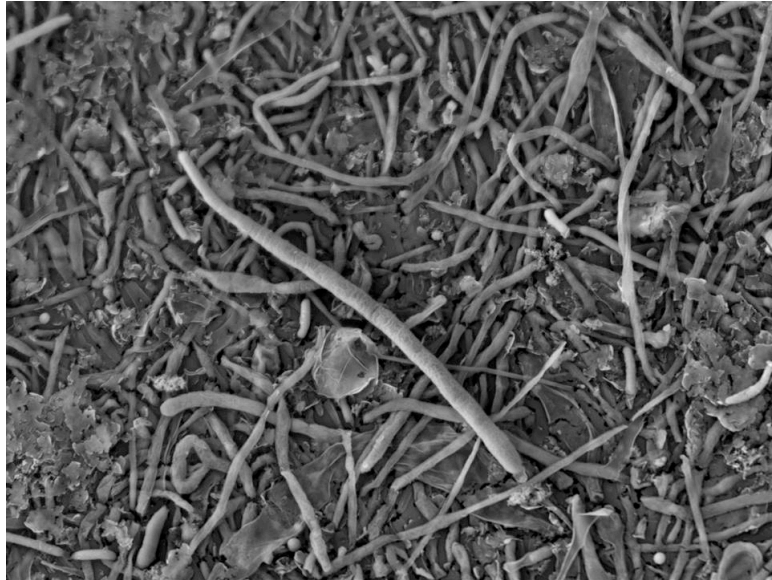


*Cultivation and genomics of extremophiles from a deep and hot  
North Sea oil reservoir*



*Master of Science Thesis in Biology*

*Specialization in Microbiology*

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*University of Bergen*

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## Preface

First I would like to thank my supervisor Professor Nils-Kåre Birkeland for all his time and help during my Master thesis work. Thank you for finding a project that I could work with when I was feeling “lost in microbiology”.

I would also like to thank the staff engineers: Marit Madsen, Helge Erikstad and Birte Tøpper for helping me out in the lab, and also all my fellow microbiology students for good advice and help in the lab.

Thanks to Oda and Natalie for a great trip to Germany and Chicago and all the joyful lunch breaks, I’ll miss you!

My brother Karstein and good friend Ida for their good help with all my Mac issues, being a technophobe is hard while writing a thesis.

Thanks to my family for encouraging me during a decade as a student. Now I’m finally coming home for good!

Thanks to sir David Attenborough, a great inspiration since I was a child for becoming a biologist in the 1<sup>st</sup> place.

Synnøve, nå e mamma endelig ferdig med oppgaven!

## Abstract

In this study, production water from the Troll B oilfield in the North Sea has been used as a source in order to isolate and characterize new extremophilic prokaryotes. Anaerobic enrichment cultures with different carbon sources and growth conditions were made in order to enrich specifically for novel groups and species of thermophilic anaerobes. Growth was obtained in enrichments incubated at 55°C with casamino acids, glucose, peptone, yeast extract, arabinose, maltose, dextrin, threonine and serine as substrates. Isolation of organisms was performed by dilution to extinction prior to identification through 16S rRNA gene sequence analyses. A total of seven strains, belonging to the *Thermotogae* and *Synergistetes* phyla, were obtained.

All the isolates but one belonged to species (*Thermovirga lienii*, *Petrotoga mobilis*, and *Thermosipoho africanus*) previously isolated from North Sea oil wells with 16S rRNA gene sequence identities above 99%. One isolate, recovered using glucose and yeast extract as substrates in presence of 5% NaCl shared 99.3% sequence identity with the Thermotogales organisms, *Petrotoga mexicana*, which has previously only been recovered from a oil field in the Gulf of Mexico.

A draft genome sequencing of this isolate, termed *Petrotoga mexicana* strain Troll was done using paired-end Illumina HiSeq sequencing performed by a commercial genome sequence provider. Ninety sequence contigs constituting a total of 2,116,100 bases were obtained. The sequencing indicates a genome length less than 2.2 Mb. The genome was annotated and analysed using Rapid Annotation Subsystem Technology (RAST), and compared to the *Petrotoga mobilis* genome, the closest genome-sequenced relative. Based on the genomic sequence, metabolic features of strain Troll were analysed and compared to *Petrotoga mobilis*. Although the main energy metabolism seems to be based on glycolysis with lactate as main fermentation product, some unexpected features, e.g. the presence of genes for a respiratory complex I, a Na<sup>+</sup>-translocating NADH-quinone oxidoreductase and carbon monoxide dehydrogenase were identified. A large number of transport systems for amino acids, peptides, sugars and minerals were also identified as well as a complete xylan degradation pathway. A difference of 2.2% in G+C content from the *Petrotoga mexicana* type strain as well as morphological differences indicates that strain Troll may represent a novel *Petrotoga* species.

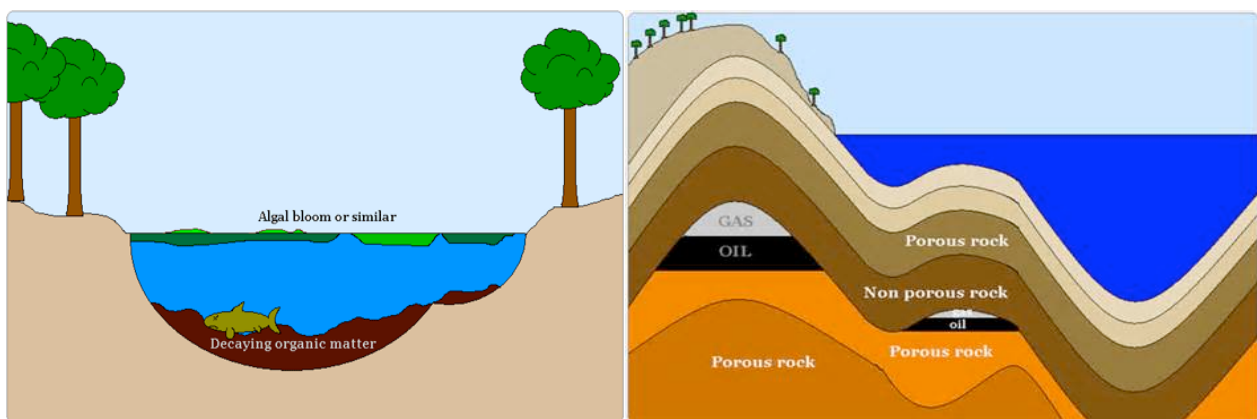
<b>1. Introduction</b>	<b>6</b>
1.1 The sterile subsurface	6
1.2 Extremophiles	7
1.3 Physiological groups that have been recovered from oil reservoirs	9
1.4 Microbial diversity in North Sea oil wells	14
1.5 Biofilms	16
1.6 In situ biodegradation of hydrocarbons	17
1.7 The Troll oil and gas field	18
1.8 The 16S ribosomal RNA gene as phylogenetic marker	20
1.9 Aims and research questions	22
<b>2. Materials and methods</b>	<b>23</b>
2.1 Production-water	24
2.2 Medium	24
2.3 Anaerobic stock solution	27
2.4 Enrichments	28
2.5 DNA extraction	29
2.6 Polymerase Chain Reaction (PCR)	31
2.7 Electrophoresis	33
2.8 GenElute™ PCR Clean-Up Kit	33
2.9 Basic Sequence Alignment Search Tool (BLAST)	35
2.10 Scanning Electron Microscope (SEM)	35
2.11 Genome sequencing	35
2.12 Rapid Annotation using Subsystem Technology (RAST)	35
2.13 Phylogenetic trees	35
2.14 Mauve	35
<b>3. Results</b>	<b>36</b>
3.1 Enrichments	36
3.2 Scanning Electron Microscopy (SEM)	39
3.3 Genome analysis statistics	40
3.4 Subsystems statistics	41
3.5 Genome comparison	42
3.6 Genes related to electron transport phosphorylation (ETP)	46
3.7 CRISPR genes and phage	49
3.8 Antibiotics and toxins	49
3.9 Xylan degradation	50
3.10 Central metabolism	52
<b>4. Discussion</b>	<b>54</b>
4.1 Cultivation	54
4.2 Strain Troll	55
4.3 Genome features and predictions	56
<b>5. Conclusion</b>	<b>59</b>
<b>6. References</b>	<b>60</b>
<b>7. APPENDICES</b>	<b>63</b>
Appendix A	63
Appendix B	65

# 1. INTRODUCTION

## 1.1 The sterile subsurface

The formation of the oil reservoirs in the North Sea took place during the Jurassic period, 200-146 million years ago. During this period, high intensity sedimentation from decaying organisms occurred in the area (Figure 1 left). The sedimentation layer got thicker, and the pressure increased due to the weight from the continuous sedimentation and the sea above. When the sedimentation layer with organic matter was pressed further downwards, the temperature increased, resulting in the formation of Kimmeridge clay, equivalent to porous rock in Figure 1, right, the most important reservoir rock in the North Sea. The high temperature and pressure transformed the organic matter into hydrocarbons that were trapped inside pores within the reservoir rock, and thus forming oil and gas reservoirs (Bryhni, 2009). Typically, the gas is trapped on top of the oil (Figure 1 right) and usually there's a layer of water below the oil.

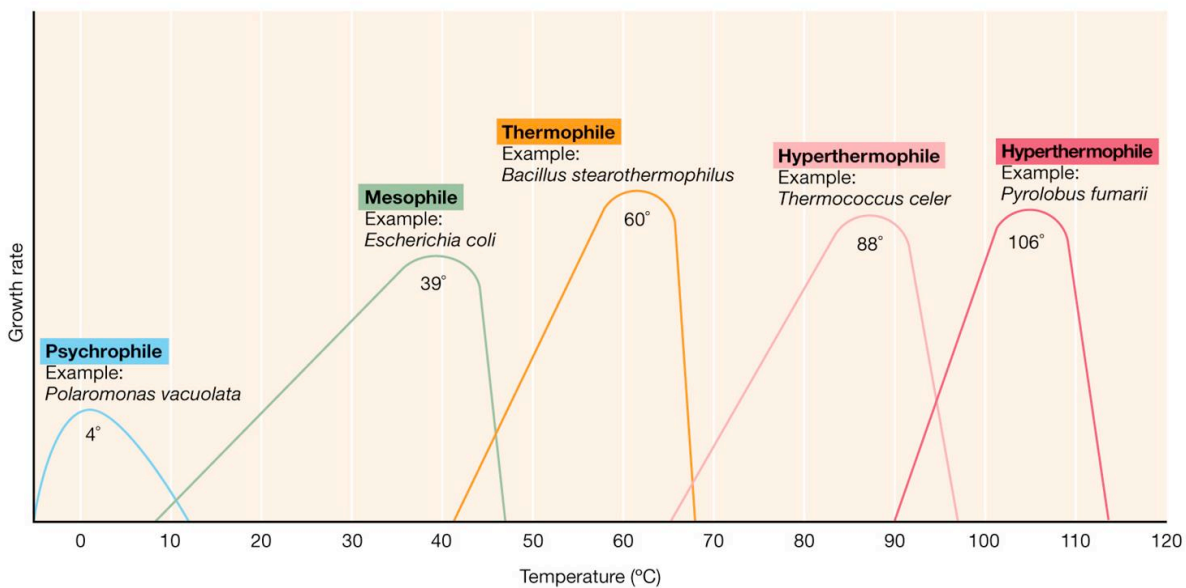
The subsurface is often referred to as the marine sediments below 10cm and terrestrial habitats below 8 meters. For a long time the subsurface including oil reservoirs were believed to be sterile, and detected bacteria were assumed to be introduced by extracting the oil via drilling equipment. It was believed to be impossible that any form of life was able to thrive in such an extreme environment like an oil reservoir where the temperature is high, anoxic conditions, low pH, and high salinity and pressure. In 1926, Edson S. Bastin was the first scientist to isolate sulfate reducing bacteria (SRB) from oil wells in Illinois, and suggested the SRB to be indigenous in oil reservoirs or introduced with ground water after their formation. He also pointed out the uncertainty regarding the sampling, that one could never exclude the possibility that the bacteria had been introduced with the equipment, and thus it was never concluded that the SRB are true indigenous oil-field microorganisms (Edson et al., 1926, Magot et al., 2000). At present, it is estimated that the total biomass of the subsurface may exceed that of the oceans and soil (Whitman et al., 1998).



**Figure 1 Left: Decaying organic matter to be covered with a thick sedimentation layer. Right: The typical layering of an oil reservoir: oil, gas and porous rock/water** (<http://www.green-planet-solar-energy.com/fossil-fuel-formation.html>).

## 1.2 Extremophiles

Extremophilic microorganisms are capable to tolerate extreme conditions relating to salinity, pressure, pH and temperature (Table 1). In high salinity environments, organisms are referred to as halophiles. Piezophiles are adapted to high pressure. Alkaliphiles are found in habitats with pH above 8, and acidophiles in pH below 5.5. The tolerance of heavy metals is another factor. Further, extremophiles are divided into different groups with regard to optimal growth temperatures (Figure 2). Prokaryotes that live in the cold are referred to as psychrophiles. Those microorganisms that prefer high temperatures, the thermophiles, thrive in environments with temperatures from 40-80°C. The hyperthermophiles thrive in environments that are extremely hot and have optimal growth temperatures above 80°C, some are capable of resisting heat up to 122°C (Madigan; et al., 2012). Organisms living in oil reservoirs are poly-extremophiles with temperatures up to 80°C, salinity, pressure beyond 150 bar, presence of heavy metals such as barium and strontium and anoxic conditions (Dahle et al., 2008)



**Figure 2** Here 5 organisms represent the four groups from left: psychrophiles, mesophiles, thermophiles and hyperthermophiles (Madigan; et al., 2012).

**Table 1 Overview of different extremophiles; their descriptive term and habitat (Madigan; et al., 2012).**

<b>Extreme</b>	<b>Descriptive term</b>	<b>Genus/species</b>	<b>Domain</b>	<b>Habitat</b>	<b>Minimum</b>	<b>Optimum</b>	<b>Maximum</b>
<b>Temperature</b>							
High	Hyperthermophile	<i>Methanopyrus kandleri</i>	Archaea	Undersea hydrothermal vents	90°C	106°C	122°C
Low	Psychrophile	<i>Psychromonas ingramhamii</i>	Bacteria	Sea ice	-12°C	5°C	10°C
<b>pH</b>							
Low	Acidophile	<i>Picrophilus oshimae</i>	Archaea	Acidic hot springs	-0,06	0,7	4
High	Alkaliphile	<i>Natronobacterium gregoryi</i>	Archaea	Soda lakes	8,5	10	12
<b>Pressure</b>	Barophile(piezophile)	<i>Moritella yayanosii</i>	Bacteria	Deep ocean sediments	500 atm	700 atm	>1000
<b>Salt (NaCl)</b>	Halophile	<i>Halobacterium salinarum</i>	Archaea	Salterns	15%	25%	32% (saturation)

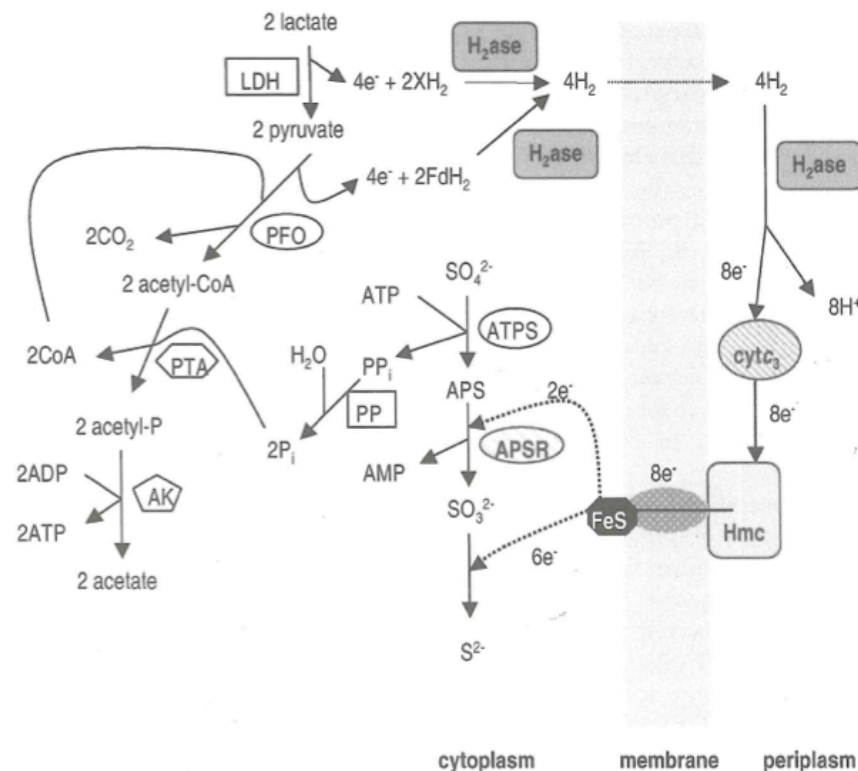


### 1.3 Physiological groups that have been recovered from oil reservoirs

#### 1.3.1 Sulfate reducing prokaryotes

Sulfate reducing prokaryotes (SRP) are found in a variety of anaerobic environments, one of them being oil reservoirs. SRP have been recovered from oil reservoirs around the world from several different lineages, the Gram-positives, *Proteobacteria*, *Thermodesulfobacterium*, *Thermodesulfobium* and *Thermodesulfovibrio*. In the Archaea domain, organisms from *Archaeoglobus* in the lineage *Euryarchaeota* have been recovered from oil reservoirs. SRP use sulfate ( $\text{SO}_4^{2-}$ ) as an electron acceptor and transfer it to sulfide (Figure 3). Most of them oxidize organic acids to acetate and/or  $\text{CO}_2$ . SRP using  $\text{H}_2$  as electron donor carry out the following energy yielding reaction:

$4 \text{H}_2 + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4 \text{H}_2\text{O}$ , which has a net yield of 1 ATP for every  $\text{SO}_4^{2-}$  reduced to  $\text{HS}^- / \text{S}^{2-}$  (Birkeland, 2005).



**Figure 3 Sulfate reducing bacteria: e<sup>-</sup> transport and energy conservation. H<sub>2</sub> from lactate is brought out of the cell, and together with external H<sub>2</sub> give up their e<sup>-</sup> that are brought back into the cell. Eight e<sup>-</sup> are brought into the cell where they reduce SO<sub>4</sub><sup>2-</sup> into S<sup>2-</sup>. Two ATP are formed, and one consumed (Birkeland, 2005).**

### 1.3.2 Methanogenic archaea

Methanogenic archaea produce methane in strictly anaerobic environments, where they usually reduce carbon dioxide with electrons from hydrogen and form methane. But they also use compounds such as methanol, formate, carbon monoxide and acetate. The methanogenesis from  $\text{CO}_2 + \text{H}_2$  starts by activating  $\text{CO}_2$  using an enzyme that contains methanofuran that reduces the  $\text{CO}_2$  to formyl (Figure 4). The formyl group continues to the methanopterin enzyme that reduces and dehydrates formyl, first into methylene and then methyl. Next a methyl transferase enzyme passes on the methyl group to an enzyme that contains CoM. This is an exergonic reaction that's involved in pumping  $\text{Na}^+$  out of the cell and generating  $\text{Na}^+$  motive force that can be used for ATP formation through a  $\text{Na}^+$ -linked ATP synthase. Further, methyl reductase, coenzyme  $\text{F}_{430}$  and CoB reduce the methyl-CoM to methane (Madigan; et al., 2012).

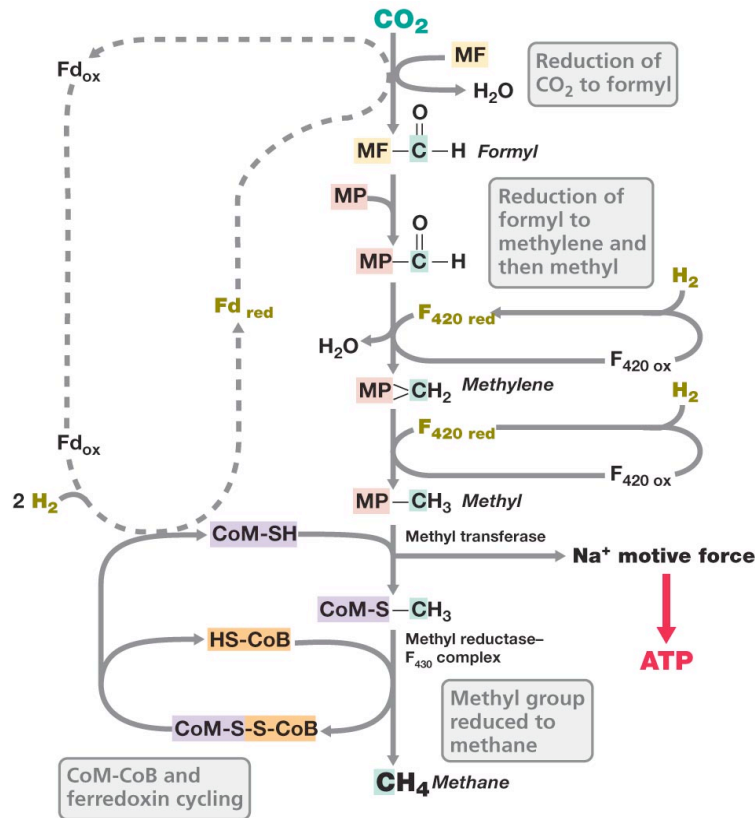


Figure 4 Methanogenesis from  $\text{CO}_2$  and  $\text{H}_2$  where  $\text{CO}_2$  is reduced in several steps; formyl, methylene, methyl and eventually methane (Madigan; et al., 2015)

Different methanogens have been recovered from oil reservoirs; *Methanobacterium thermoalcaliphilum* from oil fields in Siberia (Davydova-Charakhch'yan et al., 1992), and *Methanocalculus halotolerans* has been isolated from an oil reservoir in France (Ollivier et al., 1998).

### 1.3.3 Fermentative bacteria and *archaea*

The fermentative organisms utilize organic compounds such as carbohydrates and amino acids, and form organic acids, ammonia and hydrogen as fermentation products. They don't depend on any electron acceptor, since they have an internal red-ox balance. Some fermentative bacteria and archaea are capable to reduce sulfur compounds, such as *Petrotoga mobilis*, isolated from the North Sea (Table 2)(Birkeland, 2004).

#### *Thermotogae*

There are several species that have been identified from oil reservoirs around the world from the phylum *Thermotogae*, which is a deep branch in the phylogenetic tree of Bacteria (Figure 6). The *Thermotogae* have rod shaped cells and are strictly anaerobic and thermophilic. *Thermotogae* is one of the eldest lineages in the domain of Bacteria, thus the members are adapted to the conditions that dominated the earth at an early age, 3-4 billion years ago, with high temperatures and an anoxic environment. The *Thermotogae* have in common that they stain gram negative, and are covered with a toga that is an outer membrane that creates a large periplasmic space filled with proteins (Figure 5). In some of the genera, the cells tend to cluster together and share their toga, forming long chains measuring more than 30  $\mu\text{m}$ . The *Thermotogae* are fermentative chemoorganotrophs, catabolizing sugars and starch, resulting in fermentation products such as  $\text{CO}_2$ ,  $\text{H}_2$ , lactate and acetate (Madigan; et al., 2012).

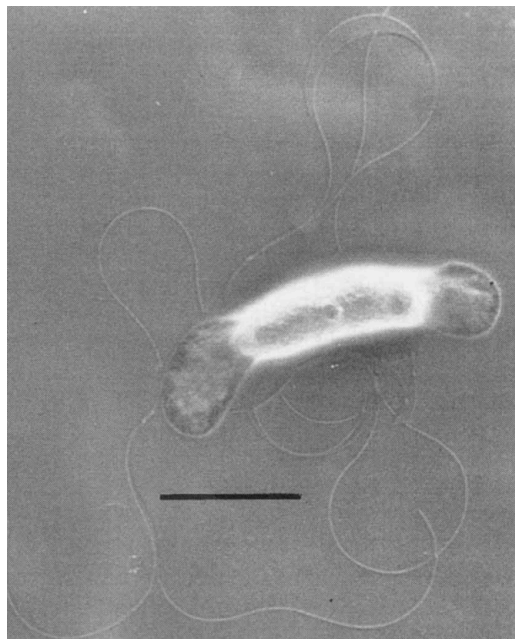
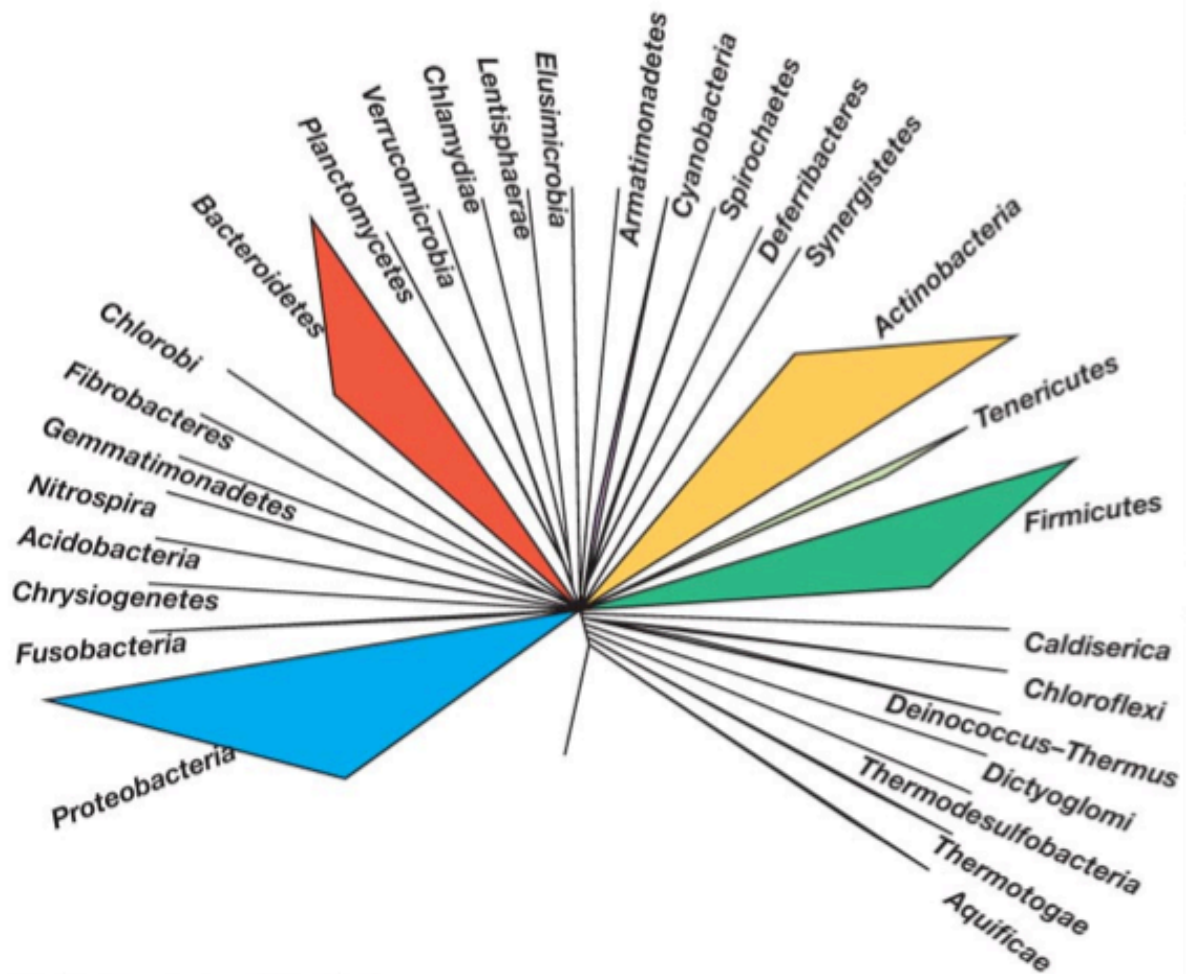


Figure 5 A scanning electron micrograph of a *Petrotoga mobilis* isolated from the North Sea with toga and flagella. Bar 2 $\mu\text{m}$  (Lien et al., 1998).



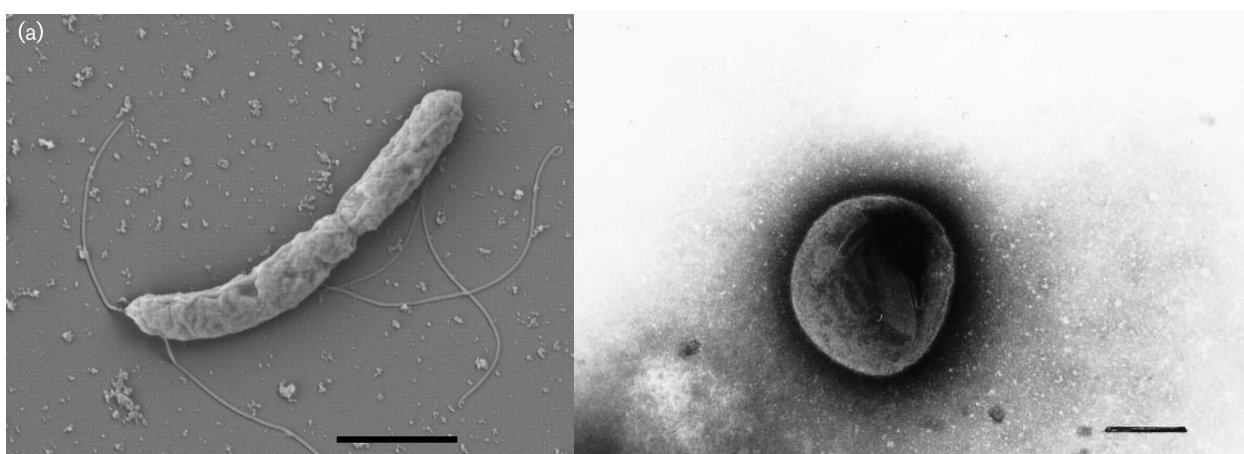
(a) Major phyla of Bacteria

Figure 6 The phylogeny of Bacteria, where *Thermotoga* is rooted second from the bottom of the Bacteria branch and Synergistetes is 11<sup>th</sup> from the root (Madigan; et al., 2012).

Members of the eleven genera in the Thermotogales are believed to have received a large number of genes from archaea by horizontal gene transfer. A genome study of the *Thermotoga maritima* in 1999 showed that as much as 24% were similar to the archaeal genes, suggesting that an extensive horizontal gene transfer has occurred at some point (Nelson et al., 1999).

## Synergistetes

*Synergistetes* was accepted as recently as in 2009 as a new phylum (Figure 6) based on 16S rRNA gene studies. The former phylum *Deferribacteres* and the family *Syntrophomonadaceae* were placed into this new phylum. The new phylum consists of the genera *Aminiphilus*, *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermanaerovibrio* and *Thermovirga*. Members of this phylum are found in a variety of different anaerobic habitats like in insect- and animal gastrointestinal tracts, periodontal tissues, wastewater treatment systems, in soil and in oil wells. So far, only members of the *Thermovirga* has been recovered from oil reservoirs. Besides being strictly anaerobic, the *Synergistetes* members are neutrophilic, rod shaped (Figure 7, left), stain Gram-negative and ferment amino acids (Jumas-Bilak et al., 2009).



**Figure 7** Fermentative prokaryots, left: *Thermovirga lienii* with flagella (bar:1 $\mu$ m). Right: *Thermococcus sibiricus* (bar 0.5 $\mu$ m) (Dahle and Birkeland, 2006, Miroshnichenko et al., 2001).

## *Thermococcus*

Different members of the archaeal *Thermococcus* have been isolated from oil reservoirs around the globe, such as *Thermococcus sibiricus* and *Thermococcus litoralis* in Siberia and Japan respectively (Miroshnichenko et al., 2001, Takahata et al., 2001) The *Thermococcus* is an obligate anaerobe, fermentative and chemoorganotroph that oxidizes proteins and some sugars. *Thermococcus* uses sulfur as an electron acceptor, which is reduced to hydrogen sulfide. The *Thermococcus* cells are spherical (Figure 7, right) and some species have a tuft of polar flagella. They thrive in temperatures from 55-95°C (Madigan; et al., 2012).

### 1.3.4 Iron reducing organisms

Mesophilic iron reducers such as *Shewanella putrefaciens*, have been isolated from oil fields. *S. putrefaciens* can in addition to iron also reduce sulfur, sulfite and thiosulfate into sulfide. H<sub>2</sub> or formate serve as electron donors and iron oxyhydroxide as the electron acceptor. From oil reservoirs in Western Siberia iron reducing bacteria from *Thermotoga* and *Thermoanaerobacter* and iron reducing archaea from *Thermococcus* were also recovered (Miroshnichenko et al., 2001). From the North Sea the thermophilic bacterium *Deferribacter thermophilus* have been isolated. *D. thermophilus* reduce both iron and manganese, and grow on substrates such as yeast extract, peptone, casamino acids and organic acids (Birkeland, 2004, Magot et al., 2000).

### 1.4 Microbial diversity in North Sea oil wells

In Norway, *Petrotoga mobilis* from the *Thermotogae* phylum was isolated in 1998. This novel species had an optimum growth temperature at 58-60°C, pH at 6.5-7.0 and grew with 3-4% NaCl and 0.7% MgSO<sub>4</sub> • 7H<sub>2</sub>O. Vitamin solution was added, and yeast extract utilized for growth stimulation. The cell size varied from a couple of µm up to 50 µm. The closest related species was found to be *Petrotoga miotherma*, that *Petrotoga mobilis* differs from with its' sub polar flagella making it motile as the name indicates (Lien et al., 1998).

*Thermosiphon africanus* was first isolated in Djibouti, Eritrea from a shallow marine hydrothermal system in 1989 (Huber et al., 1989). In 2009 *T. africanus* was isolated from an oil reservoir in the North Sea (Nesbø et al., 2009). *T. africanus* is rod shaped, and grows in chains with up to twelve cells.

*Kosmotoga olearia* was isolated from an oil reservoir in the North Sea in 2009. This new genus and species was described as non-motile rods with a sheath like structure like a toga. They stained gram negative and grew at 20-80°C, pH 5.5-8, with NaCl concentrations of 10-60 g l<sup>-1</sup> and ferment several carbohydrates, pyruvate and proteinaceous compounds. Phylogenetic analyses indicated that this was a species from the order of the Thermotogales (DiPippo et al., 2009).

From the *Synergistetes* the *Thermovirga lienii* was characterized within the family *Syntrophomonadaceae*, an organism isolated from a non-flooded North Sea oil well. *T. lienii* is a strictly anaerobic motile rod, staining gram negative, with optimum growth temperature at 58°C, salinity 2.0-3.0% and pH 6.5-7.0. It ferments organic acids, protein compounds and a selection of single amino acids. It reduces elemental sulfur and cysteine to sulphide (Dahle and Birkeland, 2006)

An archaeal methanogen recovered from the North Sea is the *Methanococcus thermolithotrophicus* (Table 2)(Nilsen and Torsvik, 1996).

These are some of the organisms isolated from the North Sea, more species are included in Table 2.

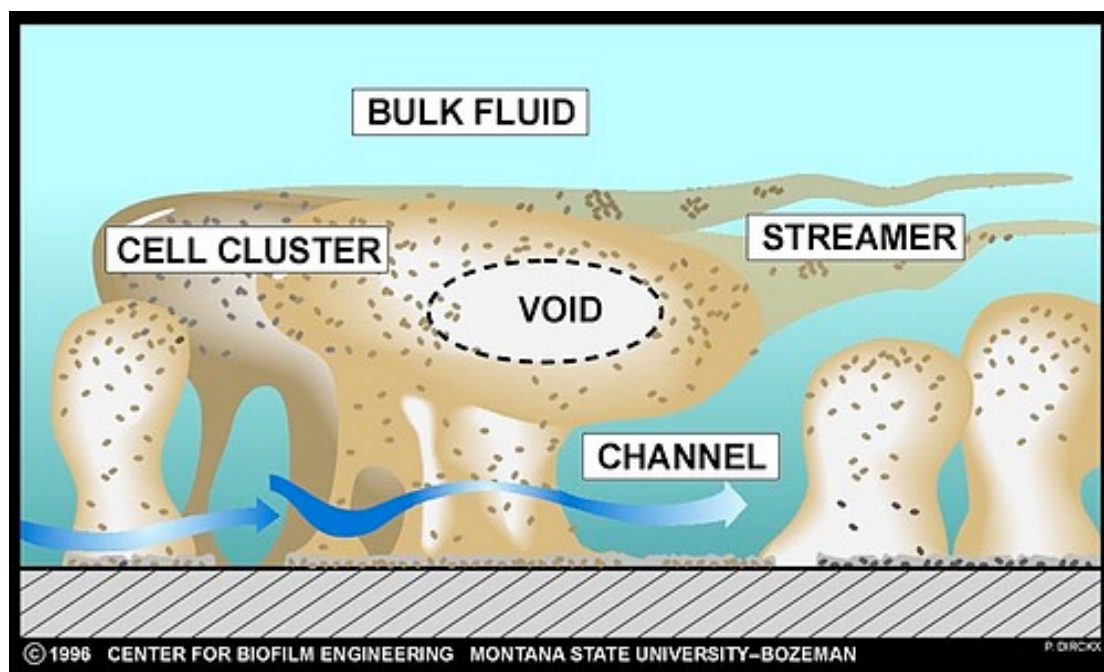
**Table 2 Organisms isolated from production water from the North Sea(Norwegian sector) Isolation and charaterization has been performed at the former Institute of Microbiology, University of Bergen.**

<b>Group</b>	<b>Isolate</b>	<b>Domain</b>	<b>Reference</b>
Sulphate Reducing Prokaryotes	<i>Desulfotomaculum thermocisternum</i> (sp. nov.)	<i>Bacteria</i>	(Nilsen et al., 1996)
	<i>Desulfobulbus rhabdoformis</i> (sp. nov.)	<i>Bacteria</i>	(Lien et al., 1998)
	<i>Desulfobacter vibrioformis</i> (sp. nov.)	<i>Bacteria</i>	(Lien and Beeder, 1997)
	<i>Desulfotomaculum</i> strains T93B T90A	<i>Bacteria</i>	(Rosnes et al., 1991)
	<i>Thermodesulfobacterium mobile</i>	<i>Bacteria</i>	(Christensen et al., 1992)
	<i>Thermodesulforhabdus norvegicus</i> (gen. nov. sp. nov.)	<i>Bacteria</i>	(Beeder et al., 1995)
	<i>Archaeoglobus fulgidus</i> strain 7324	<i>Archaea</i>	(Beeder et al., 1994)
Metanogens	<i>Metanothermococcus thermolithotrophicus</i> strain ST22	<i>Archaea</i>	(Nilsen and Torsvik, 1996)
Fermenting organisms	<i>Petrotoga mobilis</i> (sp. nov.)	<i>Bacteria</i>	(Lien et al., 1998)
	<i>Petrotoga</i> strain TBD7213	<i>Bacteria</i>	(Dahle, 2001)
	<i>Thermosipho</i> strain TBA5	<i>Bacteria</i>	(Bjørø, 2000)
	<i>Thermovirga lienii</i> (gen. nov. sp. nov.)	<i>Bacteria</i>	(Dahle and Birkeland, 2006)
	<i>Thermosipho africanus</i>	<i>Bacteria</i>	(Nesbø et al., 2009)
	<i>Kosmotoga olearia</i>	<i>Bacteria</i>	(DiPippo et al., 2009)

In a recent microbial community structure analysis of produced water from a the Troll oil field using both molecular and culture-based methods, a large number of organisms without close 16S rRNA sequence match have been identified (Dale et al., 2008 and Appendix A). Especially clones with closest match to *Thermovirga lienii*, *Caminicella sporogenes* and *Anaerophaga thermohalophila* (Dahle et al., 2008). Based on the high amount of clones, this could indicate that there is probably many undiscovered species still in these environments.

### 1.5 Biofilms

Biofilm formation is very common in oil pipelines and installations and the sulfide that is produced by the microbial communities within these biofilms has a detrimental corroding effect. Many kinds of bacteria are able to form biofilms on different surfaces, both living and non-living, where there is a stream of nutritional fluid such as on river rocks and tubes. In the oil subsurface it is believed that prokaryotes form biofilms in the pores of reservoir rocks. The bacteria are able to attach to the surface by forming a matrix of sticky polysaccharides, proteins and DNA, and by clustering together the bacteria become up to 1000 times more resistant to antibiotics, disinfection agents and detergents. The biofilm provides protection, and the cells do not have to spend valuable energy in motility since there is a constant flow of nutrients that can be assimilated. Biofilms may consist of a sole species or complex communities comprised of different species cooperating in metabolism, where each species contribute with different essential enzymes in order to degrade nutrition from the flow, benefiting from the different redox levels (Hauge, 2013).



**Figure 8** The structure of a biofilm with its channels through the cell clusters (<http://www.biofilm.montana.edu/content/biofilm-structure-labels>).

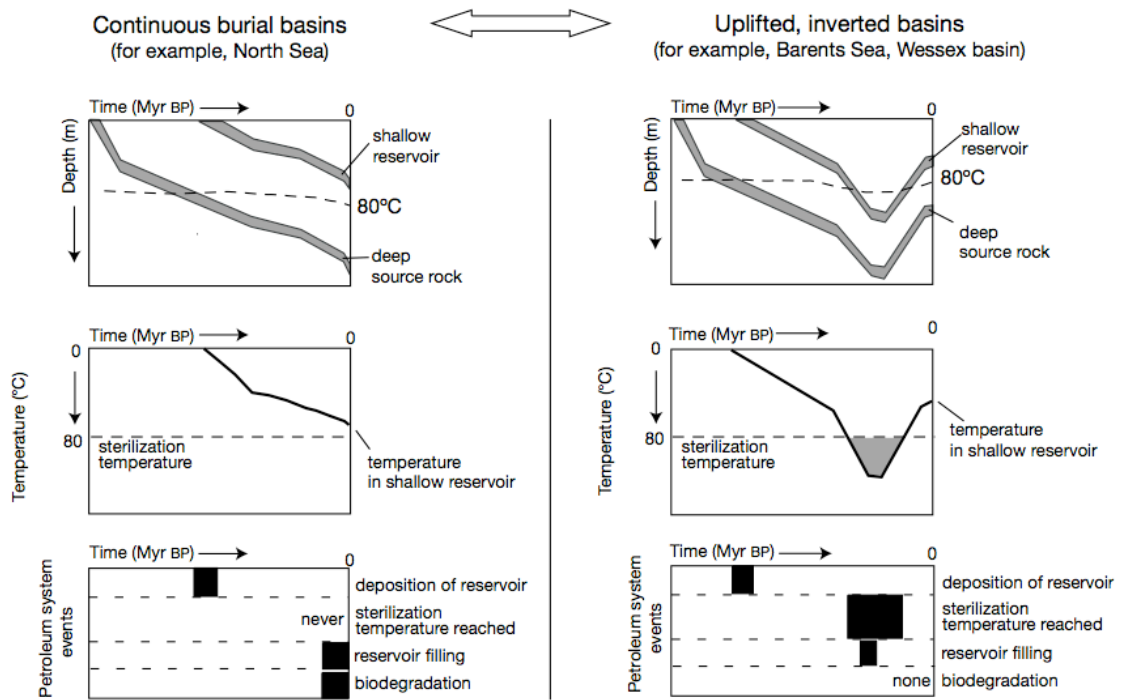


Biofilms are formed when a certain critical amount of cells are gathered and is often regulated by quorum-sensing. In the biofilm there is a network of channels so that the nutrients can flow within the biofilm and reach the cells at different levels (Figure 8). Horizontal gene transfer also frequently occurs in biofilms, and in this way neighboring bacteria can acquire beneficial properties such as antibiotic resistance (Parsek et al., 1999). Scientists are trying to find ways to prevent the formation of biofilm on surfaces that are difficult to clean physically or chemically. Nanometer thick antibacterial films have been developed at the University of Oslo, containing biologically active molecules like thiophenons. Thiophenons inhibit the communication between the bacteria thus preventing them to form biofilms in the first place. Since thiophenons do not kill the bacteria like antibiotics it is therefore believed not to contribute to any development of enhanced resistance (Kjemisk-Institutt, 2013).

### **1.6 In situ biodegradation of hydrocarbons**

Oil reservoirs with temperatures above 80°C are generally not biodegraded, because of the high temperature blocking microbial metabolism, but most oil reservoirs have temperatures below this critical point. The in situ microbial biodegradation of petroleum changes the oil's properties, the amount of saturated hydrocarbons decrease, and so does the amount of aromatic hydrocarbons, the acidity and metal content increase and thus affecting its value negatively on the market due to increased production and refining costs.

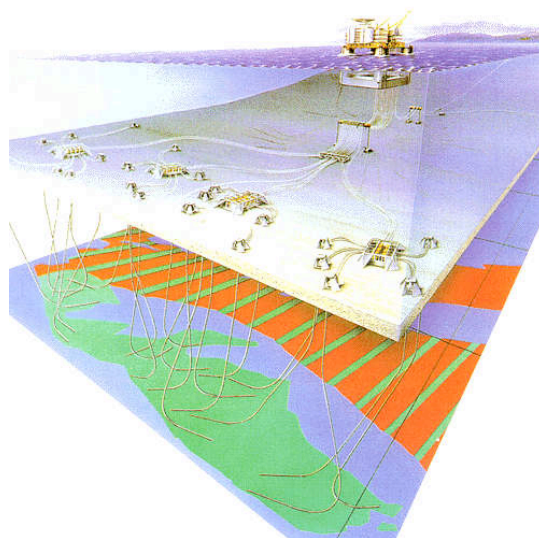
Oil density is measured as the American Petroleum Institute gravity (API), which describes the oils density compared to water. Oil with API value greater than 10 floats on water and are considered better quality oil. Oil with API values below 10 is so dense that it will sink. Oil with API gravity >36 is non-biodegraded oil, and considered high-quality. Oil with <20 API is heavy oil, and API <10 super heavy oil (Head et al., 2003). Palaeosterilization occurs when an oil reservoir has been exposed for temperatures above 80°C during the formation (Figure 9)(Wilhelms et al., 2001).



**Figure 9 Biodegraded oil reservoir to the left, and non-biodegraded oil reservoir to the right. During the formation the sterile reservoir has been exposed to temperatures above 80°C (Wilhelms et al., 2001).**

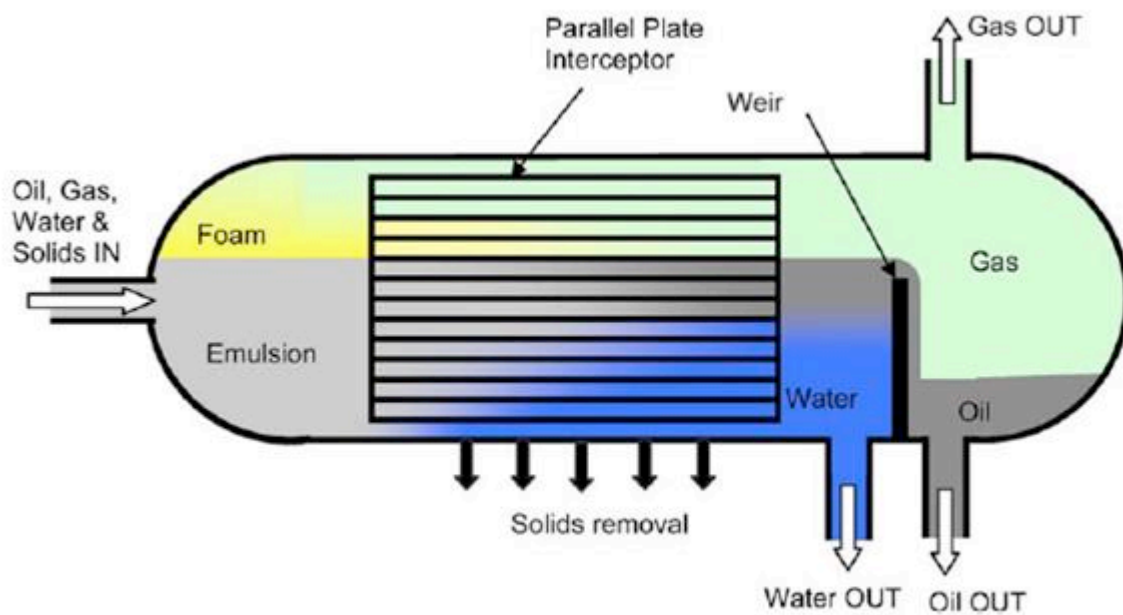
### 1.7 The Troll oil and gas field

The Troll field is located about 65 km west of Kollsnes in Hordaland at 60.645556°N; 3.726389°E (Wikipedia, 2014). This oil and gas field was discovered in 1979, and the Troll B platform started the production in September 1995. The Troll B platform is a semisubmersible platform, with a concrete substructure (Offshore-Technology).



**Figure 10 The Troll B facility with its horizontal wells within the narrow oil reservoir layer (green color) (<http://www.offshore-technology.com/projects/troll/troll1.html>).**

The oil is trapped in thin horizontal layers, ranging between 11-13 meters thickness in the Troll West gas province, and 22-26 meters thick in the Troll West Oil province (Figure 10). Due to the thin layers, it was necessary to develop advanced drilling techniques, first vertical until contact with the oil at 1600 meters below sea level, and then drill >3km horizontally within the oil layer (Statoil, 2007). When extracting the oil, a mixture of oil, water, gas and solids are brought up thru the pipes and to the surface where it's run thru an oil separator tank. The oil floats on top of the water, solids sink to the bottom and the gas is led out on the top. A parallel plate interceptor neutralizes the foam formed due to the mixing in the pipelines and also helps separating the oil from the water (Figure 11). The oil, gas and water are run through further separations in order to obtain as purified products as possible, and the water produced is either let out in the sea or reinjected into the oil reservoir in order to maintain the pressure within the reservoir (Offshoreteknikk, 2013). The Troll field is a non-flooded oil reservoir, meaning that it is not subject to seawater injection. The *in situ* temperature is 70°C.



**Figure 11 Schematic model of an oil separator tank where a mixture of oil, gas, water and solids is let in, and separated in the tank thru the parallel plate interceptor** (<http://offshoreteknikk.files.wordpress.com/2013/10/3-fase.jpg>).

## 1.8 The 16S ribosomal RNA gene as phylogenetic marker

The American scientist Carl Woese, a microbiologist at the University of Illinois, was the pioneer behind the utilization of small subunit ribosomal RNA in order to study the evolution of prokaryotes in the 1970s. The gene encoding the 16S rRNA molecule has four important features making it a useful phylogenetic marker and evolutionary clock; the gene is universal, i.e. it is found in all prokaryotes, it is functionality constant (same function in all organisms), it is highly conserved, and the gene length makes it appropriate to be used in phylogenetic relationship analysis. Still, it's not certain in what rate a DNA sequence change over time, but it's assumed that mutations occurs proportionally to time, and that most mutations are casual and do not affect the gene function (Madigan; et al., 2012, Rajendhran and Gunasekaran, 2011).

The 16S rRNA has nine variable regions, indicated with color in Figure 12, while the rest of the molecule is more invariable. In the archaea the primary structure is somewhat different, but the secondary structure is very similar to the bacterial structure. The 16S rRNA gene constitutes only about 0.1% of the bacterial genome, and most organisms carry two or more copies. In a time where complete genome sequencing is getting more common due to decreasing cost and more efficient methods, analysis and comparison of whole bacterial genomes is being done. Whole genome-based phylogeny might alter the universal phylogenetic tree that we know today, but still 16S rRNA gene sequence analysis is widely used in order to identify and make phylogenetic comparisons of organisms, and it is the most representative phylogenetic marker, so far only shown to be subject to vertical inheritance.

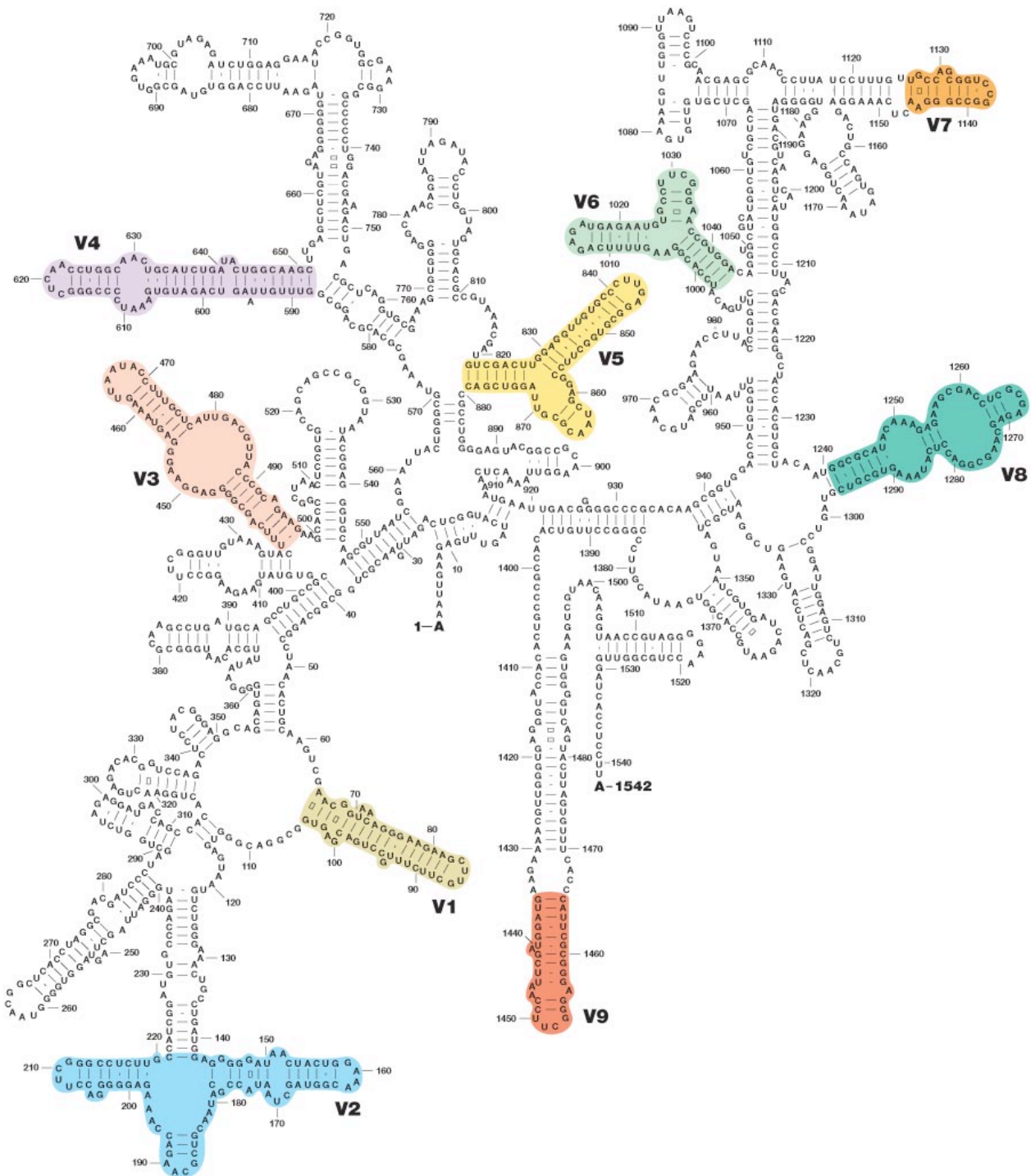


Figure 12 The primary and secondary structure of the 16S rRNA from *E.coli*. The colored regions V1-V9 indicate the regions of variability, and the colourless are conserved regions (Madigan; et al., 2015).

## 1.9 Aims and research questions

Previous analysis of production water from the Troll oil reservoir suggests that the water contains a high amount of anaerobic thermophilic prokaryotes possibly representing novel taxons (Dahle et al., 2008) (Appendix A). Because of the importance of microorganisms for in situ oil biodegradation, souring and increased corrosion it is important to unlock more biological information about these extremophilic prokaryotes. Thermophilic anaerobes also represent a potential for microbially enhanced oil recovery.

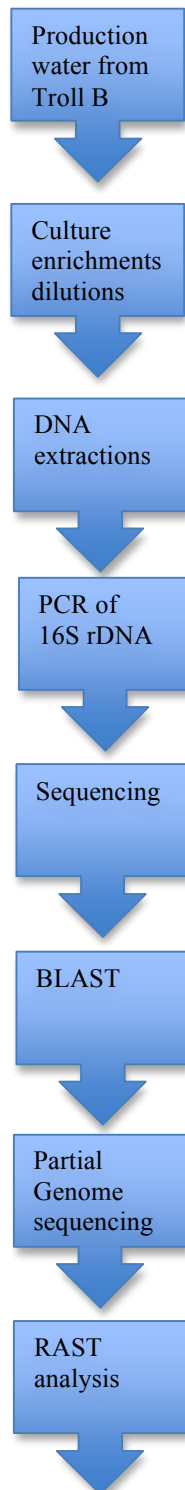
There is also a great economic interest in thermophilic organisms and their thermostable enzymes. There is a huge potential for use of thermostable enzymes in biotechnology and various industries.

One organisms that has a useful feature is *Anaerophaga thermohalophila*, isolated from an oil separation tank has been found to produce surfactants that stabilizes hexadecane/water emulsions, a feature useful in order to enhance oil recovery using microorganisms (Denger et al., 2002).

The main aim is to isolate and characterize additional thermophilic bacterial species and strains known to be present in the production water from the Troll field, such as members of Firmicutes (e.g. *Caminicella* and *Thermovirga* spp.) and Bacterioidetes (e.g. *Anaerophaga* spp.) as well as other culturable microbes. Isolates will be subjected to phenotypic, phylogenetic and molecular characterization. The results will improve our understanding of the microbial and physiological diversity in this extreme environment.

## 2. MATERIALS AND METHODS

The procedure in this study is as shown in Figure 13.



**Figure 13** Flow diagram of the thesis work.

## 2.1 Production-water

Production-water from the Troll B oil field was used for the analysis. One-litre bottles were filled with production-water at the wellhead, then corked with butyl rubber stoppers, and sent to the University of Bergen. The bottles arrived February 15<sup>th</sup> 2013, and upon arrival the bottles were flushed with N<sub>2</sub> and corked, and stored in the dark at 4°C. The formations are situated 1550-1600m below the sea floor and have a temperature of 70°C.

Produced water from the Troll field has previously been shown to contain (per liter): Na (16.4 g), Ca (1.6 g), Mg (560 mg), Ba (160 mg), Fe (14 mg), Sr (300 mg), K (420 mg) and P (<0.1% (w/v)). Sulfate and ammonium were present at concentrations of <2 and 110 mg/l respectively (Dahle et al., 2008). The pressure in the reservoir is between 130-158 bar (Garshol, 2005). There is a physical pressure contact between Troll B and Troll C and thus a reason to believe that the conditions in the two fields are similar.

## 2.2 Medium

**Anaerobic Basal medium** (Widdel et al., 1983)

1 litre distilled water

20 g NaCl

0.9 g MgCl<sub>2</sub> x 6 H<sub>2</sub>O

1.4 g MgSO<sub>4</sub> x 7H<sub>2</sub>O

0.33 g KCl

0.25 g NH<sub>4</sub>Cl

0.14 g CaCl<sub>2</sub> x 2H<sub>2</sub>O

0.45 g KH<sub>2</sub>PO<sub>4</sub>

1.0 ml trace element solution SL-10

0.5 ml resazurin (0.02%)

4 ml 0.5 M Na<sub>2</sub>S

10 ml vitamin solution

6 M HCl for pH adjustment (pH 6.7)



**Trace element solution SL-10** (Widdel et al., 1983)

10 ml 32% HCl

1.5 g FeCl<sub>2</sub> x 4H<sub>2</sub>O diluted in HCl

10 mg ZnCl

100 mg MnCl<sub>2</sub> x 4H<sub>2</sub>O

6 mg H<sub>3</sub>BO<sub>3</sub>

190 mg CoCl<sub>2</sub> x 6H<sub>2</sub>O

2 mg CuCl<sub>2</sub> x 2H<sub>2</sub>O

24 mg NaCl<sub>2</sub> x 6H<sub>2</sub>O

36 mg Na<sub>2</sub>MoO<sub>4</sub> x 2H<sub>2</sub>O

Distilled water adjusted to final volume; 1000ml

**Vitamin solution** (Widdel and Pfenning, 1981)

8 mg 4-amino benzoic acid

2 mg D(+) Biotin

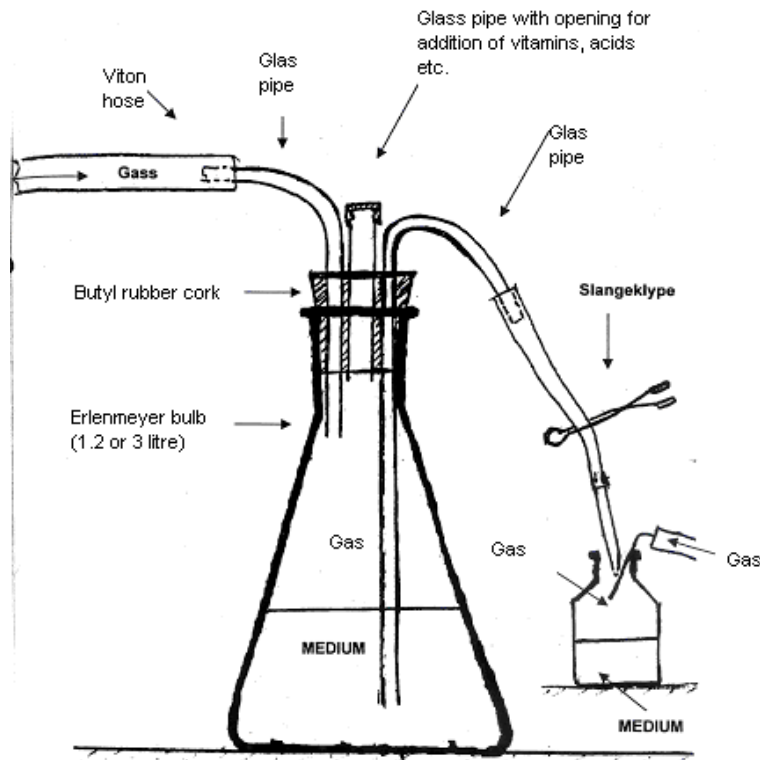
30 mg pyridoxamine hydrochloride

20 mg thiamine dichloride

20 mg nicotinic acid

10 mg Ca-D (+) pantothenate

Distilled water adjusted to final volume; 1000ml



**Figure 14 Hungate technique: preparation of anaerobic medium in an Erlenmeyer flask** (from course booklet MIK200, UiB 2012).

The anaerobic basal medium was prepared in a 2-3 litre Erlenmeyer flask. The flask was corked with a butyl rubber stopper, with three integrated tubes: one tube for draining, another for flushing and the last for adding ingredients and to control pressure within the flask (Figure 14).

The flask was autoclaved, and left to cool while flushing with  $N_2$ . When the medium reached about  $40-50^\circ C$   $Na_2S$  and vitamin solutions were added, and the pH was adjusted to 6.7 with 6M HCl.

The basal medium was drained from the flask by Hungate technique. Created overpressure in the flask due to gassing led the liquid through a silicone tube into 100ml and 50ml serum bottles respectively, that were corked with butyl rubber stoppers and capped while flushing with  $N_2$ , leaving a headspace filled with  $N_2$ .

### **Anoxic water**

Anoxic water was prepared by boiling MiliQ or distilled water for 20 minutes while flushing with  $N_2$ . A silicone tube was used in order to lead the gas down below the water surface and thus making the water anoxic. The anoxic water was stored in serum bottles that were flushed with  $N_2$  and then corked with butyl rubber stoppers.

## Dithionite

Dithionite was prepared with 24 ml of anoxic water mixed with 1.75 grams of dithionite, and then sterile filtered in (0.2  $\mu\text{m}$ ) into a  $\text{N}_2$  flushed serum bottle and corked.

## 2.3 Anaerobic stock solution

Stock solutions were made by using a measurement cylinder with a slip cork, in order to keep it anoxic after flushing with  $\text{N}_2$ . Adequate anoxic water was measured and substrates (Table 3) added to the cylinder and mixed gently. The mixture was poured into a glass beaker that was being flushed with  $\text{N}_2$ , and then taken up in a syringe and sterile filtered with a 0.2  $\mu\text{m}$  filter into flushed serum bottles that were corked and capped.

## Substrates

**Table 3 Substrates used as electron donors in enrichments, and concentration in stock solutions.**

<b>Substrate</b>	<b>Concentration</b>
Alanine	10 mM
Arabinose	10 %
Casamino acid	10 %
Dextrin	10 %
Glucose	10 %
Glycine	10 mM
Maltose	10 %
Peptone	10 %
Serine	10 mM
Threonine	10 mM
Yeast extract	10 %

## 2.4 Enrichments

For the enrichments 100 ml serum bottles were used, containing approximately 50ml of basal medium with a headspace filled with N<sub>2</sub>.

Substrates were added to the enrichments as listed in Table 4, and inoculated with 5 ml of production water.

The serum bottles were incubated at 55°C and 70°C respectively until turbidity developed, varying from a couple of days up to a week or two.

During incubation the enrichments were monitored by microscopy in order to distinguish bacterial growth from precipitation.

The enrichments were then transferred to a new bottle with medium and substrate by inoculating 1 ml of the original enrichment to the new, and were then incubated until visible bacterial growth occurred.

The 2<sup>nd</sup> enrichments were then diluted in dilution series. Seven or eight 10 ml serum bottles were prepared, inoculating 1 ml of the original enrichment into 9 ml of basal medium (1:10). The highest dilution with growth was diluted once more, and the highest dilution with growth was inoculated to a 100 ml bottle with 50 ml of medium, incubated and then utilized for 16S rRNA gene sequencing and scanning electron microscopy.

**Table 4 Cultures with respective substrate combinations**

<b>Culture</b>	<b>Substrate combination</b>
1	Casamino acids
2	Dextrine
3	Glucose + yeast extract + peptone
4	Glucose + yeast extract
5	Arabinose
6	Maltose
7	Threonine
8	Alanine
9	Alanine + glycine
10	Serine

## 2.5 DNA extraction

### GenElute™ Bacterial Genomic DNA Kit

For 16S rRNA PCR DNA was extracted with the GenElute™ Bacterial Genomic DNA Kit.

Falcon tubes (15 ml) were filled with culture, and then spun down in a centrifuge for 10-15 minutes at 7,000 x g (Eppendorf 5430R) in order to harvest cells.

The pellet was then stored in the freezer overnight. In order to extract the DNA from the cells the GenElute™ Bacterial Genomic DNA Kit was used.

The pellet was resuspended in 180 µl of Lysis Solution T. Then 20 µl of Proteinase K were added, mixed and incubated for 30 min at 55°C.

Further, 200 µl of Lysis Solution C were added, vortexed and incubated for 10 min at 55°C.

The column was prepared by adding 500 µl Column Preparation Solution to the pre-assembled GenElute Miniprep Binding Column that was placed in a 2 ml collection tube and then centrifuged at 12,000 x g for 1 min, and the eluate was discarded.

The lysate was then added 200 µl of ethanol (95-100%) and vortexed until the solution was homogenous. The lysate was then transferred to the collection tube with the binding column, and then centrifuged for 1 min at >6,500 x g. The eluate was discarded and the binding column was placed in a new collection tube.

Further, 500 µl of Wash Solution 1 were added to the column, centrifuged for 1 min at >6,500 g. The eluate and the collection tube were discarded, and the column was placed in a new collection tube.

In the second wash 500 µl of Wash Solution 1 w/ethanol was added and centrifuged for 3 min at 12,000-16,000 x g. The eluate was discarded. If necessary a second spin was performed in order to dry the column completely. The column was placed in a new 2 ml collection tube, and 80-100 µl of Elution Solution was added, and incubated for as long as 20-30 min at room temperature.

Then the tube was centrifuged for 1 min at >6,500 x g, the column was discarded, and the collection tube with the eluate was kept in the freezer at -20°C until further use.

### **DNA isolation; the CTAB method (Gannon et al., 1988)**

For genome sequencing the CTAB method was used, due to the high yield of DNA.

About 50 ml culture were placed in a falcon tube and centrifuged in order to harvest cells, and these were resuspended in 250 µl of lysozyme solution.

Then 247 µl of 0.5% SDS, and 3 µl proteinase K (20mg/ml) were added, vortexed and transferred to an 1.5 ml eppendorf tube.

The eppendorf tube was incubated at 55°C for 30 min in a water bath, and the tubes were inverted after approximately 15 minutes.

Afterwards 80 µl 5 M NaCl and 100 µl preheated (55°C) CTAB were added, mixed well and incubated for 10 min at 65°C on the heating block.

After incubation, 500 µl Chloroform : isoamylalcohol (24:1) was added and mixed, before centrifuged for 5 min at 12, 000 x g. The top phase including the DNA was then transferred to a fresh tube, and 360 µl isopropanol were added. The tube was inverted a few times and left in room temperature for one hour to let the DNA precipitate.

The tube was then centrifuged for 15 min at 12, 000 x g, and the supernatant was discarded.

The pellet was washed with 200 µl of ice-cold 70% ethanol, and then centrifuged for 8 min at 12, 000 x g. The supernatant was discarded and the pellet left to dry on a heater at 40°C, before it was resuspended in 15 µl TE buffer.

Lysozyme solution: 1 mg lysozyme/ ml TE buffer

Lysis buffer: 0.5% SDS, 20µg/ml proteinase K

CTAB: 10% (w/v) hexadecyltrimethylammoniumbromide in 0.7% NaCl

TE buffer: 10 mM Tris, 1 mM EDTA, pH 7.4

## 2.6 Polymerase Chain Reaction (PCR)

In order to amplify the DNA from the isolates, PCR was set up according to Table 6, and run with the primers listed in Table 5.

**Table 5 Universal 16S primers used in the PCR.**

Primer	Sequence 5'-3'	Supplier
27F	AGAGTTTGATCCTGGCTCAG	Sigma-Aldrich
1492R	GAAAGGAGGTGATCCAGCC	Sigma-Aldrich

**Table 6 Polymerase chain reaction setup.**

Component	25µl reaction/50µl reaction	Supplier
5x OneTaq® Standard Reaction Buffer	5µl/10µl	NEW ENGLAND BioLabs®
10 mM dNTP's	0.5 µl/1 µl	ABgene
10 µM Forward Primer	0.5 µl/1 µl	Sigma-Aldrich
10 µM Reverse Primer	0.5 µl/1 µl	Sigma-Aldrich
OneTaq® DNA Polymerase	0.125 µl/0.25 µl	NEW ENGLAND BioLabs®
Template DNA	2-3 µl*	
MilliQ water**	Up to 25 µl/50 µl	
2% BSA***	2 µl/4 µl	NEW ENGLAND BioLabs®

\*concentration <1, 000 ng

\*\*MilliQ water was filtered with a 0.2 µm filter.

\*\*\*Bovine Serum Albumin

PCR tubes were placed in a BIORAD DNA Engine® Peltier Thermal Cycler, with the programme in Table 7.

**Table 7 PCR program.**

<b>Step</b>	<b>Temperature</b>	<b>Time</b>
Initial Denaturation	96°C	3 min
30 cycles	96°C	30 sec
	55°C	30 sec
	68°C	45 sec
Final Extension	68°C	5 min
Cooling	4°C	∞

The DNA concentration of the PCR products were measured in a Qubit® fluorometer (Invitrogen™), and analysed on an agarose gel in order to control the success of the amplification and the expected size of the PCR product. A 1kb + DNA Ladder (New England BioLabs®) was used to determine the size of the PCR product.

#### **Agarose gel, 1 %**

40ml 1 x TAE buffer

0.4 grams 1% SeaKem® LE Agarose Lonza

1 µl GelRed™ Nucleic Acid Stain 10 000 x in water (Biotium).

#### **50 x TAE buffer**

242 grams Tris base

57 ml glacial acetic acid

100 ml 0.5 M EDTA (ethylenediamine tetraacetic acid, pH 8.0)

H<sub>2</sub>O until final volume 1000 ml

Buffer and agarose were gently mixed in a flask and then heated in the microwave until the agarose was completely melted. The flask was left to cool a little, before GelRed™ was added. Then the solution was poured into the agarose tray with a comb in order to form the wells, and left to polymerize for 20-30 min.



## 2.7 Electrophoresis

The agarose gel was placed in the electrophoresis tray, and covered with 1 x TAE buffer. Then PCR products mixed with 6 x Nucleic Acid Sample Loading Buffer were pipetted into respective wells, always a 1 kb + ladder (Invitrogen) in the first well in order to determine the size of the PCR product. The electrophoresis was run for approximately 45 minutes, at 70 volt, 50mA, and 3.5 W in an Invitrogen life technologies Power Ease 500. This separates the DNA fragments according to size, with smaller molecules migrating fastest. Then the gel was placed in a fluorescent gel chamber (Molecular Imager® ChemiDoc™xRST), utilizing an Image lab™ software (BioRad) for visualization of the DNA bands.

### 6 x Loading buffer

0.05 g Bromphenol blue

0.05 g xylene cyanol FF

8 g sucrose

For 20 ml m/1 x TAE

Sterile filtered

## 2.8 GenElute™ PCR Clean-Up Kit

In order to clean the PCR product, GenElute™ PCR Clean-Up Kit was used.

A GenElute plasmid mini spin column was placed in a collection tube, 0.5 ml of Column Preparation Solution was added. Then the tube was centrifuged for 30 seconds to 1 min at 12, 000 x g, and the eluate was discarded.

Then 5 volumes of Binding Solution were added to 1 volume of the PCR reaction, mixed and placed in the binding column. The column was centrifuged at 12, 000 to 16, 000 x g for 1 min, and the eluate was discarded.

Thereafter 0.5 ml of Wash Solution diluted with ethanol was added to the column, and the column was centrifuged for 1 min at 12, 000 to 16, 000 x g. The eluate was discarded and the column was centrifuged for another 2 min to remove all the ethanol. The column was then placed in a new 2 ml collection tube, and 50 µl of Elution Solution was added to the column, and incubated at room temperature for 1 min.

The column was then centrifuged for 1 minute at 12, 000 to 16, 000 x g, and the cleaned PCR product in the eluate was stored at -20°C until further use.

## Sequencing

BIG Dye reaction Table 8 was performed in order to amplify the 16S rRNA genes before sequencing, using the programme in Table 9.

**Table 8 BIG Dye reaction.**

<b>Component</b>	<b>10 µl reaction</b>	<b>Supplier</b>
BIG Dye	1 µl	
Sequencing Buffer	1 µl	
Primer (3.2 pmol)	1 µl	Sigma-Aldrich
Template	10-40 ng	
MilliQ water	Up to 10 µl	

**Table 9 BIG Dye programme.**

<b>Step</b>	<b>Temperature</b>	<b>Time</b>
Initial Denaturation	96°C	3 min
25 cycles	96°C	10 sec
	50°C	5 sec
	60°C	4 min
Hold	4°C	∞

After the BIG Dye reaction, the reaction was added 10 µl Nuclease-free water, and then analysed at the High technology centre in Bergen. The sequencing facility is performing Automated Sanger DNA Sequencing using a capillary-based Applied Biosystem 3730XL Analyzer

(<http://www.uib.no/en/seqlab/artikler/2011/09/service-overview>).

## **2.9 Basic Sequence Alignment Search Tool (BLAST)**

Sequences were Blasted at <http://blast.ncbi.nlm.nih.gov>, and tools at:

<http://emboss.bioinformatics.nl> was utilized to reverse the reverse sequences and merge them with the forward sequences. 4Peaks, a software program, was used in order to visualize the DNA sequences.

## **2.10 Scanning Electron Microscope (SEM)**

About 1 ml of culture was filtered through a 0,2 µm polycarbonate membrane filter which was subsequently fixed in 6% glutaraldehyde mixed with 1% OsO<sub>4</sub> (1:1) in cacodylate buffer (pH 7.4) for two hours and then washed with the same buffer for 10x5 min.

Then the filter was washed with dsH<sub>2</sub>O 5 x 5 min.

Afterwards, dehydration was performed using graded ethanol series. The sample was incubated with 50%, 75%, 96% and then twice in 100% ethanol for 15 min each.

Thereafter critical point drying was done in a Polaron's Critical Point Drying Apparatus utilizing liquid CO<sub>2</sub>.

After critical point drying was performed, the filters were placed on aluminium stubs, and then placed in a Sputter coater, where the filters were coated with gold before visualized in a ZEISS Supra Scanning Electron Microscope.

## **2.11 Genome sequencing**

A DNA sample of strain Troll extracted with the CTAB method was sent to GATC Biotech (<http://www.gatc-biotech.com/en/home.html>) in Germany for genome sequencing using the paired-end Illumina HiSeq sequencing. The raw sequence data were assembled *de novo* by GATC.

## **2.12 Rapid Annotation using Subsystem Technology (RAST)**

The strain Troll fasta file containing 90 contigs was uploaded to RAST (<http://rast.nmpdr.org>) in order to annotate genes and analyse metabolic pathways.

## **2.13 Phylogenetic trees**

Alignments and phylogenetic trees were constructed using Clustal X (Thompson et al., 1997).

Phylogenetic trees were visualized in TreeView (Page, 1996).

## **2.14 Mauve**

Mauve was used in order to order the contigs of strain Troll according to reference genome sequence (Darling et al., 2004).

### 3. RESULTS

#### 3.1 Enrichments

The 16S rRNA gene library previously constructed from Troll field production water (Dahle et al., 2008) included many sequences with closest match with organisms like *Thermovirga lienii*, *Caminicella sporogenes*, both belonging to the Firmicutes phylum, and *Anaerophaga thermohalophila*, belonging to the Bacteroidetes phylum. The sequence identities were however in the range from below 90 to almost 100%. This indicates a great potential for discovery of novel genera and species, possibly with novel biological properties. In this study, enrichment media with different combinations of substrates were prepared, and inoculated with production water from the Troll field. Basal medium supplemented with 5% NaCl was prepared in addition to the standard 2% NaCl basal medium in order to enrich for halophilic organisms such as *Anaerophaga thermohalophila* and *Caminicella sporogenes*. Glucose with peptone and yeast extract is a rich medium that will typically enrich for members of the *Thermotogales*. Arabinose and maltose were used in order to enrich for *Kosmotoga olearia*. Cultures with alanine and glycine were made in order to enrich for organisms with Stickland fermentation. *Thermovirga lienii* use proteinaceous substrates, so enrichments with casamino acids, threonine, alanine and serine were prepared.

Parallel cultures with substrates as indicated in Table 10 were incubated at 55°C and 70°C. No growth was detected at 70°C, which is close to *in situ* temperature. Eight out of ten cultures incubated at 55°C had visible growth within a week, and were transferred to fresh media. The serine culture failed to grow after transfer. For the other seven enrichments, organisms were successfully isolated by dilution to extinction. The reason for the failure to enrich any organisms at the *in situ* temperature can be that the organisms need high pressure to grow at this temperature. Another reason could be that these organisms are not indigenous to the oil well but originate from layers above with lower temperatures.

Microscopic examinations of the isolates revealed the presence of rod-shaped cells (isolate 1) or Thermotogales-like cell morphology (isolate 2 – 7).

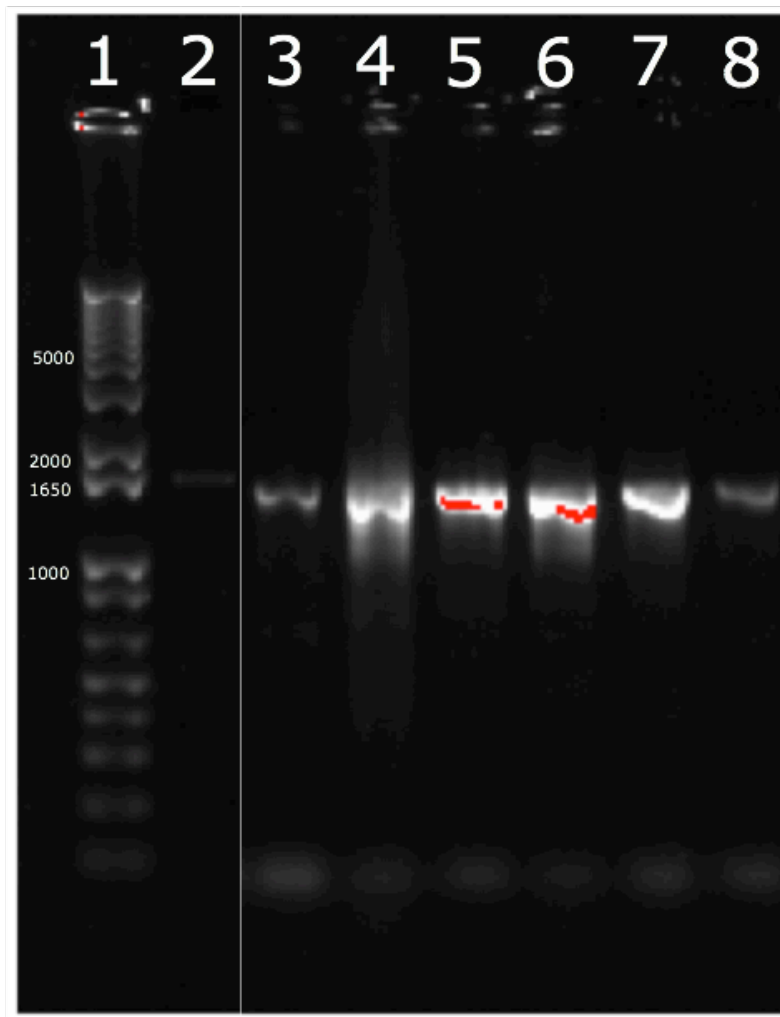
**Table 10 Overview of the enrichments/isolates and their phylogenetic affiliations.**

Isolate	Substrates	Growth	Closest match *	Identity
1	Casaminoacids	+	<i>Thermovirga lienii</i> (NC 016148.1)	99,6%
2	Dextrine	+	<i>Petrotoga mobilis</i> (NC 010003.1)	99,9%
3	Glucose, yeast extract, peptone	+	<i>Thermosipho africanus</i> (NC 011653.1)	99,6%
4	Glucose, yeast extract, with 5% NaCl	+	<i>Petrotoga mexicana</i> (NR 029058.1)	99,3%
5	Arabinose	+	<i>Petrotoga mobilis</i> (NC 010003.1)	99,3%
6	Maltose	+	<i>Petrotoga mobilis</i> (NC 010003.1)	100%
7	Threonine	+	<i>Petrotoga mobilis</i> (NC 010003.1)	99,9%
8	Alanine	-		
9	Alanine,Glycine	-		
10	Serine	+ <sup>d</sup>		

\*Accession number.

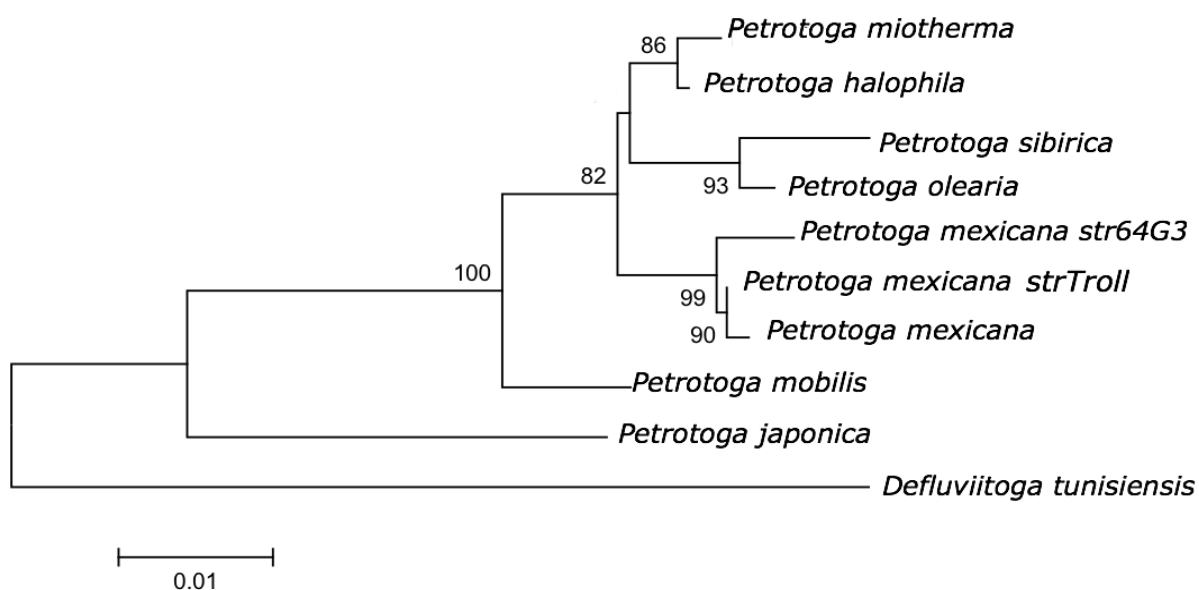
<sup>d</sup> limited growth in the first culture, and did not grow after transferred to the second enrichment

For phylogenetic analysis of the isolates, DNA was extracted and used as template for duplicate 16S rRNA in PCR reactions using universal bacterial 16S rRNA gene primers supposed to yield 1465 bases DNA products. The PCR products were analysed by agarose gel electrophoresis (Figure 15).



**Figure 15** Agarosegel with 16S rDNA PCR products; lane 1:1kb + DNA ladder, lane 2:isolate 1, lane 3:isolate 2, lane 4:isolate 3, lane 5:isolate 4, lane 6:isolate 5, lane 7:isolate 6, lane 8:isolate 7.

The PCR products were sequenced in both directions and the resulting sequences were merged. Blast searches identified four different species isolated from the seven enrichments; *Thermovirga lienii* from the *Synergistetes* phylum, and *Thermosipho africanus*, *Petrotoga mobilis* and *Petrotoga mexicana* from the *Thermotogae* phylum. *T. lienii*, *T. africanus* and *P. mobilis* have been isolated from North Sea oil fields before, and had a similarity of >99% with previous isolates. Due to this high similarity with previously well-described strains it was decided not to continue with further characterization of these isolates. *P. mexicana*, however, has previously only been recovered from an oil reservoir in the Gulf of Mexico (Miranda-Tello et al., 2004). The *P. mexicana* type strain and our isolate share 99.3% 16S rRNA gene sequence identity and thus probably belongs to the same species. Since this species has never been isolated from the North Sea, it was decided to carry out a draft genome sequence analysis of this strain, which was designated *Petrotoga mexicana* strain Troll. A phylogenetic tree based on the 16S rRNA gene sequence placed strain Troll within a *P. mexicana* clade together with two isolates from the Mexican Gulf (Figure 16).



**Figure 16** The *Strain Troll* (this study) placed in a phylogenetic tree together with other *Petrotoga* members, and rooted with *D. tunisiensis*. Accession numbers are as follows: *Petrotoga miotherma* FR733705, *Petrotoga halophila* NR\_043201, *Petrotoga sibirica* NR\_025466, *Petrotoga olearia* NR\_028947, *Petrotoga mexicana* str64G3 JQ417660, *Petrotoga mexicana* NR\_029058, *Petrotoga mobilis* NR\_074401, *Petrotoga japonica* GQ385331 and *Defluviitoga tunisiensis* DQ641495. Bootstrap values from 1000 replications are shown at nodes. Only values above 80 are indicated. Bar indicates 1 substitution per 100 nucleotides.

### 3.2 Scanning Electron Microscopy (SEM)

Since *Strain Troll* was the only new species identified from the North Sea, this was the only species that was analysed by scanning electron microscopy in order to reveal the morphology. The images from SEM showed long and slender cells measuring more than 60  $\mu\text{m}$  in length (Figure 17), twice the size of the *P. mexicana* type strain (Miranda-Tello et al., 2004).

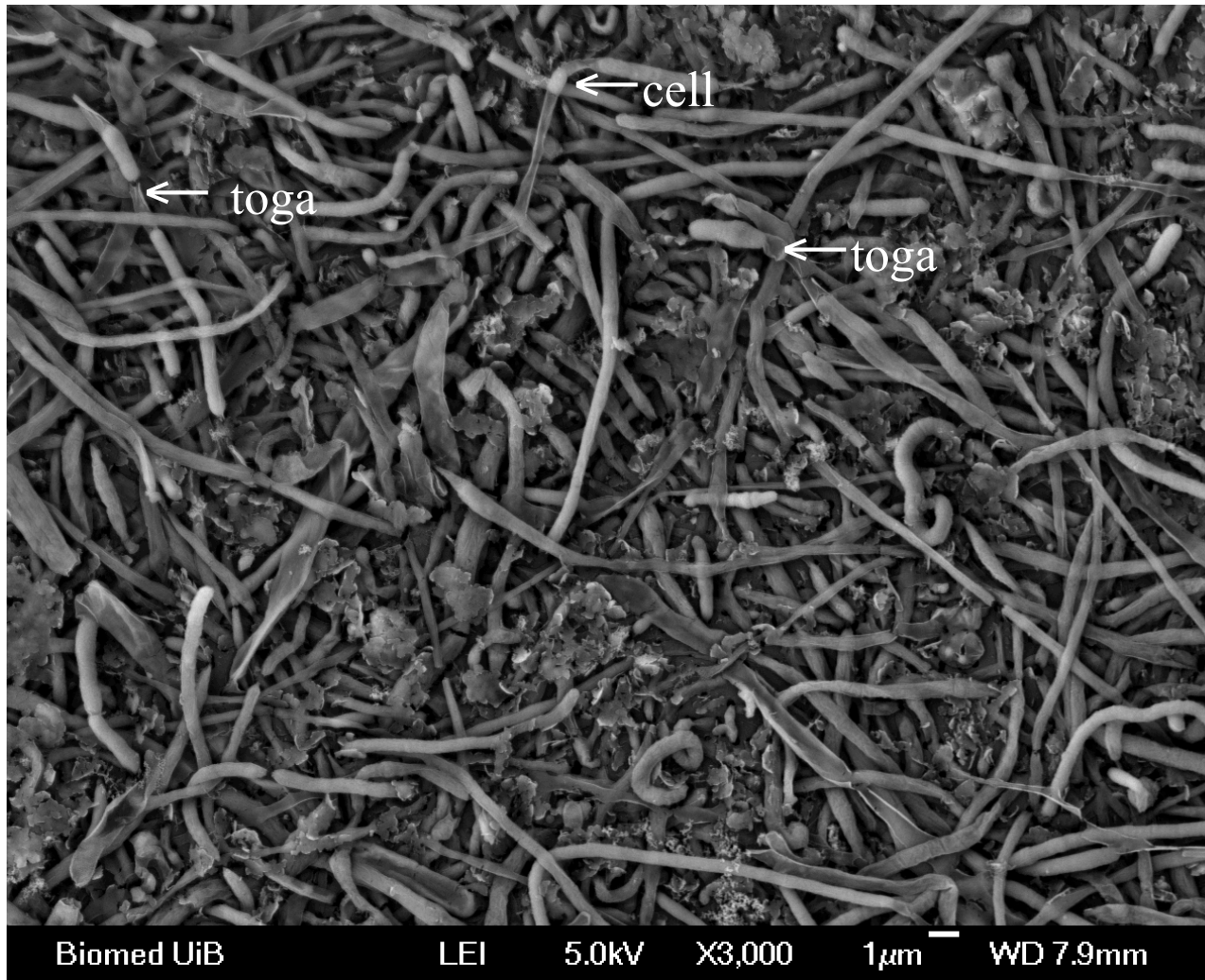


Figure 17 Scanning Electron Microscopy of strain Troll, tags indicate toga and the cell within the toga.

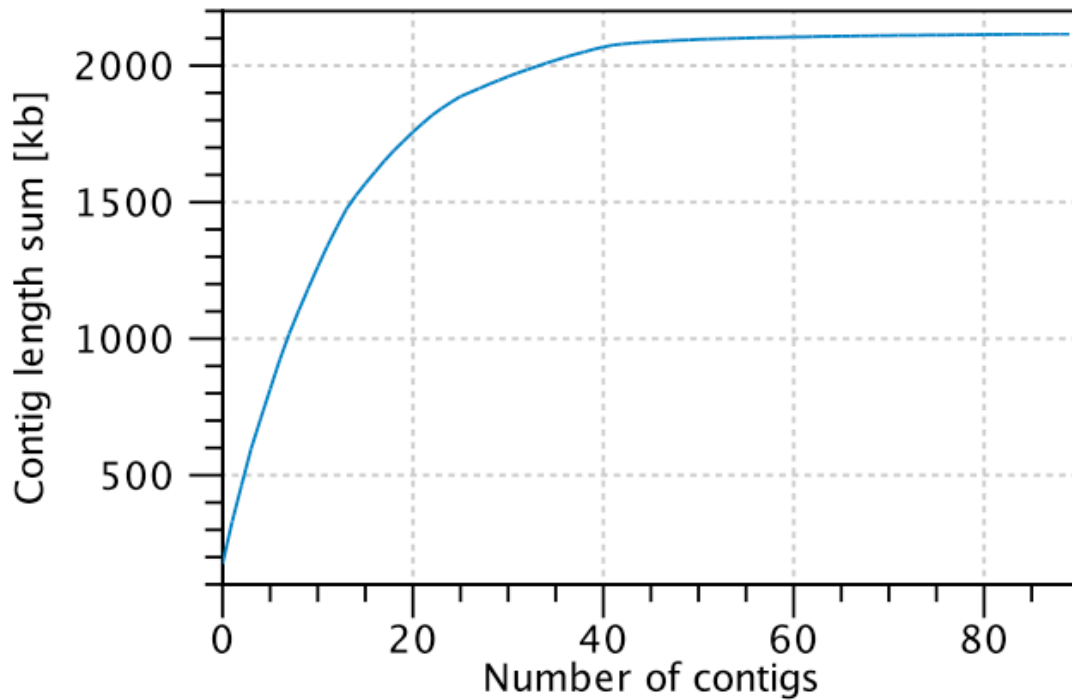
### 3.3 Genome analysis statistics

From the Illumina HiSeq genome sequence analysis, 2,399,998 sequence reads were obtained, with a total of 242,399,798 sequenced bases. The average read length was 101 bases. Following assembly, 90 contigs with an average length of 23,512 bases were obtained. The total number of bases assembled into contigs is 2,116,100.

The nucleotide analysis indicated a GC content of 33.9%. This is quite different from the type strain which was determined to possess 36.1% GC (Miranda-Tello et al., 2004).

The genome sequences were distributed over 90 contigs, which accumulated up to about 2.1 Mb (Figure 18) indicating a total genome size of less than 2.2 Mb.

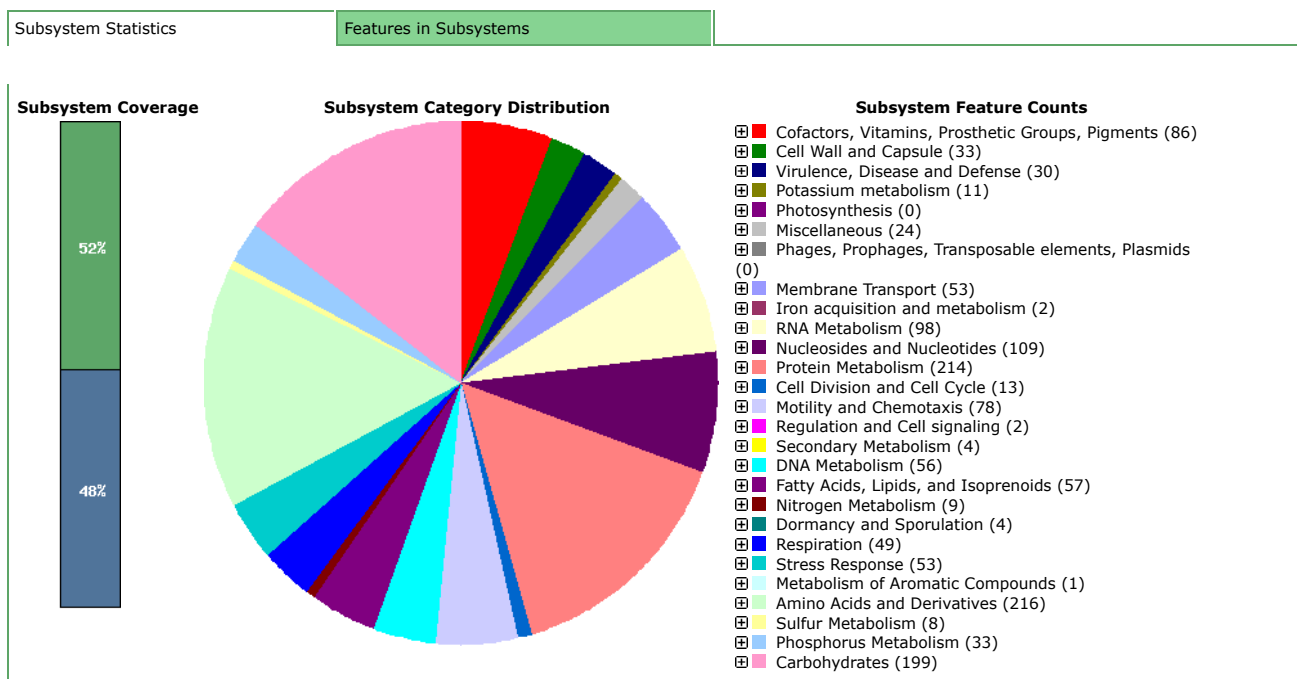




**Figure 18** Accumulated contig length sum as a function of number of contigs.

### 3.4 Subsystems statistics

The fasta file of the complete genome draft sequence of strain Troll was uploaded to the Rapid Annotation using Subsystem Technology (RAST) server. Following automatic annotation, the subsystem information showed that 52% of the 2013 coding sequences are placed in a RAST-defined subsystem (Figure 19). A large number of membrane transport features (254) were identified, including 125 ABC type features. Eleven antiport features were identified. Surprisingly, although this organism is supposed to be a fermentative bacterium, a large number (49) of respiratory features were also identified, including a respiratory complex I and a Na<sup>+</sup>-translocating NADH-quinone oxidoreductase. A total of 454 genes were listed as hypothetical, with no similarity to known gene functions.



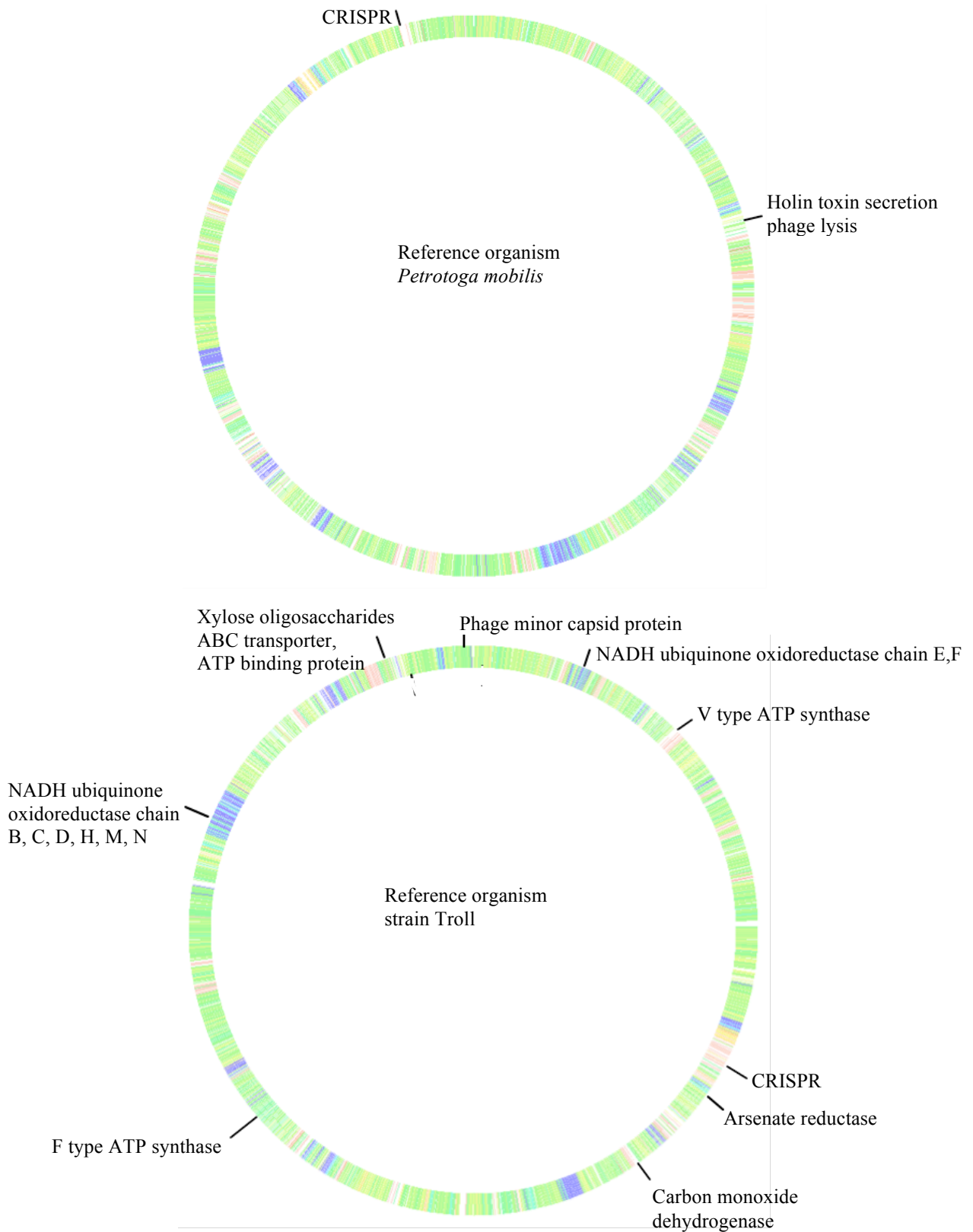
**Figure 19 From RAST, Strain Troll subsystem statistics**  
 (<http://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=204046.3>).

### 3.5 Genome comparison

The genome was compared in RAST with other available genomes. As the *Petrotoga mexicana* type strain has not yet been genome sequenced the most closely related genome was that of *P. mobilis*. *P. mobilis* was therefore used as main reference organism.

Using the Genome-to-Genome Distance Calculator at <http://ggdc.dsmz.de/distcalc2.php> (Meier-Kolthoff et al., 2013) 48.7 % overall genome sequence homology between strain Troll and *P. mobilis* was determined. This shows that strain Troll definitely is not a *P. mobilis*, but belongs to the *Petrotoga* genus. DNA:DNA hybridization threshold values between species and genera is in general accepted to be 70% and 25%, respectively.

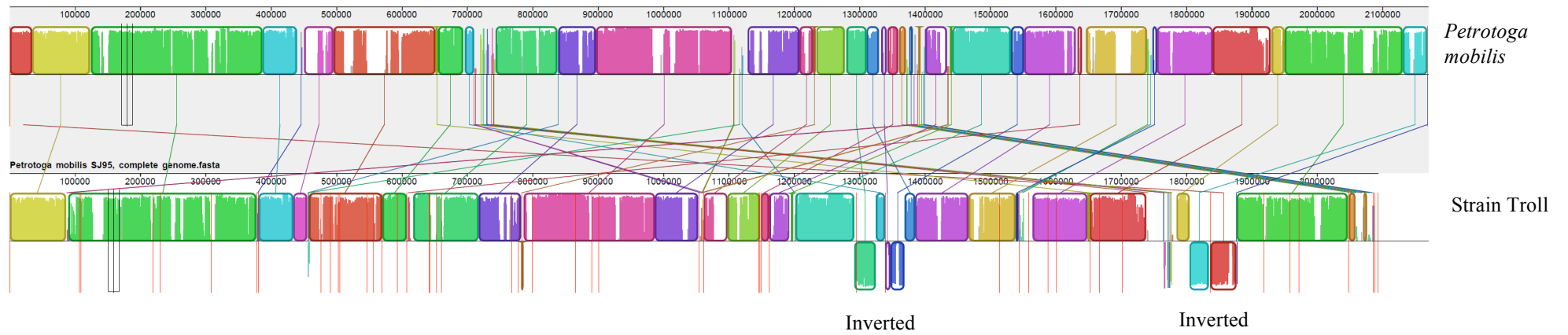
	Percent protein sequence identity															
Bidirectional best hit	100	99.9	99.8	99.5	99	98	95	90	80	70	60	50	40	30	20	10
Unidirectional best hit	100	99.9	99.8	99.5	99	98	95	90	80	70	60	50	40	30	20	10



**Figure 20** Upper genome: reference organism *Petrotoga mobilis*, lower genome: reference organism strain Troll. Colors indicate degree of similarity between the genomes, and some of the scrutinized genes are shown.

When making genome sequence comparisons in RAST, one genome is set as comparison organism and the other as reference, and vice versa, resulting in a color coded genome circle as seen in Figure 20, where *P. mobilis* is used as a reference in the upper part, and strain Troll as a reference in the lower part. Colors indicate the degree of protein sequence identity in percent, and white gaps indicate missing gene or regions of no homology. The first impression is that the genome circles are quite green, indicating high similarity; still there are several gaps, and the pink color indicates regions of low similarity. Several genes and gene clusters were identified in strain Troll that are missing in *P. mobilis*, e.g. genes encoding Carbon monoxide dehydrogenase, a V-type ATP synthase and a complete set of xylan degradation genes. Strain Troll lacks a complete CRISPR gene set.

The Mauve program was used to arrange the 90 strain Troll contigs according to the gene order and synteny of the *Petrogoga mobilis* genome, thus using the latter as a template. As can be seen from Figure 21 this resulted in a reasonably well syntentic arrangement of a large part of the strain Troll sequence blocks except for two smaller regions with inversions. This analysis confirms that our genome sequence covered most of the strain Troll genome and left only small regions uncovered.

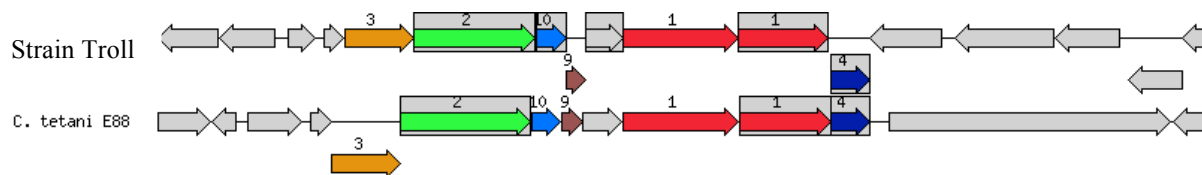


**Figure 21** The *Petrotoga mobilis* genome used as template for ordering of the strain Troll contigs. Colored lines drawn between the genomes indicate the location of the homologous sequence blocks. Some inverted sequence blocks are also indicated. The red lines indicate the division of the contigs in strTroll.

### 3.6 Genes related to electron transport phosphorylation (ETP)

The Troll strain has 49 genes encoding respiratory features (Figure 19), and 17 out of these genes are related to the two types of ATP synthases: V-type ATP synthase and F0F1-type ATP synthase, both which are members of a superfamily of H<sup>+</sup> translocation ATPases. In contrast *P. mobilis*, which is the closest related organism available for comparison, only possesses the F0F1-type genes. F0F1 is responsible of ETP-driven ATP synthesis in the cell, located in the plasma membrane of bacteria. The hydrophobic part: F0 consists of >3 transmembrane subunits that form a proton channel. The F1 has two kinds of nucleotide binding subunits (alpha and beta) and three minor subunits (gamma, delta and epsilon). The gamma subunit forms like a shaft that penetrates the hexagonal alpha beta, and rotates when F1 hydrolyses ATP.

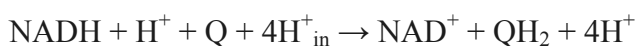
The vacuolar V-type has two subunits: Vo and V1 that are homologue to the F0 and F1. But in contrast to the F0F1, the V-type extrudes protons using ATP hydrolysis as energy. Looking at the V-type in the subsystem the ATP synthase subunit A has the closest operon homologue in the gram-positive *Clostridium tetani*, Figure 22. *C. tetani* is an obligate anaerobe, found in the intestine of animals and in soil and sewage (Madigan; et al., 2012). It uses V-type ATPase to maintain an energized cell membrane.



**Figure 22** The operon region where the V-type ATP synthase subunits are located. Arrow no. 3=subunit C, no.2=subunit I, no. 0=subunit K, 9=subunit F, 1=subunit A, 1(shaded)=subunit B and 4=subunit D.

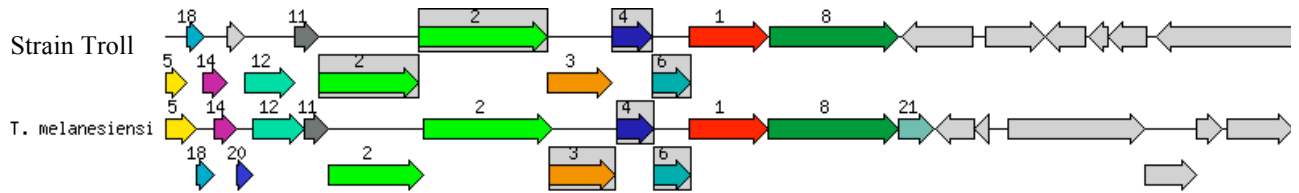
*Marinotoga piezophila* and *Thermotoga neapolitana* which also are members of *Thermotogales* possess a V-type ATP synthase.

In the strain Troll genome there are eight genes coding for NADH ubiquinone oxidoreductase forming part of the respiratory Complex I. In the Complex I the following reaction is carried out:



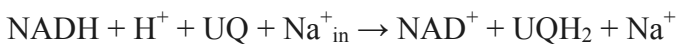
The NADH binds to the Complex I and is oxidized; this gives 2 electrons and 4 H<sup>+</sup>. The electrons are brought to ubiquinone (Q) by the flavin mononucleotide (FMN) and this alters the redox state in the protein, and causing 4 H<sup>+</sup> to be pumped out. The electrons are brought into the Quinone pool by QH<sub>2</sub>, and transferred to the Cytochrome b in the Complex 3. Strain Troll does not have the Cytochrome b homolog and if the Respiratory Complex I is active it is not clear how electrons are further transported.

The organism with the most similar operon with the NADH ubiquinone oxidoreductase genes is the *Thermosipho melanesiensis*, Figure 23. The *Petrotoga mobilis* also has NADH ubiquinone oxidoreductase but these are not part of a subsystem.

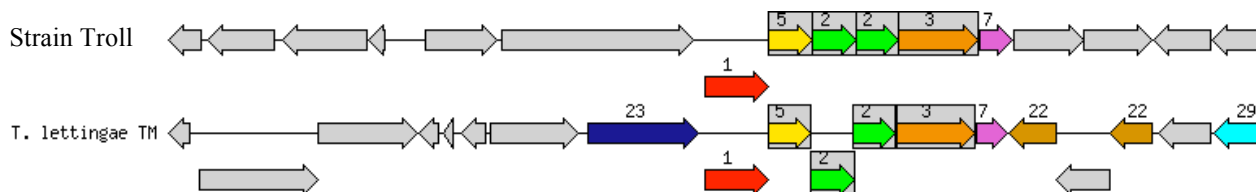


**Figure 23** The operon where the NADH-ubiquinone oxidoreductase chain is located, the organisms with the most similar operon is the *Thermosipho melanesiensis*. From the left: the two green arrows (2) are the NADH-ubiquinone oxidoreductase chain M and N respectively, the brown (3) chain H, the blue (4) chain B, light blue (6) chain C, red (1) chain D.

Both in the *P. mobilis* and the strain Troll there are five genes coding for Na<sup>+</sup> translocating NADH-quinone reductase; subunit B, C, D, E and F, Figure 24. The system carries out the following reaction:

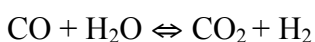


NADH is oxidized, and electrons are passed on to the ubiquinone, and the system pumps out Na<sup>+</sup> in order to create sodium motive force.

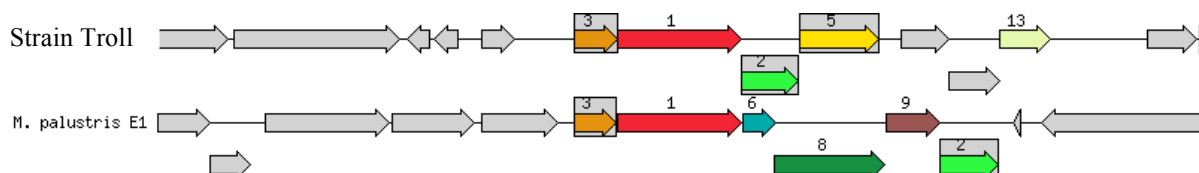


**Figure 24** The Na<sup>+</sup> translocating NADH-quinone reductase subunits are clustered together on the operon. Arrows 1, 5, 2, 2 and 3 are subunits B, C, D, E and F respectively. The organism with the most similar operon is *Thermotoga lettingae*.

Other interesting features found in strain Troll are the genes for the Carbon monoxide dehydrogenase CooS subunit, the CODH iron-sulfur protein CooF and CODH accessory protein CooC (nickel insertion). The carbon monoxide dehydrogenase enzyme catalyzes the following reversible reaction:

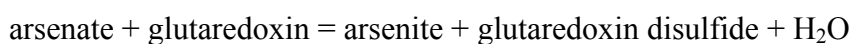


Where AH2 is an electron donor. The genes are not found in the *P. mobilis* genome, but they are typical for methanogens, homoacetogens and some sulfate reducers where CODH is a key enzyme in formation of acetyl-CoA from H<sub>2</sub> and CO<sub>2</sub>. The closest similar operon is found in the archaea; *Methanosphaerula palustris*, Figure 25. *Methanosphaerula palustris* is found in minerotrophic fen peats, and it is a mesophilic methanogen (Cadillo-Quiroz et al., 2009). Searching for the gene with BLAST shows that the strain Troll is the only member of *Thermotogales* with these genes. The closest gene match has 75% similarity and found in the sulfate reducer *Desulfobacula toluolica*, an organism found in the anoxic water near upwelling areas, and in marine sediments, which degrade aromatic compounds such as toluene found in petroleum (Raber et al., 1998, Wohlbrand et al., 2013). Other organisms with a similar gene arrangement are found both in bacteria and archaea.



**Figure 25** The operon region where the Carbon monoxide dehydrogenase genes are located, arrow 3: CO dehydrogenase iron-sulfur protein CooF, arrow 1: CO dehydrogenase CooS subunit, arrow 2: CO dehydrogenase accessory protein CooC (nickel insertion). Closest similar operon is found in *Methanosphaerula palustris* E1-9c.

Arsenate reductase is found in both *P. mobilis* and strain Troll. The Arsenate reductase catalyses the following reaction:



This reaction is an arsenate detoxification mechanism. Arsenate reductase can also be involved in respiration, by reducing arsenate to arsenite through receiving electrons from a respiratory electron transport chain.

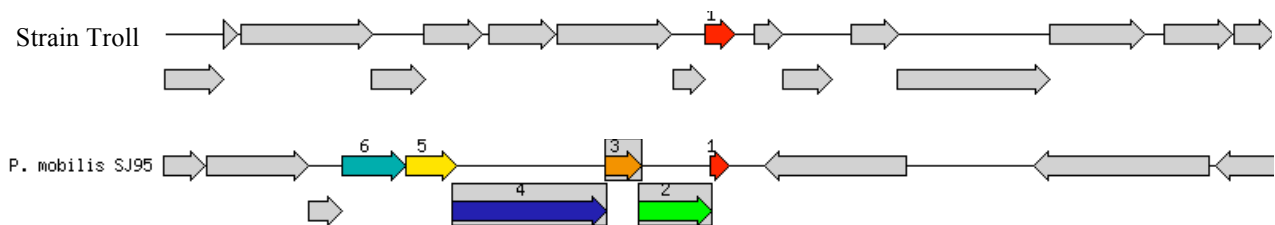
The operon with the highest similarity is found in the thermophile bacteria *Meiothermus silvanus*, *Meiothermus silvanus* is found in hot springs/hydrothermal vents and forms red-orange pigmented colonies (Tenreiro et al., 1995).

There are also two genes coding for Arsenic efflux pump protein, which pumps arsenate compounds out of the cell.



### 3.7 CRISPR genes and phage

CRISPR is short for Clustered Regularly Interspaced Short Palindromic Repeats and is primarily a virus defence system found in prokaryotes. Strain Troll has only one CRISPR repeat RNA endonuclease (Cas6) that is not active. The *P. mobilis* on the other hand has 8 CRISPR associated genes, Figure 26. CRISPRs' function as an immune system in prokaryotes, able to cut out foreign DNA from plasmids or phage, and due to this useful skill of cutting DNA, CRISPRs has been studied extensively the recent years. CRISPRs can potentially be harnessed in order to cure inherited diseases caused by single cell mutations such as cystic fibrosis and sickle cell anaemia by cutting out the mutated genes (Hwang et al., 2013). Another possibility is preventing horizontal gene transfer between prokaryotes, which is important in preventing the increasing antibiotic-resistance in pathogenic bacteria through lateral gene transfer (Marraffini and Sontheimer, 2008).



**Figure 26** The operon where the CRISPR gene is located. In *P. mobilis* there are 8 CRISPR related genes, and 6 of them are clustered together at the same operon.

Both strain Troll and *P. mobilis* have genes associated to phages but do not seem to possess complete prophages. Prophage ps3 protein 03 and Phage minor capsid protein-DNA pilot protein are present in strain Troll while in *P. mobilis* there are four genes present: Holin, toxin secretion/phage lysis, Phage integrase, Prophage ps3 protein 01 and nonessential protein; lysogenic conversion; exonuclease; blocks growth of phage lambda.

### 3.8 Antibiotics and toxins

The Troll genome has 30 genes in the Subsystem for Virulence, Disease and Defence. Below 'Resistance to antibiotics and toxic compounds' in Table 11, there are several genes encoding for heavy metal resistance, such as cobalt, copper and arsenic which was expected considering the high content of heavy metals in oil reservoirs. Many of the genes code for transporting heavy metals out of the cell.

**Table 11 Genes for resistance to antibiotics and toxic compounds**

Copper homeostasis (3)
Cobalt-zinc-cadmium resistance (3)
Aminoglycoside adenylyltransferases (1)
Arsenic resistance (5)
Copper homeostasis: copper tolerance (1)
Resistance to fluoroquinolones (2)
Multidrug Resistance Efflux Pumps (2)

### 3.9 Xylan degradation

The *P. mexicana* described by Miranda-Tello et al., 2004 ferments D-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, rhamnose, starch, sucrose, xylose, xylan and pyruvate (Miranda-Tello et al., 2004). In this study strain Troll was cultivated on a combination of glucose and yeast extract at 55°C.

Xylose is a pentose (monosaccharide), and xylan is the polysaccharide constituted of xylose that is common in plant material such as wood (Uggerud, 2005). Searching for the xylanase enzyme within the strain Troll genome revealed two xylanase enzyme; Endo-1,4-beta-xylanase A and Endo-1,4-beta-xylanase D.

In addition strain Troll has more than 20 genes involved in xylanase uptake of xylan degradation products such as Xylose oligosaccharides ABC transporter, permease protein 1. In comparison *P. mobilis* has the Endo-1,4-beta-xylanase A, and 7 other genes related to the xylan uptake. Strain Troll should be grown with xylan as substrate to verify the function of the xylan utilization genes.

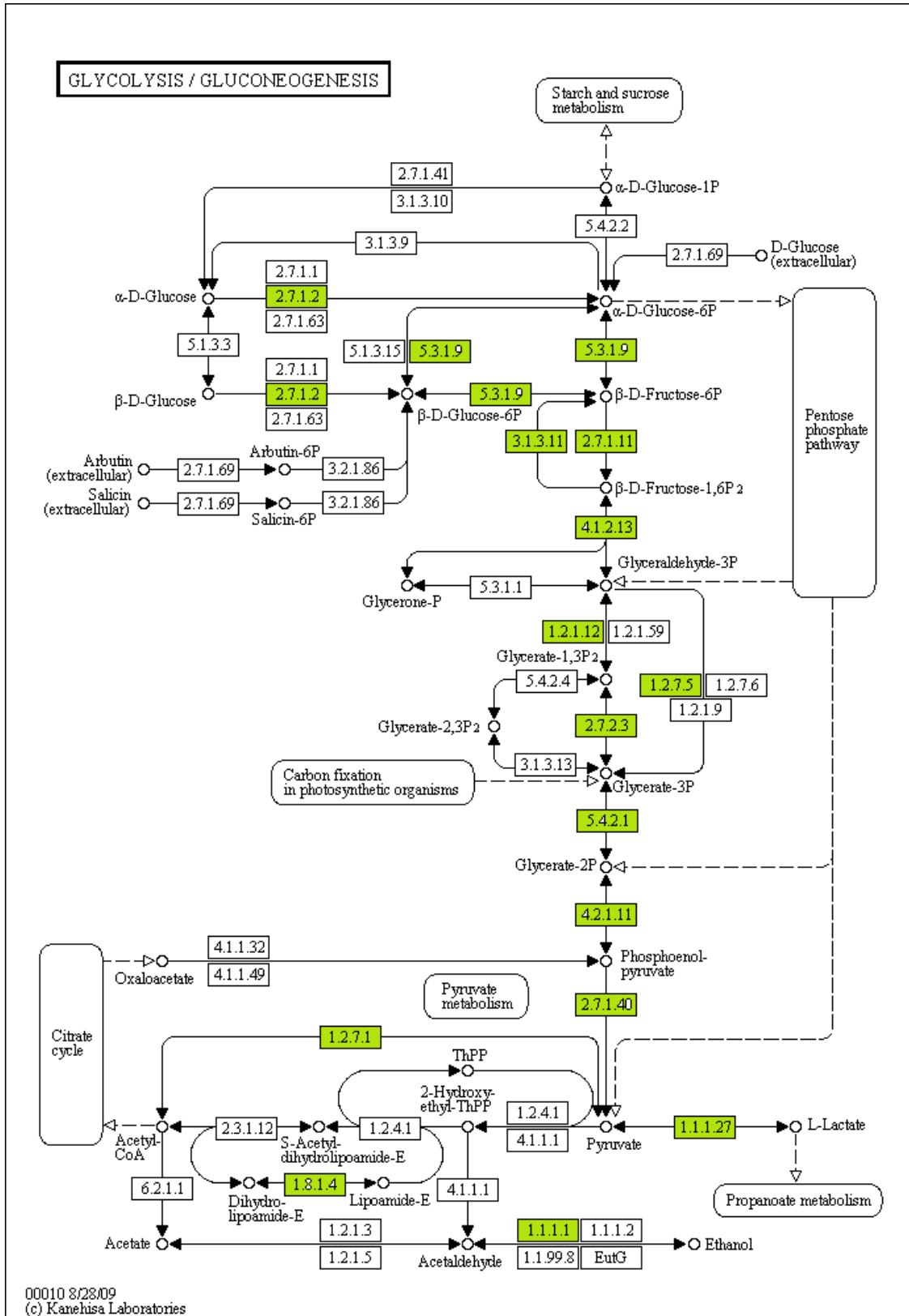
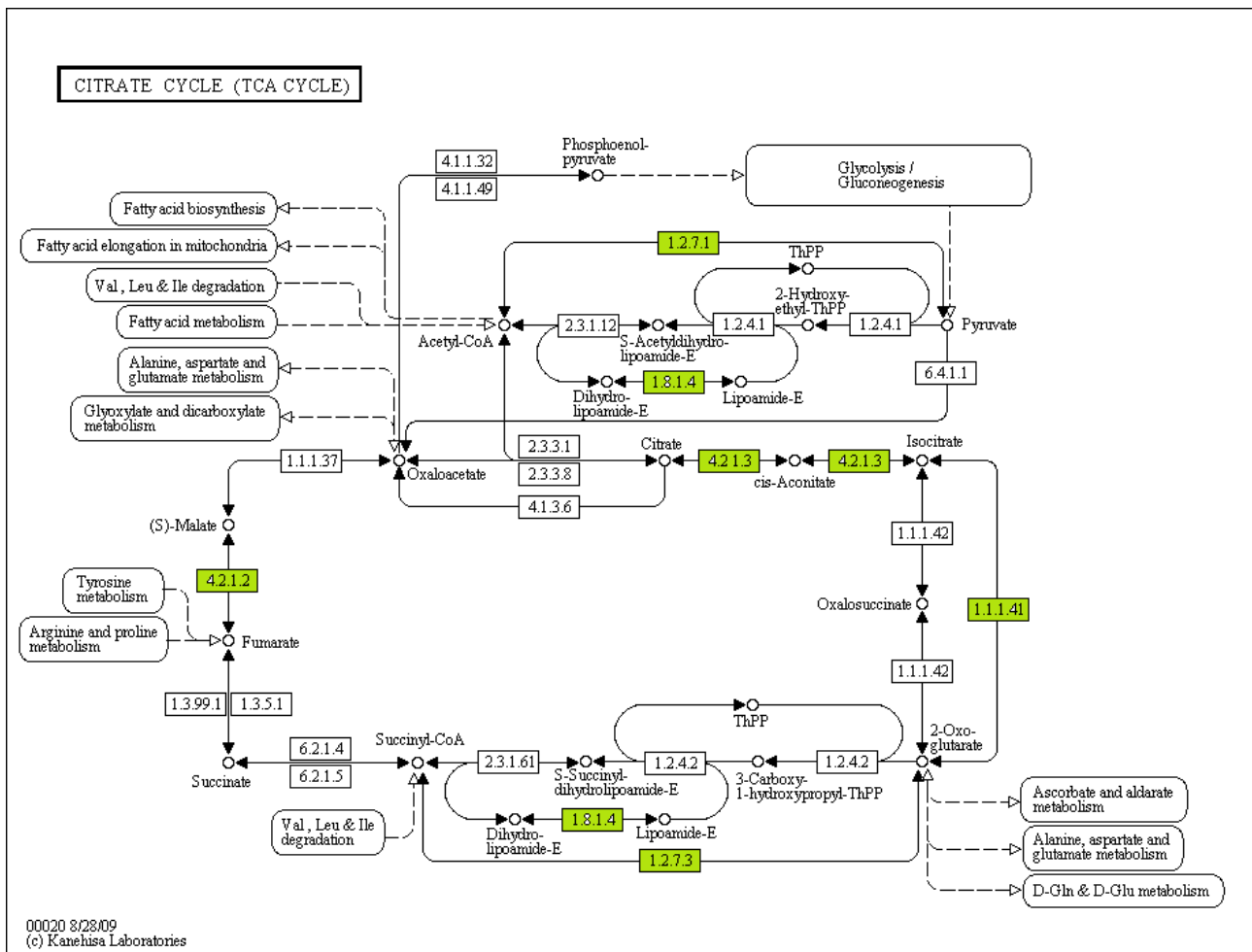


Figure 27 Glycolysis/gluconeogenesis in *Strain Troll*, green indicate present enzyme in the genome.



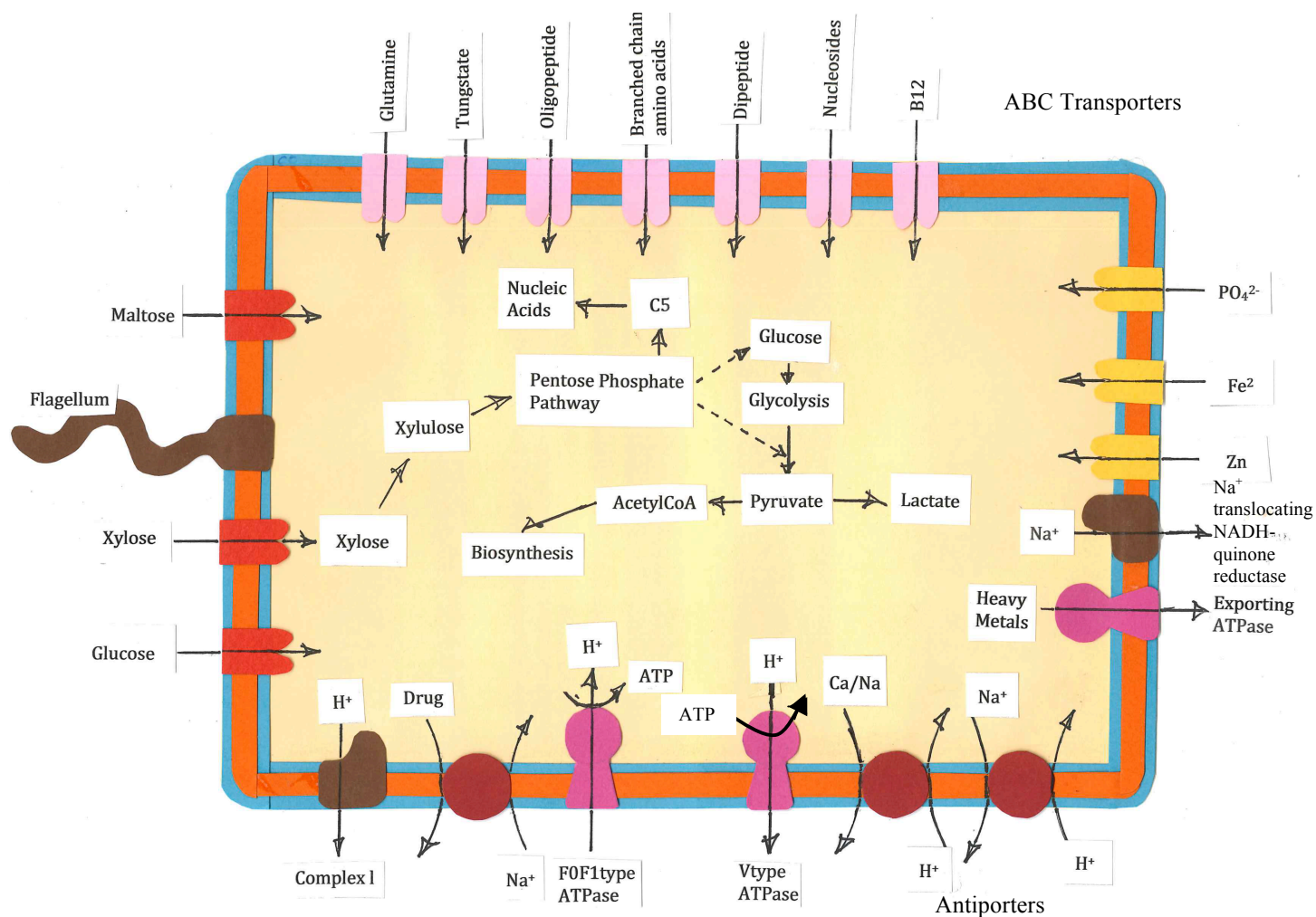
**Figure 28 Citrate cycle (TCA cycle) Green enzymes present in the strain Troll genome.**

### 3.10 Central metabolism

The map of the Glycolysis/Gluconeogenesis from the KEGG Metabolic Analysis as implemented in the RAST server (Figure 27) shows the presence of 18 genes for the glycolytic pathway. The strain also possesses lactate dehydrogenase indicating that lactate is one of the fermentation products.

Some of the citrate cycle (TCA cycle) (Figure 28) genes are present in the genome (green color) but the cycle is not complete. These enzymes probably serve biosynthetic function such as making precursors for biosynthesis of amino acids and fatty acids. There is a connection between Acetyl-CoA and Pyruvate. The enzymes necessary between Citrate and Succinyl-CoA are present.

Based on the information retrieved from RAST a hypothetical cell model can be made (Figure 29), including some of the membrane transport systems such as ABC transporters and antiporters. For respiration there are ATPases and electron donating reactions such as  $\text{Na}^+$  translocating NADH-quinone reductase and NADH ubiquinone reductase (Complex 1). Metabolic pathways such as glycolysis and Pentose Phosphate Pathway as well as their connections are included.



**Figure 29** Based on the genomic information from RAST, this model shows some of the features in the membrane transport such as ABC transport system and antiporters, and respiration features such as ATPases and central metabolic pathways.

## 4. DISCUSSION

### 4.1 Cultivation

In this study members of 4 different species of thermophilic anaerobes were isolated, and one of them an isolate (termed strain Troll) probably belonging to *P. mexicana* was isolated for the first time from a North Sea oil well. This species has only been found in oil-well water from the Mexican Gulf and has not before been detected by molecular analyses of Troll field water. The attempt to isolate a greater variety of *Thermovirga*, *Caminiella* and *Anaerophaga* species, which are known to be present in the Troll field water failed, which can be explained by a number of factors:

There are many challenges with anaerobic culture dependent analyses; e.g. keeping the enrichment cultures anoxic, selecting the right substrates, pH, salinity, and incubation temperature. In order to isolate more species, a larger variety of substrates could have been used: other carbohydrates, proteinaceous substrates and organic acids, either in several different combinations or separately. A variety of pH values could also have been used, as well as different incubation temperatures.

However, anaerobic enrichments and isolation of these types of organisms is time-consuming and very laborious, and within the time frame of this project the number of cultivation conditions had to be limited.

Another factor to be considered regarding isolation of novel bacteria is that many prokaryotes might depend on the other partner species in order to survive, like depending on a consortium in order to metabolize a given substrate. It is believed that organisms that exist in dense populations in nature often cannot grow in isolations, at least not under standard laboratory conditions. These are some of the reasons that might explain why most of the isolates were very similar to previously recovered isolates from North Sea oil wells.

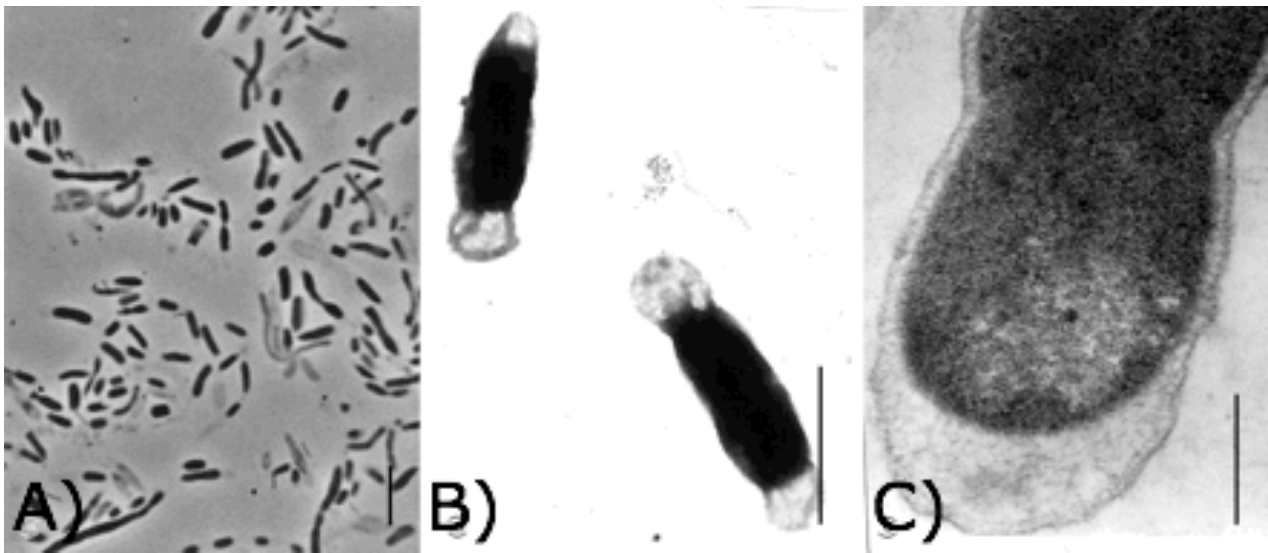
There is thus a great need for novel approaches for cultivation of a larger part of the microbial diversity that exists in these subsurface environments. Dilution to extinction is a method that selects the fastest growing organism. An alternative method, to aim for a more representative recovery of isolates would be to dilute produced water directly into gelrite shake-tubes supplemented with various growth substrates. Single colonies growing within the gelrite would in principle represent a culture resulting from a single cell, and in this way one can more easily isolate different and possibly more slow-growing species and not only the ones with the shortest generation time that tend to dominate in a liquid enrichment. Still there is a high contamination risk when picking colonies from shake-tubes as one can easily touch another colony when entering the gelrite with a needle or Pasteur pipette.

No growth occurred in enrichments incubated at 70°C, which is close to the in situ temperature. The most obvious reasons for this is that the isolates are not indigenous oil-well organisms but originate from layers with lower temperatures above the petroleum deposit and/or have been introduced to the oil wells during drilling and oil recovery processes. It is also possible that these organisms grow in the oil production systems with lower temperatures than in the reservoir. The fact that the Troll field never has been reinjected with water makes it however less likely that the isolates represent 'contaminants'. Other possible reasons are the possibility that 70°C confers too much stress for the organisms during the latent phase when they are preparing for growth after days and weeks in the fridge. Previous isolates of *P. mobilis*, *P. mexicana* and *T. africanus* can grow at 65, 65 and 77°C respectively. It is possible that our new isolate also can grow at 65-70°C. This should be tested.

A last possible explanation is that the high in situ pressure where these organisms usually grow may compensate strongly for negative temperature effects and contribute to an increased tolerance to higher temperatures (Takai et al., 2008).

#### **4.2 Strain Troll**

Because the *P. mexicana* have only been isolated from the Gulf of Mexico before, it was of interest to perform a draft genome sequence analysis of the *P. mexicana*- like isolate, strain Troll. Unfortunately the Mexican isolate has not been genome sequenced so a direct comparison between the two was not possible. The GC content difference between strain Troll and the type strain of *P. mexicana* is 2.2% suggesting a possibility that they might represent two different species, or at least subspecies. The morphology is also different, strain Troll cells appears to be long and slender rods/chains, while the *P. mexicana* forms shorter chains and rods (Figure 30). A conclusive experiment to decide whether the two represent different species will be to perform a complete DNA:DNA hybridization.



**Figure 30** Images of the *Petrotoga mexicana* isolated from the Gulf of Mexico. **A)** Phase-contrast micrograph of *P. mexicana*; bar 5 $\mu$ m. **B)** Transmission electron micrograph of cells showing toga; bar, 2 $\mu$ m. **C)** Electron micrograph of an ultrathin section showing the Gram-negative cell-wall structure; bar, 0.25 $\mu$ m (Miranda-Tello et al., 2004).

### 4.3 Genome features and predictions

The closest related organism available in RAST is *Petrotoga mobilis*. It would be interesting to compare the strain Troll genome with the *Petrotoga mexicana* isolate from the Mexican Gulf, but this comparison has to await genome sequencing of that strain.

It is interesting that a fermentative bacterium like strain Troll has the ATP synthase F and V type. The F-type ATP synthase is related to electron transport phosphorylation, while the V-type serves to create proton motive force (pmf) by pumping the H<sup>+</sup> out instead of in, thus creating a pmf which is essential in activities such as motility and transport (Madigan; et al., 2012).

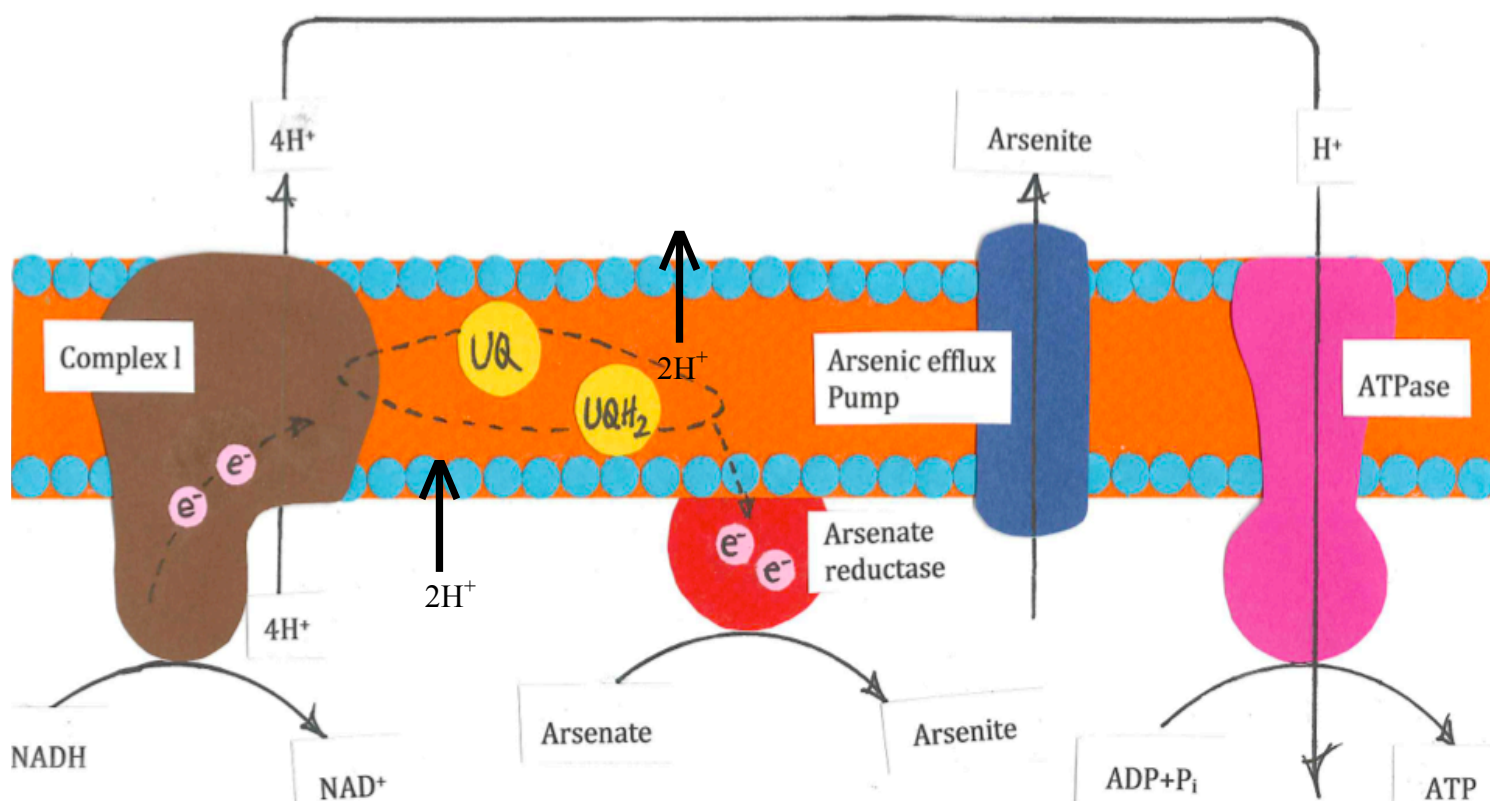
The operon where the V-type ATP synthase is located is almost identical to that of *Clostridium tetani*, (Figure 22) and horizontal gene transfer could explain this. According to (Dahle et al., 2008) *Clostridium* were also identified in their analysis of production water (Appendix A). This indicates that there are species similar to *Clostridium* present in oil reservoirs. A BLAST search for V-type genes showed that there are only two additional members of the *Thermotogales* that have similar genes; *Marinitoga piezophila* and *Thermotoga neapolitana*, making this ATP synthase scarcely distributed in the *Thermotogales*.

A carbon monoxide dehydrogenase (CODH) homolog was identified in strain Troll. We do not know the function of this enzyme in this organism or which direction the CODH reaction proceeds. The reaction can be related to anaerobic oxidation for acetate to CO<sub>2</sub>, which is common in some SRB, or H<sub>2</sub> production as a fermentative product. The opposite direction acetogenesis from CO<sub>2</sub> and hydrogen is carried out in some autotrophs. The enzyme is also involved in carbon tetrachloride



degradation II and Nitrotoluene degradation (Braunschweig, 2014).

The function of Arsenate reductase is either to detoxify or respire with arsenate. The function in strain Troll might be to respire arsenate, as this is the only reductase homolog found in this strain which can carry out an electron accepting reaction with electrons originating from the Respiratory Complex I. A simple tentative model for this possible mechanism is given in Figure 31, in which an arsenic efflux pump identified in the genome is also included. This mechanism would serve both energy yielding and detoxification purpose, but has to be verified experimentally.



**Figure 31** A hypothetical Arsenate Respiration; oxidized NADH passes two electrons on to the Complex I. These are brought further to the Arsenate reductase by the Ubiquinone. The Arsenate reductase uses the electrons in order to reduce the Arsenate to Arsenite that is pumped out via the Arsenic efflux pump.

Compared with *Petrotoga mobilis*, strain Troll has more genes related to utilization of xylose/xylan, and it might be more adapted to xylose uptake than *Petrotoga mobilis*.

Xylan utilization is as catabolic feature found in several oil-field related organisms. This is still an enigma, as this polymer is not found in this deep subsurface environment but is a component in cell

walls of plants and algae. There is a possibility that this was a prominent feature during the sedimentation of plant and algae biomass prior to formation of the oil wells and have been conserved through geological times due to slow growth and slow evolutionary rates in the subsurface.

As shown in Figures 27, 28 and 29 strain Troll possesses a complete Glycolysis and Pentose Phosphate Pathway (PPT), including ability to reduce pyruvate to lactate as a fermentation strategy. Xylose can thus be assimilated by PPT and enter the glycolysis as glyceraldehyde-3-phosphate or 2-phospho-glycerate, Figure 27. As is quite common for fermentative bacteria the TCA cycle does not appear to be complete, but only serve anabolic functions.

## 5. CONCLUSION

Considering the results in this work the members of the *Thermotogales* seem to be the easiest species to isolate from production water. This could be explained by several different reasons.

The *Thermotogales* might be more resistant to the sudden pressure drop when extracted from the oil reservoir, it could also have a shorter generation time and thus outcompeting other microorganisms present in the culture. *Thermotogales* isolated in this study also seem to have no difficulty to grow when isolated; ‘dilution to extinction’, and thus not depending on other species.

In order to determine if the *Petrotoga* sp. strain Troll is a new species or subspecies, it is necessary to compare with the genome of the *P. mexicana* type strain isolated in The Gulf of Mexico.

There are many respiration genes present in the strain Troll, despite the fact that it is considered a fermentative organism. The strain Troll has a complete Complex 1, sodium translocating NADH quinone reductase, ATP synthases and arsenate reductase, this give a strong indication that the strain Troll has some form of respiration.

Suggestions for further work would be to test for arsenate respiration in strain Troll, and the CODH enzyme activity in order to determine their function in strain Troll.

Further cultivation work could be to grow the strain Troll using xylan, and compare growth rate when using glucose. Experiments testing for the upper growth limit, salinity and pH could also be of interest. And ultimately a comparison of the genome with *P. mexicana* type strain when available.

Classic cultivation is a necessary approach for understanding of the biology of the uncultivated majority of prokaryotes identified by molecular tools. However, this approach can be very time-consuming and a great challenge.

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## 7. APPENDICES

### Appendix A

**Appendix A Closest relatives of clones obtained from the bacterial 16S rRNA gene library (Dahle et al., 2008).**

Taxonomical group	Sequence representing OTU*	No. of clones in OUT	Closest match	Closest cultivated species	%Identity to closest match /cultivated species)	Temperature optimum of closest cultivated species (°C) <sup>a</sup>	Source of closest match <sup>a</sup>
Firmicutes	TCB112x	2	<i>Halanaerobium saccharolyticum</i> X89069	<i>Halanaerobium saccharolyticum</i> X89069	89.7(89.7)		
	TCB133x	1	<i>Clostridium celerecrescens</i> X71848	<i>Clostridium celerecrescens</i> X71848	87.1		
	TCB128x	1	Uncultured bacterium AY570599	<i>Sedimentibacter hydroxybenzoicus</i> L11305	87.7(87.1)		
	TCB195x	1	<i>Orenia marismortui</i> X89073	<i>Orenia marismortui</i> X89073	94.5(94.5)	34-45	The Dead Sea
	TCB166x	1	<i>Orenia marismortui</i> X89073	<i>Orenia marismortui</i> X89073	86.3(86.3)		
	TCB170x	1	<i>Thermovirga lienii</i> DQ071273	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	89.9(89.9)		
	TCB169x	1	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	91.4(91.4)	58	Oil reservoir
	TCB152x	1	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	88.6(88.6)		
	TCB197x	4	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	99.7(99.7)	58	Oil reservoir
	TCB168x	1	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	97.8(97.8)	58	Oil reservoir
	TCB6y	3	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	99.2(99.2)	58	Oil reservoir
	TCB8y	9	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	<i>Thermovirga lienii</i> DQ071273 <sup>b</sup>	99.4(99.4)	58	Oil reservoir
	TCB198x	1	<i>Caminiella sporogenes</i> AJ320233	<i>Caminiella sporogenes</i> AJ320233	92.5(92.5)	55-60	Hydrothermal vent
	TCB15y	3	<i>Caminiella sporogenes</i> AJ320233	<i>Caminiella sporogenes</i> AJ320233	92.8(92.8)	55-60	Hydrothermal vent
	TCB136x	16	<i>Caminiella</i>	<i>Caminiella</i>	92.7(92.7)	55-60	Hydrothermal

			<i>sporogenes</i> AJ320233	<i>sporogenes</i> AJ320233			vent
	TCB199x	1	<i>Clostridium</i> <i>caminithermale</i> AF458779	<i>Clostridium</i> <i>caminithermale</i> AF458779	87.7(87.7)		
	TCB1y	1	<i>Thermacetogenium</i> <i>phaeum</i> AB020336	<i>Thermacetogenium</i> <i>phaeum</i> AB020336	94.7(94.7)	58	Methanogenic reactor
<b>δ-Proteobacteria</b>	TCB115x	1	Uncultured bacterium O11G2 AF220316	<i>Desulfomicrobium</i> <i>baculatum</i> AJ277894	99.4(85.0)		Oil reservoir
	TCB124x	1	<i>Desulfovibrio</i> <i>indonesiensis</i> Y09504	<i>Desulfovibrio</i> <i>indonesiensis</i> Y09504	98.2(98.2)	<37	Corroded oil storage vessel
	TCB4y	7	<i>Desulfomicrobium</i> <i>baculatum</i> AJ277894	<i>Desulfomicrobium</i> <i>baculatum</i> AJ277894	90.6(90.6)	28-37	Manganese carbonate ore
	TCB131x	6	<i>Pelobacter</i> <i>carbinolicus</i> X79413	<i>Pelobacter</i> <i>carbinolicus</i> X79413	96.4(96.4)	35-40	Anoxic marine mud
<b>γ-Proteobacteria</b>	TCB10y	1	<i>Agarivorans</i> <i>albus</i> AB076559	<i>Agarivorans</i> <i>albus</i> AB076559	95.9(95.9)	Mesophilic	Marine animal
Thermotogales	TCB177x	1	<i>Thermosipho</i> <i>africanus</i> DQ647058	<i>Thermosipho</i> <i>africanus</i> DQ647058	99.4(99.4)	75	Shallow hydrothermal system
	TCB116x	1	Strain TCEL2 <sup>b</sup>	<i>Thermotoga</i> <i>maritima</i> M21774	99.4(97.5)	80	Geothermal heated marine sediment
Spirochaetes	TCB129x	5	Uncultured bacterium AY667253	<i>Spirochaeta</i> <i>bajacaliforniensis</i> M71239	96.5(83.8)		Deep basalt aquifer
Bacterioidetes	TCB179x	1	Uncultured eubacterium U81730	<i>Tannerella</i> <i>forsythensis</i> AB053943	92.3(87.8)		Anaerobic digester
	TCB123x	7	<i>Anaerophaga</i> <i>thermohalophila</i> AJ418048	<i>Anaerophaga</i> <i>thermohalophila</i> AJ418048	91.6(91.6)	37-55	Oil separator tank
	TCB200x	8	Strain TC451 <sup>b</sup>	<i>Anaerophaga</i> <i>thermohalophila</i> AJ418048	94.5(92.0)	37-55	Oil reservoir
	TCB130x	1	Strain TC451 <sup>b</sup>	<i>Anaerophaga</i> <i>thermohalophila</i> AJ418048	97.9(94.5)	37-55	Oil reservoir

<sup>a</sup> Only 16S rRNA gene identities above 90% are considered

<sup>b</sup> Isolated in the study

\*Operational taxonomic unit



## Appendix B

### Fasta files of the 7 isolates.

#### Isolate 1

Merged fasta file of Casaminoacid enrichment, identified as *Thermovirga lienii*.

```
tgcagtcggacggggtgagttantgnaagctttcggggtgaagctgacttacctagtgcc
ggacgggtgagtaacgcgtgaattacctaccctagggagggggataaacgcctggaaacgg
gtgctaatacccatagggccgggaggttaaagggaggaatctgccctaggatgggttcgc
gtcctatcagctagttggtggggtaatggcctaccaaggcgaagacgggtagccggctcg
agaggatgtacggccacactggaactgagacacgggtccagactcctacgggagggcagcag
tggggaatattgggcaatgggcaagcctgaccagcgcgcgctgaggggaagaagg
cctttgggtgtaaacctctgtttgtacggggaagaagtcgggtgacggtaaccgtaCgaggaa
GccccggcaaACTacgtGccagcagccgcggtaatcgtagggggcagcgtTGtcCgGa
attactgggctaaagggcgcgagggcgatgcaaGtctgctgtgtaatgtcacggCt
caaccgtgtcattgcggtggaaactgcatactctggagtagtgagaggggagtggaattc
ccggtgtagcgggtgaaatgcgtaGatctgggaggaacaccagtgccgaaggcggctctc
tggccagttactgacgctcatgcgcgaaagctgggggagcgaaccggattaGatacccg
gtagtcccagccgtaaacgatgaatgctaggtgtgggtgtcgtGggcatctgtgccgca
GtaaacgcgataagcattccgcctggggagtagGgtcgcgaagattgaaactcaaaGgaat
tgacgggggcccgcacaagcgggtggagcacgtggtttaattcgaatgcAAaccgaaGaaCc
ttacctggGtttgacAtgtacgtggtagtgcCtgaaaGgggaGggaccatacttcGgT
atgggaGcgtacacAGGTgctGcatggCtGtcgtcAgctcgtgctgagatggtgggtt
aagccccgaacgagcgaacccctgcatctagttgccaacgggtgaagctgggcactct
agatggactgcccggcacaagccggaggaaggtggggatgacgtcaagtcacatggccc
ttatgtccagggcagacacgtgctacaatggccggtacagcgggaagcgaaggggagac
ctggagcggatctctgaaagccggtcccagttcggattgtagctctgcaactcgactacat
gaagctggaatcgctagtaatcgccgatcagccaagccgcggtgaatacgttcccgggccc
ttgtacacaccgcccgtcacaccaccgagttgggtgcacccgaagccggtnnccctaacc
ctta
```

#### Isolate 2

Merged fasta file of Dextrine enrichment, identified as *Petrotoga mobilis*.

```
tcggacgggttatagatcttcggagatataaccagtgccgaacgggtgagtaaaaggtag
ggacctgccctaaggacagagatagctactgaaacggtaggtaaacctctgataagccc
agaggggaaagtggtagacagccttaggatggacctactatccatcaggtagttggtgag
gtaaaggcttaccaagccgatgacggataaccgggtgtgagagcatggacgggtcacaaggg
cactgagacacgggcccactcctacgggagggcagcagtggggaatcttggacaatgggc
gaaagcctgatccagcgcgcgctgaaggaagaagtcacctcgggatgtaaacttctga
actaatcgaAataaaAgggtagtgagcAcaCtacagaagaaggtagAttaggaaaagtccc
ggctaaCtacgtgccagcagccgcggtgaagacgtagggggcgcgagcgttgcccggaattac
tgggtgtaaaggggacgtaggcgggtgatcaagtcactctgtgaaaagattgctcaacgat
cggcttgccgatgaaactgatcatcttgggcgtagcagaggttagacggaattacCtgagt
aggggtgaaatccgcagatacaggtagaagcgcgggtgaagaagttggtctactgggcta
cagctgacgctgaggtccgaaagccaggggagcaaacgggattagataaccgggtagtcc
tggccctaaacgatgctcactaGgtgtaGggagcgaagactctctgtgctgaagcgaac
gcgctaagtgagccactggggagtagctccgcaaggatgaaactcaaaggaattgacgg
gggtccgcacaagcgggtggagcatgtggtttaattcgaagctaaccgaaGaaCttaccA
GgGAttgacAtgtaactgAaGGTagaGaaatctactggCctaccgtaaggTaggaggTta
cacAggtggTgcacggTcgtcgtcagctcgtgccGTgaggtgttgggttaagtcccacaa
cgagcgaacccctgcaattagttaccagcaagtaaaagttggggacactaattggacagc
cgccgacgagggcggaggaagggggatgacgtcagataagcgtgccccttatactctgg
gagacacacgtgctacaatggggaggacaaaggggaagcgaagccggaaggtggagcggat
ccggaaaaactctccgtaatatggattgtaggctgaaaccgcctacatgaagctggaat
cgctagtaatcgaggtcagccaaactgcgggtgaatacgttcccgggcccctgtacacacc
gcccgtcacgccaccgagttgggaacacctgaaggcagtagcggtagg
```

### Isolate 3

Merged fasta file of Glucose+yeast extract+peptone enrichment, identified as *Thermosipho africanus*.

```
gnnnnnnngnnnnnnnnnnnnnnnnntcnnnctnnnnnnctgggtggaatcctttcgggga
ggaagccagtagactgagcggcggacgggtgagtaacgcgtaggtgacctaccctcaga
gggggataactgggggaaacctcagctaataccccatacgttccctacctataagcaggta
ggaagaaagggagcaatagctctgctgagggagggcctgcgccccatcaggtagttggt
gagtaacggctaccaagcctacgacgggtagccggctctgagaggatggccggccacaa
gggactgagacacgggcctactcctacgggaggcagcagtgggggatthttggacaatg
ggcgaagcctgatccagcagcgcgcgtgcgggacgaagcccttcggggtgtaaacgc
tgtggtgggagacgaataaggtgaggagtggaaagcctcactgatgacggtattccACTA
GaaaGCCcCggctAACTacgtGccAGCAGccGCGTAatacctaGGGggcGAGCgtTacc
cggattTACTGggCgtAaAggggGCGTAGCgGcCGTgaaagtccggTgTgaAaaCtcaC
ggCtCAccgtgggaCtgcgctggaactacacggccttgaggacggtagagggagacgga
actgCtgggtgtaggggtgaaatCCgtagatatacagcaggaacgcgggtggagaagtcggt
ctcCTgggcgttcCtgAcgCtgaGgcccgaagctaggggagcaaAccggattagatac
ccgggtagtcctagccgtaaacgatgccactaGgtgtgggggagtaattcCtccgtgct
gaagctaacgcgataagtggccgcctggggagtatgcccgcaaggggtgaaactcaagga
attgacgggacccccacacaagcGgTGgAGcgtgtggtttaattggacgctaaGccaagaa
ccttaccAGggCttgacAtgctGgTGgTactgacccgaaAgGGgagggacctgtggcggt
agtacgggagccagcacaggtggtgcacggctcgtcgtcagctcgtgccgtgaggtggtg
ggttaagtcggcaacgagcgaacccctgcccttagttgccagcggttcggccgggac
tctaaggggactgccggcagcagccggaggaaggaggggatgacgtcagatactcgtgc
cccttatgccctgggagacacacgcgctacaatgggtgggacagcgggaagcgagccagc
gatggtgagcgaagccctaaaccaccctcagttcggattgcaggctgaaaccgcctg
catgaagccggaatcgtagtaatcgcggtcagccacgcgcgggtgaatacgttcccgg
ggtttgtacacaccgcccgtcacgccaccgagttggcagcgcggcgaagatgggtntcc
anccgaaaggggnagccctattnnngnnnnnnnn
```

### Isolate 4

Merged fasta file of Glucose+yeast extract, identified as *Petrotoga mexicana*.

```
nnnnnnnnnnnnnnnnnnnnnnngtcnnnnngntatncttcttcggaagtataatca
gtggcgaacgggtgagtaaaaggtagggacctaccccgaggacagagatagctactggaa
acggtaggtaaacctctgataagcccagaggggaaagtggaaagcagccttgggatggac
ctactatccatcaggtagttggtgaggtaaaGGCTTACCAAGCcGATGACGGATAACCGG
TGTGAGAGCATGTACGGTCACAAGGGCAC'TGAGACACGGGCCCTACTcCtACggGAggCA
GCAgtGgggaATAtTGGACAATGggCGAAaGCctgatccagCgacGcCGCGTGAAGGAaG
aagtCCtCGGGatgtaaaCtTCTGAACtAATCGaATaaaagggtagtggacAcActaca
GaagaaggtagattagGaaaagTcccGctaaactacgtgccagcagccgcggtaagacgt
agggggcgagcgttggccggaatcactgggtgtaaggggacgtagggcgatgatcaagt
catctgtgaaaagattgctcaacgattggcttgcggatgaaactggtcatcttgggcgta
gcagaggtagacggaattacCtgagtaggggtgaaatccgcagatacaggtagaacgcc
ggtgaagaagttggtctactgggtacagctgacgctgaggtccgaaagccaggggagcg
aaccggattagatacccggttagtcctggctgtaaacgatgctcactaggtgtagggagc
gaaagactctctgtgctgaagcgaacgcGctaagtgagccacctggggagtacgtccgca
aggatgaaactcaaaggaattgacgggggtccgcacaagcGgtggagcatgTggtttaat
tcGaaGctaaccgaaGaaCcttACCAGGgATtgacatgtaactgAaGgTagaGAAAtcta
ctGGCctGCCcGcaagAgGgggttacacaGgtggtgcacgGTcgtcGtcAgCTcgtgcc
GTGaGgTgTtGggTTaagtCcCacAacGAGcGcAAcCccTAcgaTTaGtTAcCAGcAaGT
AaagTTGgGTAcAcTAAT'TGGACAGCCGcCGAcGAGGCGGAGGAAGGAGGGGATGACGTC
AGATaAGCGTGCCCC'TTATACtctGGGCGacacacgtgctacaatggggaggacaaaggg
aagcgaagccgggaggtggagcggatccggaaaaactctccgtaatatggattgtaggct
gaaaccgcctacatgaagctggaatcgctagtaatcgaggtcagccaaactgcgggtga
atacgttcccggccttgtacacaccgcccgtcacgccaccgagttgggaacacctgaa
ggcagtagggcaggtactggtgaaggtgggctnnnnngaggnnggcgaagtcgnnnnnnnn
nnnnnnnnnnnnnn
```

## Isolate 5

Merged fasta file of Arabinose enrichment, identified as *Petrotoga mobilis*.

```
nnnnannntcggagatatanncagtgggcgaacgggtgagtaaaaggtagggacctgcct
aaggacagagatagctactgaaacggtaggtaaacctctgataagcccagaggggaaag
tgtagacagccttaggatggacctactatccatcaggtagttggtgaggtaaaggctta
ccaagccgatgacggataaccggtgtgagagcatggacggtcacaagggcactgagacac
gggccctactcctacgggaggcagcagtggggaatccttgacaatgggcgaaagcctgat
ccagcgacgccgctgaaggaagaagtccctcgggatgtaaacttctgaactaatcgaat
aaaagggtagtgacacactacagaagaaggtagattagggaaaagtcgggtaactaCG
TGCCAGCAGCCGCGTAAGACgtagggggCgAgcgCtGcccGgAattaCtgggTgtaAAG
GggacgtaGcCGGgtgatcaaGTCatCtgNGaaaagattGctcAACgatcgGcttgCGa
tgaaaCtगतCAtcttCggGCgtaGcAGANgtaGACGgaATtacCtgaNtaGggGTGaaa
tcCgcaGaTAcANgTAagaacgCcggtgAaagaaGttggtctactGgGCTacagctGacg
CtgaGgtccGaaAgCcAggGgagcaaacCggattagatAcccggttagTcctggcCCTaa
aCGAtgctCAcTaGGtgTAGGGAgcGaaAgaCtctctgTgctgaaGCGAaCGCGCtaAgt
gaGccaCctGGggagtacgtCcgcAAGGatgaaActcAAAGGaAttgacGgGGGTCCGCA
CAaGCGgtGGAGCATgTGgttAATtCGAAGctaaccGaaGaacCTTAcCaGgGaTtGAC
ATGTAACtGAaGGTAGAGAAatcTactGGCctACCGtaAgGTAGGaggttAcAcAGGTgG
tgCACgGTCGTCTCAGctCGTgcCGtGAGgTGTtGGgTTaagtcccacaacgagcgcaa
cccctgcaattagttaccagcaagtaagttggggacactaattggacagccgccgacga
ggcggaggaaggaggggatgacgtcagataagcgtgccccttatactctgggacacac
gtgctacaatggggaggacaaaggaagcgaagccggaaggtggagcggatccggaaaaa
ctctccgtaatatggattgtaggtgaaacccctacatgaagctggaatcgctagtaa
tcgcaggtcagccaaactgcggtgaatacgttcccggccttgtagacacaccgccgtcac
ggcaccgagttgggaacacctgaaggcagtaggtaggtactggtgaaggtgggcnnnn
nnntntgntccngnntcagcnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn
```

## Isolate 6

Merged fasta file of Maltose enrichment, identified as *Petrotoga mobilis*.

```
nnnnnnnntngatcttcggagatataaccagtgggcgaacgggtgagtaaaaggtagggac
ctgccctaaggacagagatagctactgaaacggtaggtaaacctctgataagcccagag
gggaaagtggtagacagccttaggatggacctactatccatcaggtagttggtgaggtaa
aggcttaccaagccgatgacggataaccggtgtgagagcatggacggtcacaagggcact
gagacacgggccctactcctacgggaggcagcagtggggaatccttgacaatgggcgaaa
gcctgatccagcgacgccgctgaaggaagaagtcctcgggatgtaaacttctgaacta
atcgaataaaaggttagtgacacactacagaagaaggtAGGTTAGGAAaAGTCCCGct
AAcTAcGTGccAgcagCCGCGgTAAGacgTAGGGggCGAGCGTTGCCcGGAATTACTGGG
TGTAAGgGgacgtaggCggGtgatcAAGtcaTctgtgaaaagattGctcAACgatCgGc
TTgCGgaTgaAactGAtcAtcttGGgcgtagcagagGtagacggaattacCtgagTaggg
gtgaaatccgcagatAcaggtagaacgccggtgaagaagttggtctactgggctacagc
tgacGCtgaggtccgaaagccagggGagcAaAccggattagataccgggtagtcctggc
cctaaacgatgctcactaggtgtagggagcgaagActctctgtgctgaagcgaacgcgC
taagtgagccacctggggagtacgtccgcaaggatgaaactcaAaGgaattgaCGGgggt
ccgcacaagcgggtggagcatgtggtttaattCgAagctaaCgaagaaCcTtACcAGGGA
TtgacatgtaactgAAGgtagagaaatctactggcctaccgtaaggtaggaggttacaca
ggtggtgcacggtcgtcgtcagctcgtgccgtgaggtgttgggttaagtcccacaacgag
cgcaaccctgcaattagttaccagcaagtaagttggggacactaattggacagccgcc
gacgagggcggaaggggggatgacgtcagataagcgtgccccttatactctgggcga
cacacgtgctacaatggggaggacaaaggaagcgaagccggaaggtggagcggatccgg
aaaaactctccgtaatatggattgtaggctgaaacccgcctacatgaagctggaatcgct
agtaatcgaggtcagccaaactgcggtgaatacgttcccggccttgtagacacaccgcc
gtcacgccaccgagttgggaacacctgaaggcagtaggtaggtactggtgaaggtggg
cttnnngannnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn
```

## Isolate 7

Merged fasta file of Threonine enrichment, identified as *Petrotoga mobilis*.

```
nnnnnnngatcttcggagatataaccagtggcgaacgggtgagtaaaaggtagggacctg
ccctaaggacagagatagctactggaaacggtaggtaaactctgataagcccagagggg
aaagtggtagacagccttaggatggacctactatccatcaggtagttggtgaggtaaagg
cttaccaagccgatgacggataaccgggtgtgagagcatggacggtcacaagggcactgag
acacggggccctactcctacgggaggcagcagtggggaatcttgacaatgggcgaaagcc
tgatccagcagcgcgcgtgaaggaagaagtcctcgggatgtaaacttctgaactaatc
gaataaaagggtagtgacacactacagaagaaggtagattagggaaaagtcccggctaac
tacgtgccagcagccgcggtaaagcgtagggggcgagcgttGCcCGGaatTActGggtgt
aaaGgggaCgtagggcggtgatcaagtcATctgtgaaaagattgctcaacgatcggcttg
cggatgaaactgatcactctggcgtagcagaggtagacggaAttACCTgagttaggggtg
aaatccgcagatacaggtaaagaacgccgggtgaagaagttggtctactgggtacagctga
cgctgaggtccgaaagccaggggagcaaaccggattagataccgggtagtcctggcct
aaacgatgctcactaggtgtagggagcgaagactctctgtgctgaagcgaacgcgctaa
gtgagccacctggggagtagctccgcaaggatgaaactcaaaggaattgacgggggtccg
cacaagcgggtggagcatgtggtttaattcgaagctaaccgaaGaaccttaccAGGGATtg
acatgtAActGAaggtagagaaatctactggcctaccgtaaggtaggaggttacacaggt
ggtgcacggtcgctcagctcgtgccgtgaggtggtgggttaagtcccacaacgagcgc
aaccctgcaattagttaccagcaagtaaagttggggacactaattggacagcccgac
gaggcggaggaaggaggggatgacgtcagataagcgtgccccttatactctgggcgacac
acgtgctacaatggggaggacaaaggggaagcgaagccggaagggtggagcggatccgaaa
aactctccgtaatatggattgtaggctgaaaccgcctacatgaagctggaatcgctagt
aatcgcaggtcagccaaactgcggtgaatacgttcccgggccttgtacacaccgccgctc
acgccaccgagttgggaacacctgaaggcagtaggtaggtactggtgaaggtgggcnt
nnnnnnn
```