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Physiological and phylogenetic studies of thermophilic, hydrogen and sulfur oxidizing bacteria isolated from Icelandic geothermal areas

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie

University of Akureyri
Department of Natural Resource Science
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Declarations

I here by declare that I am the only author of this thesis and it is the product of my own research.

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Hildur Vésteinsdóttir

It is here by confirmed that this master thesis is satisfactory to M.Sc.- degree from the Faculty of Business and Science, department of Natural Resource Science.

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Dr. Jóhann Örlygsson

Abstract

Four thermophilic hydrogen oxidizing bacteria were isolated from various hot-springs in Grensdalur, Hveragerði, SW-Iceland. The strains were investigated with respect to phylogenetics, physiology, hydrogen uptake rates, biomass yield and sulfur metabolism.

Phylogenetic studies of the isolates were done with both partial and full 16S rRNA analysis. Two true thermophilic strains were isolated, strain 16A and D10 growing optimally at 70 - 75°C, closely related to *Hydrogenobacter* species, showing 96% and 95% homology with *Hydrogenobacter hydrogenophilus*, respectively. The strains were cultivated under hydrogen oxidizing- (HOX), sulfur oxidizing- (SOX) and both hydrogen and sulfur oxidizing- (HOX+SOX) conditions.

Kinetics of hydrogen oxidation, sulfate formation and generation times were investigated under all growth conditions. Both of the true thermophilic strains could grow under HOX conditions and are potential candidates for single cell protein production. In addition both strains were sulfur oxidizers and produced sulfate as end product which resulted in a low pH at the end of the experimental times. During both HOX and SOX conditions, a simultaneous oxidation of both hydrogen and thiosulfate occurred, although at lower rate compared to pure HOX and SOX conditions.

The other strains isolated were moderate thermophiles. Strain 16C was identified as new species within the genus *Hydrogenophilus*, most closely related to *H. thermoluteolus* (95.6%) and strain 6C was identified as new species within the genus *Thiomonas* closely related to *Tm. thermosulfata, Tm. perometabolis* and *Tm. intermedia* (94.7 – 97.3% homology). Detailed results on various growth parameters were investigated for both moderate thermophilic strains, under various growth conditions (chemolithotrophic, heterotrophic, mixotrophic), concerning hydrogen uptake rate, biomass formation, sulfate production and utilization rates of organic compounds as well as growth rates. 16C was a very effective hydrogen oxidizer but could not utilize any of the sulfur compounds investigated. The strain could grow mixotrophically on hydrogen and several organic compounds. Strain 6C showed very versatile physiology. It could oxidize hydrogen and thiosulfate and grew also mixotrophically on hydrogen and/or thiosulfate with several organic compounds. This strain is a potential bacterium has a potential of being useful in bioremediation, i.e. removal of hydrogen sulfide.

Key words: hot-spring, thermophilic, hydrogen oxidizing, sulfur oxidizing, single cell protein, bioremediation

Útdráttur

Fjórar hitakærar vetnisoxandi bakteríur voru einangraðar úr heitum hverum úr Grensdal við Hveragerði. Stofnarnir voru rannsakaðir m.t.t. erfðafræðilegs skyldleika, lífeðlisfræði, vetnisupptökuhraða, lífmassamyndunar og brennisteinsefnaskipta.

Skyldleikarannsóknir á stofnunum voru gerðar með bæði hlut- og fullraðgreiningu á 16S rRNA. Tveir hitakærir stofnar voru einangraðir, 16C og D10 sem vaxa best við 70 – 75°C. Þeir sýndu mestu sampörun við *Hydrogenobacter hydrogenophilus* (96 og 95%). Vöxtur var athugaður við mismunandi frumbjarga aðstæður; vetnisoxandi (HOX), brennisteinsoxandi (SOX) og bæði vetnis- og brennisteinsoxandi (HOX + SOX).

Vaxtarhraði, vetnisoxunarhraði og hraði á myndun súlfats voru rannsökuð nákvæmlega við allar aðstæður. Báðir hitakæru stofnarnir gátu vaxið við HOX aðstæður og hugsanlega hægt að nýta sem einfrumuprótein framleiðendur. Einnig oxa báðir stofnarnir þíósúlfat og vetnissúlfíð sem leiddi til lækkunar á sýrustigi í ræktunarvökvum þeirra. Í viðurvist bæði vetnis og þíósúlfats sýndu báðir stofnarnir HOX og SOX efnaskipti á sama tíma þó svo að lægri vetnisupptaka og hægari súlfatmyndun væri en við hreinar HOX og SOX aðstæður.

Hinir tveir stofnarnir voru lághitakærir. Stofn 16C var greindur sem ný tegund innan ættkvíslarinnar *Hydrogenophilus*, næst í skyldleika við *H. thermoluteolus* (95.6%) og stofn 6C greindist sem ný tegund innan ættkvíslar *Thiomonas,* skyldastur *Tm. thermosulfata, Tm. perometabolis* og *Tm. intermedia* (94.7 – 97.3% skyldleiki). Nákvæm lífeðlisfræðileg gögn eru birt um báða lághitakæru stofnana undir margvíslegum vaxtarskilyrðum (ófrumbjarga, frumbjarga, mixótrópískt); vetnisupptökuhraði, hraði lífmassa myndunar og súlfatmyndunar auk niðurbrotshraða á lífrænum efnum sem voru notuð sem hvarfefni í tilraununum. Stofn 16C var mjög öflugur sem vetnisoxandi en sýndi ekki brennisteinsefnaskipti. Stofninn óx "mixótrópískt" á vetni og nokkrum lífrænum efnasamböndum. Stofn 6C var með mjög fjölbreytt efnaskipti. Hann gat oxað vetni og þíósúlfat og einnig óx hann mikótrópískt á vetni og/eða þíósúlfati með nokkrum lífrænum efnum. Hugsanleg not fyrir slíkan stofn eru í lífhreinsun á brennisteinsefnasamböndum eins og t.d. brennisteinsvetni.

Lykilorð: heitir hverir, hitakær, vetnisoxun, brennisteins oxun, einfrumuprótein, lífhreinsun.

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1. Background and research objective

The present study is based on two earlier projects. One, supervised by Ólafur Friðjónsson at Prokaria ehf, was based on the study of three mesophilic hydrogen oxidizing (HOX) bacteria, *Wautersia eutropha*, *Hydrogenomonas pseudoflava* and *Acidovorax facilis*. This research emphasised on genetic engineering and expression of heterologous proteins with the main objective to use autotrophic, mesophilic HOX bacteria as protein source for industrial enzymes (Fridjonsson, 2004; Kihn, 2004). The second project was bioprospecting study on thermophilic HOX bacteria with the main aim to produce enrichment cultures as a source for single cell protein to be used as a feed ingredient in the fish farming industry (Reynisdottir, 2007). In the latter project more than 60 samples were taken from three different geothermal areas in Iceland with the main aim to isolate HOX bacteria. The main work was done on enrichment cultures since heterotrophic contaminations were very persistent. This study is based on four of the HOX enrichment cultures from Reynisdottir (2007). Four pure cultures of HOX bacteria were isolated from the enrichment cultures and characterized. This project is a co-operation between the University of Akureyri, the engineering company VGK-Hönnun hf. (now Mannvit hf.) and the biotechnology company Prokatin ehf and funded by The Icelandic Centre for Research (Rannís), The ministry of Industry (The Biotech net), the University of Akureyri and the KEA fund.

The four enrichment cultures from previous study (Reynisdottir, 2007) were chosen because of their phylogenetic analysis (two new species) and their metabolic potential. Two of the isolates from the enrichment cultures were moderately thermophilic (50°C) and two were true thermophiles (60-70°C). One of the moderately thermophilic isolates was isolated from enrichment culture that had promising hydrogen uptake rates and biomass yield under HOX conditions. Genetic analysis had also revealed a new species. The isolated strain (16C) was closely related to *Hydrogenophilus* and is described in chapter 5 (**I**). The other enrichment culture was chosen because genetic analysis had revealed a new species within the genus *Thiomonas* and because of its hydrogen oxidizing capacity, but until now no *Thiomonas* sp. have been reported to be able to oxidize hydrogen. Members within this genus are better known for their sulfur metabolism (Moreira & Amils, 1997; Shooner *et al*., 1996)

Strain 6C was isolated from this enrichment culture. Phylogenetic analysis revealed that the strain was closely related to *Thiomonas* and is described in chapter 6 (**II**). The true thermophilic strains were isolated from enrichment cultures with both HOX and sulfur oxidizing (SOX) metabolism. Both of these strains were closely related to *Hydrogenobacter* (16A and D10).

In this study HOX capacity of the strains isolated was reviewed and compared to previous results from Reynisdottir (2007). In addition sulfur metabolism of the strains was studied. The main reason for the study of sulfur metabolism is the previously described capacity of using sulfur bacteria in various bioremediation processes, especially the use of sulfur bacteria to grow on reduced sulfur compounds like hydrogen sulfide (Chen *et al*., 2004).

Here, physiological and phylogenetic characterization are performed on four isolates, one hydrogen oxidizing and three hydrogen- and sulfur oxidizing, and discussed in relation to other bacteria. Characterizations of two strains are presented as two manuscripts of scientific papers.

2. Introduction

Geothermal areas are limited to few places around the world were volcanic activity is present. Iceland is considered to be one of the earth's hot spots because of the amount of geothermal energy coming to the surface. The geothermal areas have been divided into two categories depending on the temperature, high temperature areas and low temperature areas (Kristjansson & Stetter, 1992; Arnórsson, 1995; Kristjánsson & Alfreðsson, 1986). The high temperature areas are within the active volcanic areas with temperatures above 200°C at the depth of 1000 m (Kristjansson & Stetter, 1992; Arnórsson, 1995). The main characteristics of the high temperature areas are sulfur rich and clayish hot-springs and fumaroles. In these areas water is often scarce and often very acidic which can lead to transformation of the surrounding rocks with various precipitations (Kristjánsson & Alfreðsson, 1986; Kristjansson & Stetter, 1992). The low temperature areas are located outside of the active volcanic areas with temperature below 150°C at the depth of 1000 m (Kristjansson & Stetter, 1992; Arnórsson, 1995). The main characteristics of the low temperature areas are clear water pools and springs with temperature from 20-100°C. These pools and springs are most often alkaline with pH between 8 and 10 and often with silica around the edges of the hot-springs (Kristjánsson & Alfreðsson, 1986).

The microbial flora in hot-springs is especially interesting due to the unique thermophilic properties of the organisms present. A variety of lithotrophic and heterotrophic micro-organisms have been isolated from hot-spring communities. The basis of life in the hot-springs is the primary production, the conversion of carbon dioxide into biomass (Spear *et al*., 2005). At lower temperatures cyanobacteria and eukaryotic algae are common and are the main primary producers. At higher temperatures the species composition changes (Kristjánsson & Alfreðsson, 1986), and above 70°C photosynthesis is not known to occur (Spear *et al*., 2005). Instead of phototrophic bacteria, various chemolithotrophic sulfur- and hydrogen oxidizing bacteria are present (Madigan *et al*., 2003; Kristjánsson & Alfreðsson, 1986). The sulfur- and hydrogen bacteria are capable of fixing carbon dioxide into biomass using either the Calvin cycle or reverse citric acid cycle (TCA) and are thus primary

producers and the basis of the ecosystems at higher temperatures (Aragno & Schlegel, 1992; Aragno, 1992b).

One of the most important natural resources in Iceland is the geothermal energy. It has been harnessed in many different ways, mainly for house heating, but also for power generation, greenhouse farming and various industries (Arnórsson, 1995). One of the main effects of harnessing geothermal power is the emission of gases with the geothermal steam. The main gases of concern are the greenhouse gases carbon dioxide (CO_2) and methane (CH_4) together with sulfur gases which are mainly in the form of hydrogen sulfide (H_2S) which is toxic. When H_2S is released it is often oxidized to SO_2 causing acidification of rain and soil, which is of great concern (Kristmannsdóttir *et al*., 2000). Natural flow of gases from geothermal areas is through fumaroles and hot springs. After harnessing, the produced fluid is in almost all cases much more than natural outflow from the field initially and the natural flow increases. The consequence of harnessing geothermal areas are thus increased steam flow from natural sources as well as the produced steam from the power plants. (Kristmannsdottir *et al*., 2000). The gas emitted from the power plants is a clear waste of energy and also pollution in the form of greenhouse- and sulfur gases.

Our interest is in utilizing the gas emitted from geothermal power plants and at the same time decrease the amount of H_2S and CO_2 emitted to the atmosphere. The main energy sources for chemolithotrophic bacteria found in the geothermal gases are hydrogen and hydrogen sulfide. Carbon dioxide is also present to support autotrophic growth. Examples have shown that bacteria are effective in biological deodorization based on their fast oxidation rates of hydrogen sulfide and good removal efficiency (Chen *et al*., 2004; Jensen & Webb, 1995). The main advantage in using chemolithotrophic thiobacilli-like bacteria is their simple nutritional requirements. These bacteria have been studied regarding to removal of H_2S from various gases and have given promising results (Jensen & Webb, 1995).

2.1 Thermophilic prokaryotes and their habitats

Thermophiles are organisms with optimum growth temperature above 45°C. Both eukaryotes and prokaryotes are able to live at temperatures of 45°C or above, but when the temperature reaches above 65°C only prokaryotes can thrive (Madigan *et al.*, 2003).

Originally, studies did not focus on the survival of thermophilic bacteria even though the excistence of thermophilic organisms was well known. There were two types of observations that lead to the discovery and cultivation of thermophilic bacteria. Firstly, it was discovered by standard bacterial culture procedures (mainly in the canning industry) that heat tolerant, spore forming bacteria were the cause of many problems. The second type of research resulted from ecological studies of organisms living in geothermal habitats. Because of the visible attractive colors of the phototrophic organisms the focus was mainly on organisms that rarely grow above 60-65°C. Later, because of careful observations, organisms living above these temperatures were discovered. The exsistence of thermophilic organisms had though been known from the early years of bacteriology (Brock, 2001).

Scientists have argued on where to put the boundaries between thermophilic and mesophilic organisms. It has been suggested that the lower boundaries should be with optimum temperature of 55-60°C because temperature just below these are very common in the nature and higher temperatures are mostly limited to geothermal areas. Other reason is that *Eukaryotes* are known to live at these temperatures (Kristjánsson & Alfreðsson, 1986; Kristjansson & Stetter, 1992).

Aragno (1992b) has classified thermophilic prokaryotes into several groups depending on their optimum temperature:

- Facultative thermophile: any thermophile whose temperature range extends largely in the mesophilic range (i.e. below 45°C).
- Thermotolerant: any organism with a temperature optimum ≤45°C but can grow at a temperature >45°C.
- Moderate thermophile: any organism with a temperature optimum between 45 and 60°C.
- Stricht thermophile: any organism with a temperature optimum between 60 and 90°C.
- Extreme thermophile: any organism with temperature optimum ≥90°C.

Thermophiles belong to two phylogenetically very different domains of life, *Bacteria* and *Archaea* (Stetter, 1999). It is generally agreed that in most hydrothermal environments at temperatures between 50 and 90°C *Bacteria* is dominating in the communities of micro-organisms. In terrestrial hot-springs with pH between 6 to 8 and

temperature between 60-68°C, one group of *Bacteria* is dominant, the *Aquificales* which represents genuses like *Hydrogenobacter*. In environments with temperature above 90°C *Archaea* are dominating (Reysenbach & Shock, 2002) e.g. *Pyrolobus fumarii* that has a temperature optimum of 106°C and can live at temperature up to 113°C (Kristjánsson & Alfreðsson, 1986; Madigan *et al.*, 2003). Extreme thermophilic archaea do not grow below 60°C (Huber & Stetter, 1998). Thermophilic bacteria have also been isolated from soil and hot composts. Composts are self-heating, aerobic, solid-phase, during which organic waste materials are biologically degraded (Beffa *et al*., 1996b; Blanc *et al*., 1999). The temperature can reach up to 65-80°C because of the biological degradation resulting in rapid transition from a mesophilic to a thermophilic community. The thermogenic phase is then followed by a slow temperature decrease where the diversity of micro-organisms increases (Blanc *et al*., 1999).

The main reason for making it possible for thermophiles to thrive at high temperatures is the stability of their enzymes and proteins. The enzymes in thermophiles are more stable at high temperatures because of slight difference in amino acid sequences in proteins as compared with mesophilic bacteria. The stability of proteins is a result of increased number of ion pairs i.e. ionic bonds, between the positive and negative charges of various amino acids. In addition, thermophiles produce solutes such as di-insitol phosphate, diglycerol phosphate and manosylglycerate which help to stabilize proteins against denaturation. There are not only enzymes and proteins that have adapted to the high temperature but also the cytoplasmic membrane. Thermophiles have lipids rich in saturated fatty acids resulting in firmer cell membranes. The cell membranes of hyperthermophiles, most of which are *Archaea*, also differ from the bacterial cell membranes. They are made of isoprene units (5 carbons each) in repeated chains instead of fatty acids. The isoprene units are bound with ether linkages instead of ester linkages as in bacterial and eukaryotic cell membranes. Another difference between bacterial and archaeal cell membranes is that *Archaea* do not have lipid bilayer as *Bacteria*. Their membrane is a single layer resulting in a lower risk of the membrane to be torn apart as compared to two-layer membrane (Madigan *et al.*, 2003)

The cells of hyperthermophiles are very small and they seem to have no insulation against the heat. Therefore all the cell components have to be heat resistant.

Since *Bacteria* and *Archaea* are so different, they might use different strategies for heat adaptation (Stetter, 1999).

Investigations on thermophilic micro-organisms have shown that they have high growth rates and generation time as short as 1 h (Madigan *et al.*, 2003; Goto *et al.*, 1977; Goto *et al*., 1978). The maximum growth themperature known is 113°C but the maximal growth termperature at which microbial life can exist is considered to be found somewhere between 113 and 150°C. Deep sea smoker fluids of 200-350°C have proven to be sterile (Stetter, 1999).

Detection and isolation of new thermophilic microorganisms has increased for the last decades or ever since thermophilic bacteria were first discovered in the 1960´s (Brock, 1994). This is mainly because of progress in molecular methods, such as amplification of the 16S rRNA gene, which allows detection of DNA sequences of many different organisms at a time. Despite these great progresses only a small fraction of all microorganisms have been isolated and characterized (Reysenbach & Shock, 2002). The genetic data collected using these molecular methods can be found in large databases such as the National Center for Biotechnological Information (NCBI or GenBank) (Altschul *et al.*, 1990) and the Ribosomal Database Project (RDP II) (Cole, *et al*., 2007).

2.1.1 Geothermal areas

Geothermal areas are limited to few places around the world where volcanic activity is present. These areas are favourable habitats for thermophilic organisms where they offer stability in heat in contrast to soil or compost (Kristjánsson & Alfreðsson, 1986; Madigan *et al.*, 2003). The temperature in soil can reach up to 70°C due to sunlight at midday but cools fast and few centimeters under the suface the temperature can be much lower (Amend & Shock, 2001). The temperature of the hot-springs in geothermal areas is usually very constant, not varying more than 1-2°C over many years even though the temperature between the hot-springs can vary greatly, ranging from 20°C up to above 100°C depending on location and pressure (Madigan *et al.*, 2003). In addition to relatively stable temperatures, geothermal areas offer wide range in acidity, oxidation/reduction states, solute concentrations, gas compositions, mineralogy and nutrition to support growth of both chemolithotrophs and chemoorganotrophs. This variety in growth parameters results in enormous genetic and metabolic diversity

present (Amend & Shock, 2001). Different geothermal areas can be categorized as follows:

- Hot terrestrial springs with temperature up to boiling point.
- Hydrothermal vents in the ocean floor where the temperature can reach 350°C or higher.
- Steam vents with temperature up to 500°C (Madigan *et al*., 2003).

Subterranean hot springs or boreholes have also been studied for microbial life. There turned out to be diverse communities of bacteria and archaea (Marteinsson *et al*., 2001; Kimura *et al*., 2005).

The main characteristics of geothermal areas is extremely low oxygen concentrations. Consequently, most of the known species of thermophiles are classified as obligate or facultative anaerobes, though aerobic and microaerobic isolates are also known (Amend & Shock, 2001).

Terrestrial hot springs are found in most geothermal areas in the world, in the western United States, New Zealand, Iceland, Japan, Italy, Indonesia, Central America, Russia and Central Africa (Madigan *et al.*, 2003).

2.1.2 Microbial flora in Icelandic hot-springs

The microbiology of Icelandic hot-springs has been studied by many scientists but Iceland is considered to be one of the largest geothermal areas in the world. There is a great difference in physical and chemical composition, both within the geothermal areas as well as between different areas, resulting in great variety of bacterial communities. Geothermal areas in Iceland can be divided into two different categories as stated previously:

High temperature areas are located at active volcanic areas. The main visible characteristics of these areas are sulfuric and clayish hot-springs as well as steam vents (100-200°C at the depth of 1000 m). The pH of these hot-springs is usually acidic (pH 2-4) and organic matter is scarce. Concentration of dissolved inorganic matter is thus high. Even thought these conditions seem to be hostile for micro-organisms, one millilitre of liquid from these hot-springs may contain as much as 100 million micro-

organisms (Kristjánsson & Alfreðsson, 1986). High temperature areas in Iceland are thought to be approximately 30 and cover over few hundreds of km^2 (Arnórsson, 1995).

Low temperature areas are on the border of the active volcanic areas. The temperature in these areas is below 150°C at the dept of 1000 m. The main visible characteristics of these areas are clear water pools and springs with temperatures from 20-100°C. These hot-springs are generally alkaline with pH between 8 and10 and often with silica precipitation around the edges of the hot-springs (Kristjánsson & Alfreðsson, 1986). Low temperature areas in Iceland are thought to be over 250 with over 600 hot-springs and pools (Hveravefsíðan, 2008b).

Molecular methods have been used to analyze the microbial composition in hot-springs in Icelandic geothermal areas. 16S rRNA genes from environmental samples have been extracted, cloned and analyzed (Marteinsson *et al*., 2004; Pétursdóttir *et al*., 2006; Þórðarson & Pétursdóttir, 2002). These studies revealed bacterial species belonging to the phylum of *Aquificae, Deinococcus-Thermus* and *Proteobacteria* as well as *Cyanobacteria, Chloroflexi, Spirochaeta, Acidobacteria, Firmicutes, Nitrospirae, Planctomyces* and more (Pétursdóttir *et al*., 2006).

Species composition varies greatly between different hot-springs but usually there are only one or two dominating species. Studies have also shown that in extreme environments where temperature is above 80°C, the pH is between 2 and 3 or sulfide is present in high concentrations, the variation in the number of species is lower than in neutral to alkaline environments on the accont of phototrophic organisms (Marteinsson *et al*., 2004; Þórðarson & Pétursdóttir, 2002).

Growth of algae and bacteria is characteristic at low temperature areas. At temperatures from 30 to 45°C, cyanobacteria and eukaryotic algae are common. Above 45°C the micbrobial diversity decreases and only two genuses of cyanobacteria are present, *Mastigocladus* and *Phormidium*. In these areas anaerobic phototrophs such as *Cloroflexus* species are common as well as *Thermus. Thermus* species are heterotrophic, gram-negative, rod shaped bacteria that are common in alkaline hot-springs with temperature from 50-85°C. In addition sulfur- and hydrogen-oxidizing bacteria have been found in these low temperature areas as well as sulfate reducers and methanogens (Kristjánsson & Alfreðsson, 1986).

At temperatures between 60-80°C, species composition is diverse but bacteria of the family *Aquificales* are often dominating. *Chloroflexus* species are also common in

the presence of sunlight, but otherwise chemolithotrophic sulfur- and hydrogen oxidizing bacteria are present (Marteinsson *et al.*, 2004; Kristjánsson & Alfreðsson, 1986). In the high temperature areas many species of anaerobic archaea and bacteria with optimal temperature around 85-95°C have been isolated from clayish hot-springs with pH below 4.0. Examples are species such as *Thermoproteus, Thermophilum, Desulforococcus* and *Sulfolobus* (Kristjánsson & Alfreðsson, 1986; Marteinsson *et al*., 2004). Research have shown that in hot-springs with low sulfide concentration *Chloroflexus* are dominating while in the hot-springs with high sulfide concentration *Aquificales* were dominating at the same temperature (Skirnisdottir *et al.,* 2000).

Most hot-springs have some run off because of overflow and form streams which cool down as it runs further away from the hot-spring, forming thermal gradient. These gradients make it possible for various species with different temperature optimum to inhabit these areas resulting in diverse microbial flora (Madigan *et al.,* 2003). The overflow provides constant temperatures and mineral nutrients which allows dense microbial mats to be formed (Lowe *et al*., 1993). The appearance and types of bacteria in the microbial mats seems to vary depending on sulfide concentration, pH, temperatures and other chemical and physical factors (Skirnisdottir *et al*., 2000). The top layer of these mats are usually phototrophic, thermophilic bacteteria, such as *Cyanobacteria*, which are the primary producers. In the lower layers, where decomposition of the primary producers occurs, anaerobic thermophiles are dominating. The bacteria in the lower layers can be hydrolytic, fermentative, methanogenic and sulfate reducing. These mats create optimal conditions for various thermophilic bacteria (Lowe *et al.*, 1993).

2.1.2.1 Grensdalur

Grensdalur is a part of the Hengill area in SW-Iceland. Grensdalur is on the boundaries of being called high temperature geothermal area and has a variety of different hot-springs with great variations in temperatures, pH and mineral concentrations. Among the hot-springs there are sulfide rich, neutral hot springs which can be found in Iceland but are relatively rare in other geothermal systems in the world (Háskólasetrið, 2007; Skirnisdottir *et al.,* 2000). Geothermal areas in Iceland have been categorized into 8 categories and 47 subcategories. A good example of the diversity in habitats for thermophilic bacteria in Grensdalur, is the fact that eleven of these subcategories can be

found there, e.g. clear hot pools, mud pools, silica hot-springs and geysers (Jóhannesson & Sæmundsson, 2005).

Ever since 1930, scientist have been interested in the microbial flora in the hot-springs located in Grensdalur. It consists of a great variety and the emphasis has been on many types of microbes. The first studies dealt with the upper limits of growth of cyanobacteria (Thorkelsson, 1930; Binder *et al*., 1972; Castenholz, 1969). Later, studies aimed more towards the composition of microorganisms in the hot-springs and especially the methanogens present (Binder *et al*., 1981), the distribution of *Thermus* sp. and microbial ecology in thermal gradient from hot-springs (Kristjánsson & Alfreðsson, 1983). Experiments towards the phylogeny and physiology of SOX bacteria have also been done (Kristjánsson & Alfreðsson, 1986). The University of Iceland and Technological Institute of Iceland have for many years studied the microbial flora in Grensdalur. The result of these studies is a strain collection with over 200 strains. These strains belong to different genera (16S rRNA sequencing) and some are new strains that have to be investigated further (Háskólasetrið, 2007).

2.2 Chemolithotrophy

The term "chemolithotrophy" is used to describe the energy metabolism of bacteria that use inorganic compounds as energy source (Kelly & Wood, 2006). The concept of chemolithotrophy was first proposed by Sergei Winogradsky after his studies of nitrifying bacteria and SOX bacteria. From these studies he concluded that the nitrifying bacteria derived their carbon for cell synthesis from CO_2 in the air and were thus autotrophs and SOX bacteria using inorganic compounds as energy sources were chemolithotrophs. Neither of these concepts was accepted at that time, but today chemolithotrophy and autotrophy are very important processes and can even support growth and existence of higher organisms (Madigan *et al*., 2003).

Bacteria can derive energy from different sources. It can be obtained from light (photosynthesis), organic carbon (respiration and fermentation) and from inorganic substrates (chemolithotrophy) (Madigan *et al.*, 2003). Chemolithotrophy describes the process when bacteria obtain their energy by oxidation of inorganic compounds (Kelly & Wood, 2006).

The reduced inorganic compounds commonly used by chemolithotrophs are hydrogen (H_2), sulfide (HS^-/H_2S), sulfur (S^0), ammonium (NH_4^+), nitrite (NO_2^-) and ferrous iron (Fe^{2+}) (Gottschalk, 1979). There are many sources for these inorganic compounds. The burning of fossil fuels, industrial wastes, agriculture and mining are the main sources for reduced sulfur-, nitrogen- and iron compounds. Many inorganic compounds utilized by chemolithotrophs are also waste products from other bacteria, e.g. from chemoorganotrophs that gain energy from organic compounds. For instance, hydrogen is a waste product formed by bacterial fermentation of organic matter and sulfide is formed by anaerobic reduction of sulfur or sulfate (Madigan *et al.*, 2003).

When compared to chemoorganotrophs, which utilize organic compounds as both carbon and energy source, chemolithotrophs cannot gain carbon from an organic energy source. Therefore carbon must be assimilated from another source which is in most cases CO_2. For most chemolithotrophs CO_2 is the only carbon source required for growth (Madigan *et al.*, 2003). These organisms, capable of fixing CO_2 by chemosynthesis, are referred to as chemolithoautotrophs (Stetter, 1999). Thus the term autotrophy refers to the ability of bacteria to utilize CO_2 as a sole carbon source (Madigan *et al.*, 2003). It has been suggested that the term autotrophy should be used for all organims that get their cellular carbon from one substrate, such as methane, methanol and formate in addition to CO_2 (Kelly & Wood, 2006). The use of the term autotrophy in the following text refers only to the use of CO_2 as a carbon source.

Until the 1970‘s it was believed that organic compounds were toxic for chemolithotrophs and that all cell carbon had to come from CO_2. Subsequently, organisms proved to be able to use organic compounds for assimilation. Chemolithotrophs which can assimilate organic compounds and use them as energy and carbon source are thus facultative chemolithoautotrophs (Kelly & Wood, 2006). The term mixotrophy is also known and applies to when inorganic and organic compounds serve as energy and/or carbon sources simultaneously. An example of mixotrophic growth by HOX bacteria is when organic substrate is serving as an energy and carbon source and hydrogen is also provided and used as energy source (Bowien & Schlegel, 1981). The third group are organisms which use inorganic energy substrates and organic carbon for assimilation. These are referred to as chemolithotrophic heterotrophs. These abilities blur the boundaries between lithoautotrophy and heterotrophy. For that reason scientist have agreed that it is preferable to distinguish

energy and carbon metabolism, especially among the chemolithotrophs (Kelly & Wood, 2006).

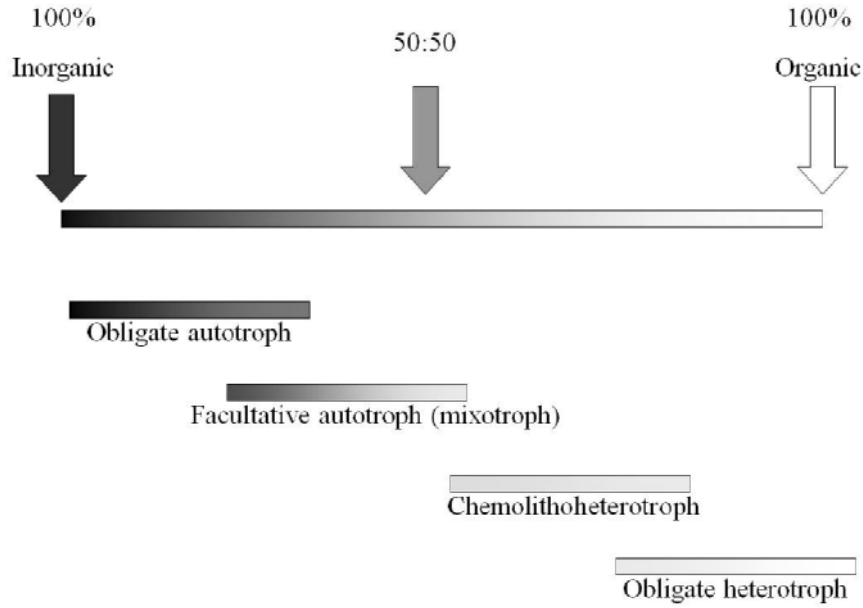


Figure 1. A spectrum originally put up to describe the spectrum of the four physiological types of the colourless sulfur bacteria, but can be applied to other chemolithotrophic species (Robertson & Kuenen, 2006).

Figure 1. shows how overlapping occurs depending on the availability of organic or inorganic substrates. When inorganic substrates are dominant and no organic substrates are found obligate autotrophs are dominating. However, when organic substrates are in high concentrations obligate heterotrophs will be dominant. Between these two circumstances overlapping occurs where both organic and inorganic substrates are available followed by changes in bacterial composition (Robertson & Kuenen, 2006).

2.3 Energetics of inorganic oxidations

Energy is defined as the ability to do work. In microbiology the energy is measured in kilojoules (kJ). Gibbs free energy (G) is the energy released that is available to do useful work (Madigan *et al.*, 2003). In all respiratory processes and energy-conserving metabolism the transfer of hydrogen from a state more electronegative than that of the H^+/H_2O couple to that of water is a fundamental process (Kelly & Wood, 2006).

All chemosynthetic organisms gain energy by catalysing various organic and inorganic oxidation/reduction (redox) reactions. These reactions have to be thermodynamically favoured to serve as energy sources (Amend & Shock, 2001). The $\Delta G^{0'}$ in table 1 stands for the change in "Gibbs energy" under standard conditions (pH 7, 25°C, 1 atm), i.e. the energy yield of few reactions. When $\Delta G^{0'}$ is negative the reaction is exergonic and yields energy in a spontaneous process. If the $\Delta G^{0'}$ value is positive the reaction is exergonic and will not happen spontaneously and is not thermodynamically favoured (Madigan *et al*., 2003). As the temperature rises the reaction rates increase and at some elevated temperature abiotic reaction rates are so fast that there is no benefit for organisms to catalyse the reaction (Amend & Shock, 2001). In chemolithotrophic bacteria oxidation reaction occurs only if the reactions create sufficient energy to support ATP synthesis and electron transport. The oxidation can also only occur if the organisms have the suitable enzymes and metabolic systems to be able to oxidize substrates to gain energy (Kelly & Wood, 2006).

Table 1 shows few examples of chemolithotrophic reactions and estimated energy yield. When oxygen is used as an electron acceptor a number of inorganic compounds are able to provide sufficient energy for ATP synthesis (Madigan *et al*., 2003).

Table 1. Gibbs energy yields and estimated numbers of mol ATP per mol substrate of few oxidation reactions from inorganic substrates (Madigan et al, 2003; Kelly and Wood, 2006).

Electron donor [a]	Electron acceptor	Type of chemolithotrophy	$\Delta G^{0'}$ (kJ/reaction)	Number of electrons	$\Delta G^{0'}$ (kJ/2e$^-$)	Mol ATP /mol [b*]
H_2	O_2	Hydrogen bacteria	-237.2	2	-237.2	2-3
H_2	CO_2	Methane bacteria	-35	-	-	<0.25
HS^-	O_2	Sulfur bacteria	-209.4	2	-209.4	1
$S_2O_3^{2-}$	O_2	Sulfur bacteria	-818.3	8	-204.6	2.8
S^0	O_2	Sulfur bacteria	-587.1	6	-195.7	1-3
NH^{4+}	O_2	Nitrifying bacteria	-247.7	6	-91.6	1 or 2
NO^{2-}	O_2	Nitrifying bacteria	-74.1	2	-74.1	1
Fe^{2+}	O_2	Iron bacteria	-32.9	1	-65.8	0.5

a: values obtained from Madigan *et al*., 2003
b: values obtained from Kelly and Wood, 2006
*: estimated mol ATP synthesised per mol substrate

Reducing power in chemolithotrophs is either obtained directly from inorganic substrates or by reverse electron transfer reactions (Madigan *et al.*, 2003). In all the oxidation reactions in table 1, except for the hydrogen oxidation, electron transport is coupled to the cytochrome system of the bacteria. The NAD^+ reduction requires the energy dependent flow from cytochromes. This dependence results in a biochemical hindrance to the growth of such chemolithotrophs since most of the energy is used to produce NADH and less goes to the formation of biomass (Kelly & Wood, 2006)

2.4 Hydrogen

Molecular hydrogen is a very important and widespread metabolite in the world as a growth substrate providing energy and as a reductant. During the era of prebiotic evolution, which lead to forming of cellular life, the earth had a reducing atmosphere where level of molecular hydrogen was believed to be as high as 1%. The first life forms are considered to have been based on hydrogen metabolism e.g. methanogens or sulfate reducers. When oxygen started to accumulate in the atmosphere due to oxygenic photosynthesis the level of hydrogen decreased causing hydrogen dependent micro-organisms to retreat to restricted habitats (Schwartz & Friedrich, 2006).

Hydrogen can be formed in many different ways. Abiological formation of hydrogen can occur e.g. in reaction between water and silicon molecules at high temperatures, in reaction between water and hot ferrous rocks (FeO and Fe_3O_4) (Aragno & Schlegel, 1992), through reactions between dissolved gases in the system C-H-O-S in magmas, decomposition of CH_4 to carbon and H_2 at temperatures above 600°C and reaction between CO_2, H_2O and CH_4 at elevated temperatures in vapour (Morita, 2000). Hydrogen is also produced biologically by various fermentative organisms that dispose excess reducing power from their metabolism by releasing molecular hydrogen. Production of hydrogen is not restricted to anaerobic conditions but also produced by nitrogenase which is the key enzyme of nitrogen fixation in nitrifying bacteria when it fixes nitrogen from the atmosphere. This process is the major source for hydrogen in soils (Morita, 2000). At the same time soil is the most important global sink for hydrogen and accounts for more than 90% of the total consumption (Schwartz & Friedrich, 2006).

Hydrogen concentrations today in the atmosphere are very low (0.5 to 1.0 ppm) but may vary depending on locations. At higher altitude the concentration increases and vice versa at lower altitudes (Morita, 2000). An exception are the geothermal areas where hydrogen occurs frequently in concentration of several percent (v/v) of the total dry gas. Hydrogen emission from terrestrial solfatara in Italy have been measured as high as 4.8% (Aragno, 1992a; Conrad *et al*., 1985). Due to anthropogenic sources concentration of hydrogen seems to be increasing at a rate of 3.2 ppbv per year (Morita, 2000).

2.4.1 The "knallgas reaction"

Hydrogen consumption is a widespread phenomenon in natural environments. It can occur both aerobically, with oxygen as final electron acceptor, and anaerobically, with e.g. nitrate, sulfate or ferric iron and carbon dioxide as final electron acceptors (Madigan *et al.*, 2003). Hydrogen consuming bacteria are very widespread and are found within the orders of *Aquificales, Nostocales, Stigonomatales, Acitnomycetes, Bacillales, Clostridiales*, *α*-, *β*-, *γ*- and *ε*-Proteobacteria and the *Thermotogales*. Most of the hydrogen consuming archaea are anaerobic, such as methanogens and sulfate reducing bacteria (Schwartz & Friedrich, 2006). Different pathways of hydrogen consumption are presented in figure 2.

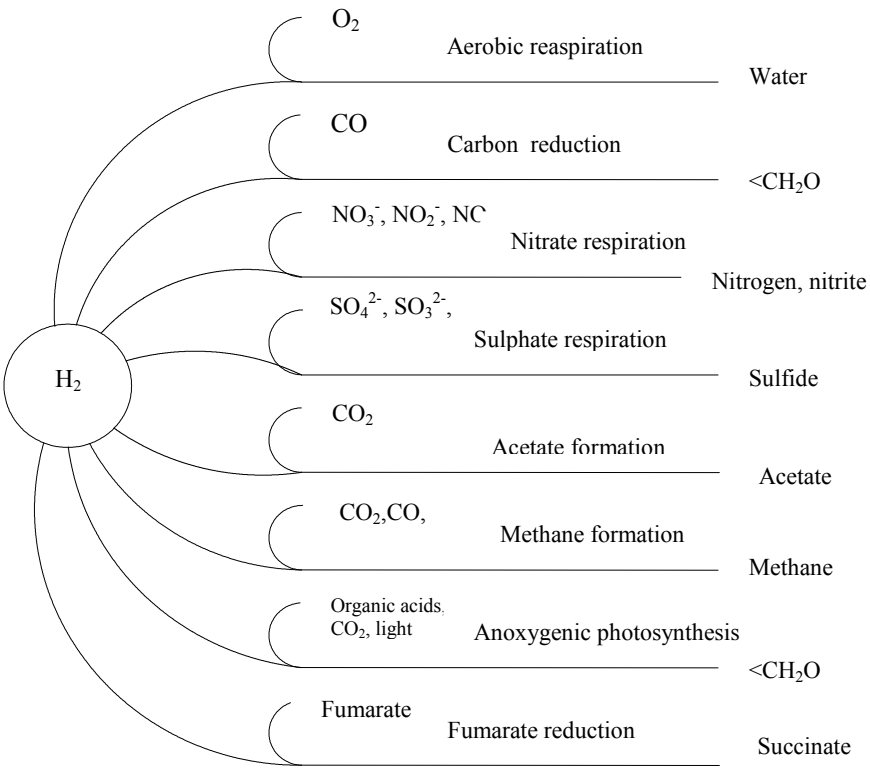


Figure 2. Different processes of hydrogen utilization by various bacteria in the presence of different electron acceptors (Aragno & Schlegel, 1992).

The aerobic hydrogen oxidation is often referred to as the "knallgas reaction" i.e. the reduction of oxygen using hydrogen as energy source:

$$H_2 + O_2 \rightarrow H_2O \qquad \Delta G^{0'} = -237 \text{ kJ}$$

This reaction is very exergonic and results in formation of at least one ATP leading to formation of proton motive force. These reactions are catalysed by the enzyme hydrogenase which transfers the electrons from the hydrogen to a quinone acceptor. The enzyme plays a key role in binding hydrogen to use it either to produce ATP or as reducing power for autotrophic growth. From the hydrogenase the electrons are transferred through series of cytochromes and eventually reduce oxygen to water (Bowien & Schlegel, 1981) (Fig. 3).

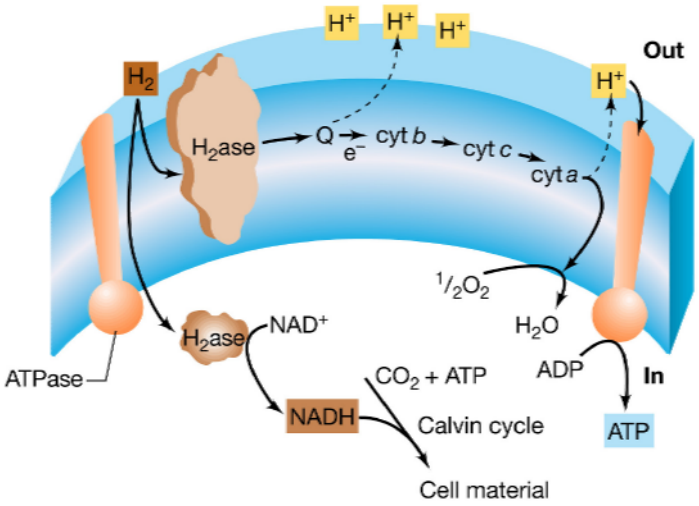


Figure 3. The oxidation of H_2 through hydrogenase, resulting in formation of water and cell material (Madigan et al., 2003)

All knallgas bacteria have hydrogenase, and some more than one. One of these hydrogenase is membrane bound and another is soluble in the cytoplasm. The membrane bound enzyme is involved in energetics while the soluble enzyme takes up hydrogen and reduces NAD^+ to NADH directly. Hydrogen has such a low reduction potential (-0.42 V) that reverse electron flow as a means of making reducing power is unnecessary (Madigan *et al.*, 2003). Many micro-organisms use this reaction for their metabolism.

2.5 Hydrogen-oxidizing bacteria (knallgas bacteria)

Aerobic, chemolithoautotrophic, HOX bacteria are often referred to as "the knallgas bacteria". This is a group of physiologically defined diverse group of bacteria from several taxonomic units. The main characteristic of this group is the ability to utilize gaseous hydrogen as electron donor with oxygen as electron acceptor and to fix carbon dioxide into cell material via the Calvin cycle (ribulose biphosphate cycle) or the reverse citric acid cycle (TCA) (Aragno & Schlegel, 1992; Madigan *et al.*, 2003). The "knallgas bacteria" strictly differs from other bacteria that utilize hydrogen in that way that other HOX bacteria do not fix CO_2 into cell material. Furthermore it differs from anaerobic hydrogen utilizing bacteria that utilizes e.g. sulfate, elemental sulfur or carbon dioxide as electron acceptors but not oxygen like the "knallgas bacteria". Most of the "knallgas bacteria" are facultative autotrophs, i.e. can also grow heterotrophically

using various organic compounds as electron donors and carbon sources (Aragno & Schlegel, 1992).

The best studied representatives of the "knallgas bacteria" are within the genera of *Wautersia* and *Pseudomonas.* Many more are found and have been studied, both gram negative and gram positive. Among hydrogen-oxidizers are bacteria that belong to the β- and γ-Proteobacteria, few moderately thermophilic but most are mesophilic species. Additionally, thermophilic bacteria within the *Aquificales* can oxidize hydrogen (Madigan *et al.*, 2003).

2.5.1 Physiology

Wautersia eutropha is one of the classical "knallgas bacteria" and is the best studied HOX bacteria. It has been used as a model for studying aerobic hydrogen oxidation (Madigan *et al.*, 2003; Schwartz & Friedrich, 2006).

Wautersia eutropha utilizes mixtures of hydrogen and carbon dioxide but can additionally utilize various organic substrates. When cultured on both hydrogen and organic compounds it utilizes both simultaneously. The metabolic pathways of *W. eutropha* have been well characterized and have helped greatly in understanding the metabolism of other HOX bacteria (Schwartz & Friedrich, 2006). The hydrogen metabolism is dependent to hydrogenase enzymes. *W. eutropha* has two energy generated hydrogenases that catalyses the hydrogen oxidation. One is membrane bound which feeds electrons into the respiratory chain via a cytochrome and one soluble tetrametric that couples the oxidation of hydrogen to reduction of NAD^+. The membrane bound enzyme is found in most of the HOX bacteria studied so far and is the basis of hydrogen oxidation. The soluble enzyme has only been found in very few HOX bacteria (Schwartz & Friedrich, 2006). The bacteria that have only the former type of enzyme cannot transfer electrons to NAD^+ for reduction and thus need a reverse electron transport for biosynthesis (Friedrich & Schwartz, 1993). Because hydrogenase is oxygen sensitive enzyme, most hydrogen bacteria grow best under microaerophilic conditions (5-10% oxygen concentration). It is also important to have nickel and/or iron present in the media because hydrogenases are metalloenzymes and are dependent on these metals (Madigan *et al.*, 2003).

Wautersia eutropha and most HOX bacteria use the Calvin cycle to fix carbon dioxide from the atmosphere. The Calvin cycle is the most widespread system known

for fixation of carbon dioxide. The key enzyme in the cycle is ribuolse bisphosphate carboxylase (RubisCO) which catalyses the first step of the cycle. Then it goes through series of enzymatic reactions (Bowien and Schlegel, 1981; Shively *et al.,* 1998). The final products of the Calvin cycle with the hexose are 12 molecules of 3-phosphoglyceric acid at the expense of 18 molecules of ATP and 12 of NADH. The hexose that is produced is used as cell material but the 3-phosphoglyceric acid is used to form new molecules of RubisCO (Shively *et al.*, 1998; Madigan *et al.*, 2003).

Alternative mechanism for carbon fixation found in thermophilic hydrogen oxidizing bacteria is the reverse citric acid cycle (TCA). As the name indicates the mechanisms uses the same steps as the citric acid cycle but in reverse order. Three molecules of CO_2 are used to form pyruvate which then forms hexose to make cell material. For every 3 molecules of CO_2, 5 molecules of ATP and 12 H^+ are needed. This pathway is thought to be an early form of autotrophy because it has been discovered in bacteria that are placed in the early branches of the phylogenetic tree of bacteria such as *Hydrogenobacter* (Madigan *et al.*, 2003).

2.5.1.1 Biomass formation

Many research have been done to study biomass formation in various HOX bacteria (Repaske & Mayer, 1976; Goto *et al.*, 1977; Bongers, 1970; Repaske, 1961; Ishizaki *et al.*, 2001). Different autotrophic bacteria have been tested for the optimization of gas ratios between hydrogen, oxygen and carbon dioxide. These ratios have turned out to vary substantially (Packer & Vishniac, 1955; Bongers, 1970; Takeshita & Ishizaki, 1996; Kwak *et al.*, 2006).

When energy is obtained from the "knallgas reaction" and carbon dioxide is fixed, two steps are needed:

$$2H_2 + O_2 \rightarrow 2H_2O \qquad (1)$$

$$2H_2 + CO_2 \rightarrow (CH_2O) + H_2O \qquad (2)$$

The first reaction generates ATP through hydrogen oxidation and leads to the formation of proton motive force (PMF). The second reaction shows the formation of biomass by means of the Calvin- or reverse TCA cycles. When the reactions are coupled together:

$$6H_2 + 2O_2 + CO_2 \rightarrow (CH_2O) + 5H_2O \qquad (3)$$

The final products of this reaction are biomass (cell material) and water (Madigan *et al.*, 2003).

No other nutrients but the gases are taken into account in these equations, such as nitrogen or phosphate. The exact composition of biomass is not known but the major elemental demands of *Wautersia eutropha* have been reported to be:

$$21.36H_2 + 6.21O_2 + 4.09CO_2 + 0.7NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.39}N_{0.76} + 18H_2O \quad (4)$$

Clearly, substantial amounts of hydrogen are needed to form biomass and considerable amounts of water are formed (Ishizaki & Tanaka, 1990; Takeshita & Ishizaki, 1996).

2.5.2 Distribution

Mesophilic HOX bacteria are widespread in the environment. They can be found in soils, fresh waters, superficial layers of sediments, activated sludge, hot composts, landfill percolating waters and geothermal areas. They can even be found in environments where the hydrogen concentration is not high enough to allow hydrogen uptake. This is one of the main reason that most mesophilic bacteria isolated from these environments are facultative autotrophs and can grow heterotrophically. They are able to utilize hydrogen when it becomes biologically available, i.e. when the concentration of H_2 increases for some reason for instance because of sudden fermentation activity. It is considered that HOX bacteria depend more on heterotrophic metabolism for survival but can switch to hydrogen metabolism when the conditions are favourable (Aragno, 1992a).

The main sources for thermophilic HOX bacteria are geothermal areas around the world. These areas are rich of hydrogen and reduced sulfur compounds but organic matter is scarce. These conditions are though suitable for obligate lithoautotrophs that can also utilize other compounds present, i.e. carbon monoxide, ferrous iron and more (Friedrich & Schwartz, 1993). Another important factor in the geothermal areas is the oxygen which is commonly present in very low concentrations. The main source of oxygen in geothermal fluids is through interaction with the atmosphere. This results in low oxygen concentrations creating good conditions for microaerobic bacteria. In areas where the temperature is above 90°C the solubility of oxygen in the water is almost none, since it decreases with increased temperature, and the gases are constantly

bubbling through the geothermal fluids the conditions become almost anaerobic. These geothermal gases are prone to oxidation and react with the small amounts of oxygen present, creating even more anaerobic conditions. This prohibits the growth of hydrogen oxidizing bacteria and anaerobic hydrogenotrophs become dominant (Aragno, 1992a; Stetter, 1999).

Thermophilic bacteria have also been isolated from cold non-geothermal habitats such as soil, drainage and composts (Aragno, 1992a; Beffa *et al*., 1996a). These habitats are likely to harbor HOX bacteria because concentration of hydrogen can be relatively high as a result of fermentation by anaerobic bacteria. The transient phase between the aerobic and the anaerobic layers has most of the abilities to be good habitats for microaerophilc HOX bacteria because oxygen is present in low concentrations from the top layer and hydrogen produced by the lower layer (Aragno, 1992a).

The main ecological factors that determine the presence or absence of HOX bacteria are pH, temperature, water and stability of the habitat. The diversitiy in species compositions is due to the diversity in the habitats (Aragno, 1992a; Aragno, 1992b).

2.5.3 Thermophilic hydrogen-oxidizing bacteria

Research on thermophilic, hydrogen-oxidizing bacteria have increased significantly in the recent years and many new species and genera have been observed. By using molecular techniques it has revealed that HOX bacteria are in many cases dominating in thermal environments, especially at temperatures below 80°C and neutral pH (Reysenbach *et al*., 2005).

Goto and coworkers (1977) isolated and described the first two isolates of moderately thermophilic, hydrogen oxidizing bacteria of the genera *Pseudomonas* and *Flavobacterium* (Goto *et al.*, 1977; Goto *et al.*, 1978). These strains were later reassigned as *Hydrogenophilus thermoluteolus* (Hayashi *et al.*, 1999). The first thermophilic, obligate hydrogen oxidizing bacteria described was *Hydrogenobacter thermophilus* in the 1980's (Kawasumi *et al.*, 1984).

Most of the thermophilic HOX bacteria belong to the family of *Aquificacaea* (Huber *et al.*, 1998; Nakagawa *et al.*, 2005). The family of *Aquificacaea* belongs to the order of *Aquificales* and represent a deep phylogenetic branch within the tree of life (16S rRNA). Their chemolithotrophic way of life makes them the primary producers of

bacterial ecosystem at high temperatures. Most of them perform the "knallgas reaction" (Huber & Eder, 2006)

At higher temperatures from 75-90°C *Aquificaceae* species like *Thermocrinis* are dominating while *Hydrogenobacter* and *Hydrogenobaculum* species are found at temperatures from 60-80°C (Reysenbach *et al.*, 2005). Most of the thermophilic HOX bacteria have been isolated from geothermal areas but with few exceptions. Temperature and pH seem to be the main factors controlling the species composition. Some of the thermophilic bacteria are neutrophilic while others grow at acidic pH (Aragno, 1992a). Most of the thermophiles are obligate chemolithoautotrophs but few species are able to utilize organic compounds for growth (Huber *et al.*, 1998; Nakagawa *et al.*, 2005; Reysenbach *et al.*, 2005). Carbon dioxide is most commonly fixed with the reductive TCA cycle but not with the Calvin-cycle like most mesophilic HOX bacteria. Some of these species are also able to use alternative electron donors, such as reduced sulfur compounds instead of hydrogen (Reysenbach *et al.*, 2005).

Known hydrogen oxidizing species within the *Aquificales* are *Hydrogenobacter* (Kawasumi *et al.*, 1984), *Thermocrinis* (Huber *et al.*, 1998), *Hydrogenobaculum* (formerly *Hydrogenobacter*) (Seigo & Suzuki, 1993; Stöhr *et al.*, 2001b), *Sulfurihydrogenibium* (Takai *et al.*, 2003), *Persephonella* (Götz *et al.*, 2002), *Hydrogenothermus* (Stöhr *et al.*, 2001b) and *Aquifex* (Huber *et al.*, 1992).

2.5.3.1 *Hydrogenophilus*

One of the main outcome of present study was the isolation of a new species closely related to *Hydrogenophilus* species. The genus *Hydrogenophilus* was created in 1999 by Hayashi and co-workers (Hayashi *et al.*, 1999). The genus belongs to the *β*-Proteobacteria which represent a very diverse group of bacteria considering metabolics, morphology and ecology. The members of this phylum are e.g. purple non sulfur phototrophs, chemolithotrophs, chemoorganotrophs, methylotrophs and nitrogen fixing bacteria (Kersters *et al*., 2006).

As mentioned earlier the first isolates of *Hydrogenophilus* were isolated from soils around hot springs. The isolates were initially assigned to the genera *Pseudomonas* and *Flavobacterium* (Goto *et al.*, 1977; Goto *et al.*, 1978). Further studies assigned these two isolates to the new genus *Hydrogenophilus* gen. nov. (Hayashi *et al.*, 1999). Known species within *Hydrogenophilus* today are *H. thermoluteolus* isolated from

geothermal site in Japan (Goto *et al.*, 1977; Goto *et al.*, 1978; Hayashi *et al.*, 1999) and *H. hirschii* isolated from hot spring in Yellowstone National Park (Stöhr *et al.*, 2001a). On databases there are however several *Hydrogenophilus* species unpublished, indicating that there may be more species emerging in near future.

Hydrogenophilus sp. are gram negative, rod shaped, moderately thermophilic bacteria, chemolithotrophic using molecular hydrogen as energy source and carbon dioxide as a carbon source. Members of *Hydrogenophilus* use the Calvin cycle to fix CO_2 into cell material (Hayashi *et al.*, 1999).

2.6 Inorganic sulfur compounds

Sulfur compounds are common throughout the world. They are found in soils e.g. in the form of sulfide (FeS_2) or sulfate ($CaSO_4*2H_2O$) minerals, in the atmosphere e.g. in the form of sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) and oceans are rich of sulfate (Kleinjan, 2005). Sulfur compounds are also found in natural fresh waters. In aerated waters the sulfur is in the form of sulfate but in anaerobic waters as sulfide (H_2S and HS^-). The sulfur compounds can be in relatively high concentration providing good habitats for SOX bacteria (Druschel *et al.*, 2003).

The sulfur compounds circulate between soil, ocean, air and living matter in geochemical and biological sulfur cycles. The geochemical sulfur cycle represent evaporation, sediments and rain but the biological sulfur cycle stands for oxidation and reduction of sulfur compounds by living organisms (Kleinjan, 2005). The sulfur cycle is described in the following chapter.

2.6.1 The sulfur cycle

The sulfur cycle compromises an interrelated set of oxidation/reduction reactions of inorganic and organic sulfur compounds through several common intermediates (Sorokin, 2003; Kleinjan, 2005).

The sulfur cycle has both sedimentary and gaseous phases. The bulk of the sulfur of the Earth is bound in sediments and rocks in the form of minerals, often gypsum ($CaSO_4$) and pyrite (FeS). From the sediments the sulfur is carried to the

ecosystem as salt solution, formed by weathering, decomposition and by oxidation and reduction of bacteria.

In the gaseous phase of the sulfur cycle the sulfur compounds enter the atmosphere from several sources. The main source is volcanic activity, but fermentation, burning of fossil fuels, exchange at the surface of the ocean and gases released by decomposition are also sources for sulfur compounds (Smith & Smith, 2003). Sulfate reducing bacteria produces sulfide with anaerobic reduction of sulfate (SO_4^{2-}). This reduction can either occur via dissimilatory reduction where inorganic compounds are reduced into other inorganic compounds, or when inorganic compounds bind to organic compounds, e.g. proteins. If the inorganic compound binds to organic compound the sulfide is formed by mineralization of the organic compound (Kleinjan, 2005).

The anaerobic sulfur reducing bacteria are present in many habitats but there are limits of the sulfide production. The reducing bacteria are dependent on organic matter or hydrogen as energy sources and if these materials are not available there is no production of sulfide. The production rate can increase rapidly by the addition of organic matter. This is important because if for instance a huge amount of organic matter is dumped e.g. in the sea, production rate of sulfide in the sediments can increase significantly causing pollution. Sulfide is considered to be pollutant because it binds with the iron in the cytochromes or other iron compounds in the cells. In the environment sulfide is detoxified when it binds with iron, forming insoluble precipitation of FeS (Madigan *et al.*, 2003).

One of the major reactions in the sulfur cycle is the biological oxidation of hydrogen sulfide to sulfate (Friedrich *et al.*, 2001). When the sulfur enters the atmosphere as sulfide it quickly oxidizes chemically or is biologically oxidized by sulfur bacteria (Smith & Smith, 2003; Madigan *et al.*, 2003). The biological oxidation occurs in the soil on the boundaries between the anoxic and aerobic layer where oxygen from the aerobic layers is present and the sulfide rises from the anaerobic layers. The biological oxidation can also occur in the anaerobic layers where sunlight is present by phototrophic bacteria. The product of biological oxidation is sulfate (SO_4^{2-}) (Madigan *et al.*, 2003) and the product from chemical oxidation is sulfur dioxide (SO_2). The sulfate from the oxidation lowers the pH in the environments by forming sulfuric acid but the sulfur dioxide is soluble in water and is carried back to the surface as rainwater in the

form of weak sulfuric acid (H_2SO_4), often referred to as acid rain (Smith & Smith, 2003). Figure 4 shows simple version of the biological sulfur cycle.

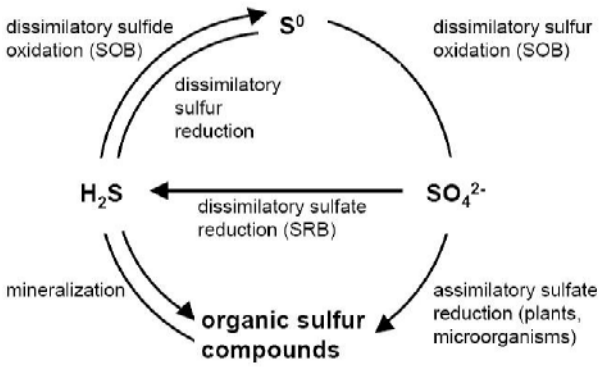


Figure 4. The biological sulfur cycle. SOB: sulfur oxidizing bacteria, SRB: sulfate reducing bacteria (Kleinjan, 2005).

The gaseous phase of the sulfur cycle causes circulation of sulfur on a global scale. When the sulfur re-enters the surface it is taken up by plants and incorporated through series of metabolic processes or utilized by bacteria as energy source and is released again as hydrogen sulfide or sulfate (Smith & Smith, 2003; Madigan *et al.*, 2003).

In natural ecosystems the sulfur cycle should be in balance. That means that the amount of sulfide oxidized should be corresponding to the amount of sulfate reduced. This occurs in synthrophic communities of bacteria in which H_2S produced by sulfate reducing bacteria is re-oxidized by SOX bacteria (Kleinjan, 2005).

2.6.2 Oxidation of inorganic reduced sulfur compounds by bacteria

A number of sulfur compounds can serve as electron donors in oxidation of inorganic sulfur compounds by bacteria. Some of them are normally present in geothermal sites (H_2S, elemental sulfur and SO_2) while others (thiosulfate and various polythionates) do not exist as minerals and would not be present if it wasn't for biological oxidation (Aragno, 1992b). Biological oxidation of sulfide (H_2S) and sulfur is common in soil and water and is the major reaction in volcanic and other extreme environments. The oxidation reactions in these ecosystems are performed by prokaryotes of the domains *Archaea* and *Bacteria* (Friedrich *et al.*, 2005). Many inorganic reduced sulfur

compounds can be used as electron donors for the SOX bacteria. The most widespread are sulfide (H_2S), elemental sulfur (S^0) and thiosulfate ($S_2O_3^{2-}$). Usually the final product of oxidation of these compounds is sulfate (SO_4^{2-}) (Madigan *et al.*, 2003; Aragno, 1992b) but incomplete oxidation can also occur. In incomplete oxidation the production of intermediates is dependent on factors such as microbial species involved, pH, concentration of sulfur compounds and oxygen partial pressure (Shreiber & Pavlostathis, 1998).

The oxidation of the most reduced compound, H_2S, occurs in stages. In the first step elemental sulfur is formed. Some H_2S oxidizing bacteria keep the elemental sulfur inside the cells as an energy reserve to use when the sulfide has been depleted. Then it can than get additional energy from oxidation of elemental sulfur to sulfate. In other cultures cells secrete the sulfur particles from the cells (Madigan *et al.*, 2003; Masau *et al.*, 2001). When sulfur is provided as electron donor or it accumulates outside of the cells the microorganisms must grow attached to the sulfur particles because of its insolubility to obtain the atoms of sulfur needed. It is thought to occur that way that sulfur is dissolved by the action of membrane or periplasmic proteins that probably reduce S^0 to HS^-, which is then transported into the cell and used as an electron donor (Madigan, *et al.*, 2003). In oxygen limiting conditions sulfur is the end product of the oxidation instead of sulfate (Kleinjan, 2005). The next step in the oxidation is the formation of SO_3^{2-}, a six electron oxidation. If S^0 is the substrate, sulfide is the first product because the sulfur reduces to H_2S which then oxidizes to SO_3^{2-} (Madigan *et al.*, 2003; Masau *et al.*, 2001). When thiosulfate is the electron donor it splits up in S^0 and SO_3^{2-} (Kelly, 1999), both of which are eventually oxidized to SO_4^{2-} (Madigan *et al.*, 2003; Masau *et al.,* 2001). Oxidation of SO_3^{2-} to SO_4^{2-} can occur in two different ways. The most widespread mechanism uses the enzyme sulfite oxidase which transfers electrons directly to cytochrome *c* and ATP is produced by proton motive force (PMF). The other way includes the enzyme adenosine phophosulfate reductase (APS). This enzyme is a key enzyme in the metabolism of sulfate reducing bacteria but in this case the function is reverse. This metabolism produces one high energy phosphate bond when AMP is converted to ADP (Madigan *et al.,* 2003). Because of these two different pathways in oxidation of sulfur compounds there is a possibility for different intermediates to be formed. Some bacteria can only take part in some of the steps, e.g.

only oxidize thiosulfate to sulfate. Thus the oxidation sometimes is a result of oxidations carried out by many bacteria (Kleinjan, 2005; Masau *et al.*, 2001).

On the basis of physiological and biochemical data two groups of chemolithotrophic SOX bacteria have been described. One is a group of SOX bacteria capable of utilizing polythionates while the other group incapable of doing so. Two major pathways have been described; the sulfur oxidizing pathway (PSO) not involving polythionates and the S_4 intermediate pathway (S4I) involving polythionates (Friedrich *et al.*, 2001; Kelly *et al.*, 1997; Masau *et al.*, 2001). The PSO-pathway is present e.g. in some *Paracoccus* species. Among facultative heterotrophs also capable of autotrophic growth this pathway is prevalent. The S4I pathway is present in all obligately chemolithotrophic thiobacilli and other 'true' thiobacilli species (Kelly *et al.*, 1997).

Only few enzymes have been demonstrated to catalyze key reactions in the two pathways. In the S4I pathway the tetrathionate syntase (or thiosulfate oxidizing enzyme) is the key enzyme. This enzyme oxidatively condenses two thiosulfate ions to make $S_4O_6^{2-}$. Among SOX chemolithotrophs sulfite oxidase is the key enzyme, converting sulfite to sulfate. In some bacteria the enzyme APS reductase also catalyzes this reaction. One enzyme, rhodanese, has been reported to be universal among bacteria whether or not they are sulfur oxidizers but it seems, at least in some SOX bacteria, to affect the cleavage of thiosulfate into sulfite and a sulfane product (sulfur) (Kelly *et al.*, 1997).

All the electrons from the oxidation of reduced sulfur compounds are used for energy transformation of the respiratory chain and for autotrophic carbon dioxide reduction (Friedrich *et al.*, 2005). The electrons are fed into the electron transport chain by oxygen which leads to PMF used for the production of ATP. NADH from the oxidation of thiosulfate and sulfur in this mechanism has to be produced with energy consuming reactions of reverse flow since the electron donors have more electropositive E_0' than NAD^+/NADH (Madigan *et al.*, 2003; Aragno, 1992b).

Much work has been done to clarify the pathways of oxidation of sulfuric compounds to sulfate and to establish the mechanisms and efficiency of the coupling of the energy released to growth of the bacteria. A factor that has impeded the understanding of sulfur oxidation mechanism was the finding that two or more oxidation pathways exist and the fact that the ability to utilize this source of energy is widespread among diverse genera of Proteobacteria and the extremely

thermoacidophilic archaea. The difference in oxidation of Proteobacteria can appear e.g. in different energy conservation or different oxidation mechanisms. The archaea on the other hand achieve the same oxidation products but use enzyme systems that are unrelated to the bacterial systems (Kelly, 1999).

Estimates of the efficiency of energy production from sulfur compounds have been made from thermodynamic calculations and measurement of growth and carbon dioxide assimilation (Kelly & Wood, 2006). To estimate the efficiency of energy conservation in sulfur oxidation the relationship between the free-energy change (energy output) from the oxidation of the substrates and the free-energy requirement (energy input) for carbon dioxide fixation to support the autotrophic growth has been used. The main point in these calculations is the Gibbs free energy of formation of the substrate and compound (ΔG_f^0 in kJ mol^{-1}) which has been calculated in literature for many compounds (Madigan *et al*., 2003). The standard free energy changes in the few reactions in sulfur oxidation have been calculated and are shown in table 2.

Table 2. Free energy changes for inorganic sulfur oxidations and for thiosulfate cleavage reactions (taken or calculated, using free energies of formation) (Kelly, 1999).

Reaction	**ΔG^0 [kJ (mol S-substrate)$^{-1}$]**
$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$	-738.7
$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2HSO_4^-$	-761.4
$S_2O_3^{2-} \rightarrow S^0 + SO_3^{2-}$	+26.8
$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$	-6.1
$H_2S + 2O_2 \rightarrow H_2SO_4$	-714.1
$S^{2-} + 2O_2 \rightarrow SO_4^{2-}$	-658.8
$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$	-732.6
$S^0 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$	-507.4
$S^0 + 1.5O_2 + OH^- \rightarrow HSO_4^-$	-598.7
$H_2S + 0.5O_2 \rightarrow S^0 + H_2O$	-209.3
$S^{2-} + 0.5O_2 + 2H^+ \rightarrow S^0 + H_2O$	-151.4
$HS^- + 0.5\ O_2 \rightarrow S^0 + OH^-$	-145.2
$S^0 + O_2 + H_2O \rightarrow SO_3^{2-} + 2H^+$	-249.4
$S^0 + O_2 + H_2O \rightarrow HSO_3^- + H^+$	-290.6
$H_2SO_3 + 0.5O_2 \rightarrow H_2SO_4$	-204.1
$SO_3^{2-} + 0.5O_2 \rightarrow SO_4^{2-}$	-258.0
$HSO_3^- + 0.5O_2 \rightarrow HSO_4^-$	-228.2

2.7 Sulfur-oxidizing bacteria

The physiological group of aerobic, SOX bacteria compromises mesophilic as well as thermophilic *Bacteria* and *Archaea* (Aragno, 1992b). There is a long history in studying inorganic sulfur compounds as electron donors for chemolithotrophic and phototrophic bacteria. The sulfur bacteria were recognized in the 19th century and the filamentous sulfur bacteria were the subject of Winogradskys studies in 1887 (Kelly *et al.*, 1997). His initial research on chemosynthesis were on these filamentous sulfur bacteria, particularly on a community of *Beggiatoa* with other micro-organisms. The sulfur bacteria were demonstrated to oxidize hydrogen sulfide to sulfur which accumulated in the cells and was later oxidized to sulfate (Karavaiko *et al.*, 2006). However, Martinus Beijernick and Cornelis van Niel did the real pioneering studies on SOX bacteria and isolated and named the two first species, *Thiobacillus thioparus* and *Thiobacillus denitrificans*. Their research layed the foundation of the role played by sulfur compounds in the photosynthesis of the anaerobic sulfur phototrophs (Kelly *et al.*, 1997).

There is a wide variety of SOX bacteria, consisting of different groups and genera. Each bacterium has different properties in relation to source of energy, sulfide oxidizing pathway, size and shape and the location in storing intermediate sulfur. The classification of these bacteria is not simple. Different bacteria belonging to the same family can have different properties. The most important classification is the distinction between phototrophic (purple and green) and chemotrophic (colourless) SOX bacteria. Phototrophic SOX bacteria gain energy from sunlight to reduce CO_2 to carbohydrates while chemotrophic SOX bacteria gain energy from chemical aerobic oxidation of reduced inorganic sulfur compounds. The phototrophic bacteria uses reduced sulfur compounds as electron donors under anaerobic conditions while the chemotrophic bacteria oxidizes reduced sulfur compounds as electron donors (Kleinjan, 2005). Other characteristic of the colourless sulfur bacteria, unique to this group is big cell size and intracellular deposition of elemental sulfur (Karavaiko *et al.*, 2006).

2.7.1 Physiology

As mentioned earlier all bacteria which are referred to as SOX bacteria are able to oxidize inorganic reduced sulfur compounds to obtain energy in the presence of

electron acceptor (e.g. oxygen or nitrogen oxides) although environmental factors may vary (e.g. pH and temperature). Chemolithotrophic SOX bacteria are split up in different classes according to the optimal pH; those living at neutral, acidic (Madigan *et al.*, 2003) and alkaline pH's (Friedrich *et al.*, 2005). Aragno (1992b) divided the SOX bacteria into three groups according to their metabolism. The first group contains the "knallgas bacteria", isolated under HOX conditions, which are also able to grow with reduced sulfur compounds as electron donors. Good examples of bacteria belonging to this group are bacteria belonging to the phylum *Aquificae*, such as *Aquifex, Hydrogenobacter* and *Hydrogenobaculum* (Miyake *et al*., 2007). The next group contains strictly thermophilic sulfur-oxidizing bacteria (e.g. the genus *Thermothrix*) and in the last group there are moderately thermophilic, thiobacilli-like bacteria (Aragno, 1992b). One more group has been defined containing moderately thermophilic, strongly acidophilic SOX and iron oxidizing bacteria (Skirnisdottir *et al.*, 2001).

As mentioned earlier different SOX bacteria have different metabolic pathways in the sulfur oxidation resulting in different intermediates and oxidation products (Friedrich *et al*., 2001). The most common electron donors are hydrogen sulfide, elemental sulfur, thiosulfate and polythionates. Electrons from these compounds enter the respiratory chain at an intermediary level. The reduction of NAD(P) implies a reverse electron flow with energy (ATP) consumption. In general, the end product of the oxidation is sulfate, which forms sulfuric acid causing the pH in the media or surroundings to drop (Aragno, 1992b). Heterotrophic SOX bacteria are different from the chemolithotrophic being only able to partially oxidize inorganic sulfur compounds. The final products in such oxidations are polythionates which results in rising but not drop in the pH in the medium or surroundings (Robertson & Kuenen, 2006).

Chemolithoautotrophic SOX bacteria generally fix carbon dioxide through the Calvin cycle even though there are some exceptions, like *Hydrogenobacter*, that fix carbon dioxide through reverse TCA cycle (Aragno, 1992b).

Not all sulfur oxidizing bacteria are able to couple sulfur oxidation to growth. Studies have shown clear evidence of sulfur oxidation i.e. decrease in pH and accumulation of sulfate in the medium but no growth detected by measurement of the optical density. Example of these kind of sulfur oxidizers is *Hydrogenophilus thermoluteolus* (Miyake *et al*., 2007).

2.7.2 Distribution

SOX bacteria are widespread in nature and are frequently found in soil, sediments and freshwater and marine environments, geothermal or non-geothermal (Robertson & Kuenen, 2006). Most geothermal environments provide suitable habitats for SOX bacteria being rich of reduced sulfur compounds (Odintsova *et al.*, 1996; Aragno, 1992b). However, it has been shown that the sulfur compounds do not have to be present in high concentration, but more important is to have simultaneous presence of an electron donor (sulfur compound) and electron acceptor (e.g. oxygen or nitrogen oxides) (Robertson & Kuenen, 2006; Karavaiko *et al.*, 2006). Some research data has shown that the concentration of sulfide may effect the microbial composition, where different species are dominating in high sulfide concentration and low concentration (Nakagawa & Fukui, 2003).

In hot springs the growth of SOX bacteria is often limited by the low solubility of oxygen at high temperatures. Other factor that limits bacterial growth is abiotic oxidation of the sulfur compounds (Odintsova *et al.*, 1996). That is why SOX bacteria are often present in narrow zones where sulfide and oxygen coexist such as for example stratified lakes, the interface between anaerobic sediment and aerobic water, anaerobic pockets and hot-springs (Robertson & Kuenen, 2006). Of all the reduced sulfur compounds present, sulfide is most important and, because of its oxidation state it gives the most energy (Madigan *et al.*, 2003).

2.7.3 Thermophilic sulfur-oxidizing bacteria

The first SOX bacteria isolated from geothermal area were *Sulfomonas* and *Thiobacillus termitanus*, strains that are now lost. As mentioned earlier Aragno (1992b) has divided thermophilic sulfur oxidizing bacteria into three categories, "knallgas" bacteria also able to oxidize sulfur compounds, strictly thermophilic sulfur oxidizers belonging to the genus *Thermothrix* and moderately thermophilic thiobacilli like species.

The first isolated strictly thermophilic SOX bacteria isolated was *Thermothrix thioparus*, a neutrophilic bacteria growing between 60 and 80°C (Aragno, 1992b; Caldwell *et al.*, 1976; Brannan & Caldwell, 1983). More recently new thermophilic SOX bacteria have been isolated, e.g. within the genuses *Sulfurihydrogenibium* (Takai

et al., 2003; Aguiar *et al*., 2004), *Hydrogenobacter* (Takai *et al*., 2001), *Thiobacter* (Hirayama *et al*., 2005) and more. Other well known sulfur chemolithotrophs are thiobacilli like species, *Paracoccus*, *Xanthobacter*, *Sulfolobus, Acidianus* and *Desulfurolobus* (Kelly, 1999).

2.7.3.1 *Thiomonas*

One of the main outcome of present investigation was the isolation of a new species closely related to the genus *Thiomonas*. The genus *Thiomonas* was proposed by Moreira and Amils (1997) to accommodate four former *Thiobacillus* species, *T. thermosulfatus, T. perometabolis, T. intermedius* and *T. cuprinus*. The classification is based on the ability of these bacteria to grow autotrophically, heterotrophically as well as mixotrophically and their phylogenetic relation (Moreira & Amils, 1997). Later two other species have been assigned to the genus *Thiomonas, Tm. delicata* (Katayama-Fujimura *et al.*, 2006), the former *Thiobacillus delicatus* (Katayama-Fujimura *et al.*, 1984) and *Tm. arsenivorans* (Battaglia-Brunet *et al.*, 2005) but the latter one has not yet been accepted. Recently, Kelly and co-workers (Kelly *et al*., 2007) made a suggestion for the reassessment of the phylogenetic relationship of *Tm. cuprina* with other *Thiomonas* species, but *Tm. cuprina, Tm. arsenivorans* and *Tm. delicata* only differ in three nucleotides from full 16S rRNA analysis. These species are thus indistinguishable but are still considered to be three species based on their physiological characteristics.

Since the genus *Thiobacillus* was first described in 1904 all gram negative, non-phototrophic rod shaped bacteria capable of utilizing sulfur compounds as energy source were classified as *Thiobacillus* species (Robertson & Kuenen, 2006). In addition almost all species within the "former" *Thiobacillus* genus possess the enzyme ribulose biphosphate carboxylase and can thus fix atmospheric carbon dioxide. Many of the former *Thiobacillus* species are also able to grow heterotrophically on various organic compounds. The variety in metabolism, G + C genomic composition and DNA-DNA hybridization values lead to further phylogenetic analysis. This revealed that *Thiobacillus* species were distributed to three subclasses of the Proteobacteria, α, β and γ. These results showed that the ability to oxidize sulfur compounds is found in many groups of gram negative bacteria and sulfur oxidation together with rod shaped morphology could not be used to include new species to the genus *Thiobacillus.* To complicate the situation even more some photosynthetic and hydrogen oxidizing

bacteria can also grow autotrophically by using sulfur compounds as energy source (Moreira & Amils, 1997).

Bacteria belonging to the new genus *Thiomonas* belong to the *β* subclass of Proteobacteria (Robertson & Kuenen, 2006). They are gram-negative, rod shaped and motile (Moreira & Amils, 1997; Kelly & Wood, 2006). They are facultative chemolithoautotrophic growing optimally in mixotrophic media containing both reduced sulfur compound and organic substrate. Heterotrophic growth occurs on various organic compounds such as yeast extract, peptone, some sugars and amino acids. Chemolithotrophic growth is in the presence of reduced sulfur compounds, such as sulfide, sulfur, thiosulfate and tetrathionate (Moreira & Amils, 1997; Battaglia-Brunet *et al*., 2005; Kelly & Wood, 2006). In the absence of organic substrate *Thiomonas* sp. can grow autotrophically using sulfur compounds (London, 1963; Shooner *et al.*, 1996).

The first *Thiomonas* species, *Tm. intermedia* (London, 1963; London & Rittenberg, 1967) were isolated from soils but more recently strains have been isolated from mining sites where soluble metals are present in relatively high concentrations (Battaglia-Brunet *et al*., 2005) and from activated sludge (Chen *et al.*, 2004). *Thiomonas cuprina* has been demonstrated to be able to grow using variety of sulfide ores (Huber & Stetter, 1990) and some *Thiomonas* species are able to oxidize Fe(II) (Coupland *et al.*, 2004; Johnson and Hallberg, 2005) and arsenic (Bruneel *et al*., 2003; Battaglia-Brunet *et al*., 2002).
Recent studies have also shown that *Thiomonas* sp. closely related to *Thiomonas perometabolis*, *Tm. thermosulfata* and *Tm. intermedia* has given promising results in biological deodorization based on its fast oxidation rates of H_2S and its removal efficiency (Chen *et al.*, 2004).

Only one strain within the genus *Thiomonas* is moderately thermophilic, *Tm. thermosulfata* with optimum temperature of 50°C (Shooner *et al.*, 1996). Others are mesophilic with temperature optimum from 30 – 37°C (Battaglia-Brunet *et al*., 2005; London & Rittenberg, 1967; London, 1963; Huber & Stetter, 1990).

2.7.3.2 *Hydrogenobacter*

One of the main outcome of the present study was the isolation of two strains within the genus Hydrogenobacter. The genus *Hydrogenobacter* was initially suggested by

Kawasumi and co-workers (1984) when the bacterium was first isolated from hot spring in Japan (Kawasumi *et al.*, 1984) and Kamchatka (Kryukov *et al.*, 1983). Later, *Hydrogenobacter* species have been isolated from hot springs in Iceland and Italy (Krisjánsson *et al.*, 1985; Aragno, 1992b).

Species of the genus *Hydrogenobacter* are gram negative, rod shaped and most strains are chemolithoautotrophic using hydrogen or reduced sulfur compounds as electron donors. The species are thermophilic with optimum growth temperatures from 65-72°C (Kawasumi *et al.*, 1984; Kryukov *et al.*, 1983; Krisjánsson *et al.*, 1985; Aragno, 1992b; Stöhr *et al.*, 2001b). Two *Hydrogenobacter* sp. ve been isolated able to grow on sulfur compounds but not on hydrogen (Skirnisdottir *et al.*, 2001; Takai *et al.,* 2001).

Phylogenetic analysis based on 16S rRNA sequencing has revealed that *Hydrogenobacter* is on the same lineage as the genera *Aquifex*. These genera are distinctly sperated from all other bacteria and form the lowest phylogenetic branch in the phylogenetic tree of bacteria (Hreggvidsson *et al*., 1995; Shima *et al*., 1994).

2.8 Sulfur-oxidizing "knallgas bacteria"

Several species of "knallgas bacteria" are able to use reduced sulfur compounds, instead of hydrogen as electron donors (Aragno, 1992b). These bacteria have often been isolated as hydrogen oxidizers but later it has been discovered that the isolates can also oxidize sulfur compounds (Miyake *et al*., 2007; Stöhr *et al.*, 2001b).

During the last decade studies using culture-dependent isolation techniques or culture independent molecular analytical methods have demonstrated that thermophilic, chemolithotrophic, hydrogen- and/or sulfur-oxidizing bacteria within the order *Aquificales* and *β*-Proteobacteria are widespread in terrestrial hot-springs at high temperature with neutral to alkaline pH (Huber *et al*., 1998; Stöhr *et al*., 2001; Hirayama *et al*., 2005).

Good example of sulfur-oxidizing "knallgas bacteria" are *Hydrogenobacter* species (Aragno, 1992b) which all have been demonstrated to be able to utilize both hydrogen and sulfur as electron donors except for two, who are not able to utilize hydrogen as electron donor (Skirnisdottir *et al.,* 2001; Takai *et al*., 2001). Other

examples for sulfur-oxidizing "knallgas" bacteria are *Hydrogenovibrio marinus* and some *Aciditiobacillus* species (Miyake *et al*., 2007).

In general growth under SOX conditions is much more microaerophilic than under HOX conditions. Therefore, it is probable that similar organisms would be isolated from enrichment isolated under SOX conditions with low partial pressure of oxygen (Aragno, 1992b).

3. Research of the present study

All the enrichment cultures chosen in the present study showed HOX capacity, but some were also very interesting because of their SOX metabolism. Hereafter, a brief summary of the purification processes for the four enrichment cultures are presented. Chemolithotrophic (HOX, SOX) and mixotrophic metabolism of the isolates are described and hydrogen oxidizing capacity compared to results from previous study (Reynisdottir, 2007). Chapter 2 and 3 are manuscripts of characterization papers on strain 16C (*Hydrogenophilus*) and 6C (*Thiomonas*), respectively.

3.1 Strain 16C

3.1.1 Purification of enrichment culture

After several enrichments and end point dilutions on a sample from a hot spring under HOX condition the cultures were sent for partial 16S rRNA analysis. The moderate thermophilic culture 16C was analysed for 743 nucleotides revealing bacteria closely related to *Hydrogenophilus denitrificans* (97%). Before full 16S rRNA analysis was performed the enrichment cultures were re-inoculated several times to maintain growth. The results from the full 16S rRNA analysis indicated that instead of *Hydrogenophilus denitrificans* it seemed that a heterotrophic contaminant, *Meiothermus sylvanus*, was now the dominating species present. To find out if the (HOX) bacteria were still present in the cultures denaturing gradient gel electrophoresis (DGGE) was done on both enrichments (Reynisdottir, 2007). The results from the DGGE analysis indicated both chemolithotrophic and heterotrophic bacteria. In addition to *M. sylvanus, Anoxybacillus amylolyticus* was shown to be present. It was clear that heterotrophic contaminants were persistent under the growth conditions used.

In the study now presented, colonies were isolated from the mixed culture under HOX conditions. This was done by using solid media on Petri dishes inoculated in boxes containing hydrogen, carbon dioxide and oxygen. From enrichment 16C, three colonies were obtained, which were then re-inoculated into liquid medium. This process was repeated and colonies obtained again. These colonies were submitted for partial

16S rRNA analysis which all indicated the presence of *Hydrogenophilus* species to be present (Fig 5.). Hence, pure cultures of *Hydrogenophilus* were obtained (**I**).

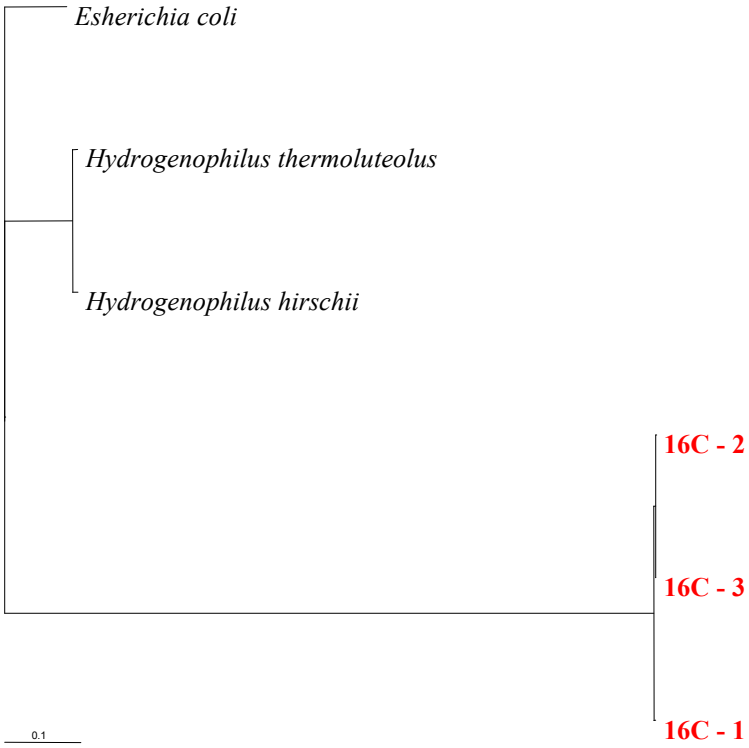


Figure 5. Phylogenetic tree showing relation of the three pure cultures isolated to Hydrogenophilus thermoluteolus (AB009828) and Hydrogenophilus hirschii (AJ131694) with E. coli (J01859) as outgroup.

3.1.2 HOX and SOX metabolism

Hydrogenophilus sp. have been well reported to be able to utilize hydrogen (Goto *et al.,* 1977; Goto *et al*., 1978; Hayashi *et al.,* 1999; Stöhr *et al*., 2001). Enrichment culture 16C, wherefrom strain 16C was isolated, was a very active HOX enrichment culture showing hydrogen uptake rate of 1.64 mmol H_2 L^{-1} h^{-1} and 20.5 mmol H_2 L^{-1} h^{-1} in batch and fed-batch cultures, respectively (Reynisdóttir, 2007). The biomass yield was 3.18 g mol^{-1} H_2^{-1} and 4.52 g mol^{-1} H_2 under batch and fed batch conditions, respectively. Pure culture of *Hydrogenophilus* showed uptake rate of 0.49 mM H_2 L^{-1} h^{-1} and biomass yield of 3.11 g mol^{-1} H_2^{-1} in batch culture (**I**).

Strain 16C could not grow on thiosulfate (**I**) but preliminary experiments showed that the strain was able to oxidize hydrogen in the presence of hydrogen sulfide but no production of sulfate was detected (data not shown).

3.1.3 Mixotrophic growth

Strain 16C was able to grow on various organic compounds tested (**I**) in the absence and presence of yeast extract. Without yeast extract the best growth was observed on butyrate. Other substrates that strain 16C utilized were various complex substrates like casamino acids, beef extract, tryptone as well as, propionate, lactate and pyruvate. Weak growth was observed on crotonate and peptone. In the presence of yeast extract more profound growth was observed on most of the substrates mentioned above. Additionally growth was observed on fructose, glucose, acetate and malate in the presence of yeast extract.

Studies on mixotrophic growth characteristics of strain 16C were done using butyrate as carbon source and hydrogen as electron donor. The hydrogen and butyrate uptake rates were 0.15 mmol L^{-1} h^{-1} and 0.18 mmol L^{-1} h^{-1}, respectively. The doubling time under mixotrophic conditions was slower than compared to stricht chemolithotrophic and heterotrophic conditions, or 18.2 h (μ_{max} 0.039) compared to 10.1 h and 6.6 h (**I**).

3.2 Strain 6C

3.2.1 Purification of enrichments

As in the case of enrichment 16C, culture 6C was sent for 16S rRNA analysis after several enrichments under HOX conditions and end-point dilutions. The sequence of the enrichment culture was analysed (744 nucleotides) and contained bacteria phylogenetically closely related to *Thiomonas* sp. (97%). Before full 16S rRNA analysis was performed, the cultures were re-inoculated several times to maintain growth. Again, heterotrophic contaminations were now the dominating bacteria, instead of *Thiomonas* species, *Meiothermus sylvanus* was mainly present and further DGGE analysis revealed heterotrophic contamination by *M. sylvanus* and *Anoxybacillus amyloloyticus*. Therefore, further end-point dilutions were performed under HOX conditions and four colonies were obtained. Partial 16S rRNA analysis revealed that three colonies represented *Thiomonas* sp. and one was most closely related to *Hydrogenophilus* sp. (Fig 6.). Finally a full 16S rRNA analysis was performed on one of the colonies that had showed homology with *Thiomonas* sp. from enrichment 6C, indicating 97.3% relationship with *Thiomonas intermedia* (**II**).

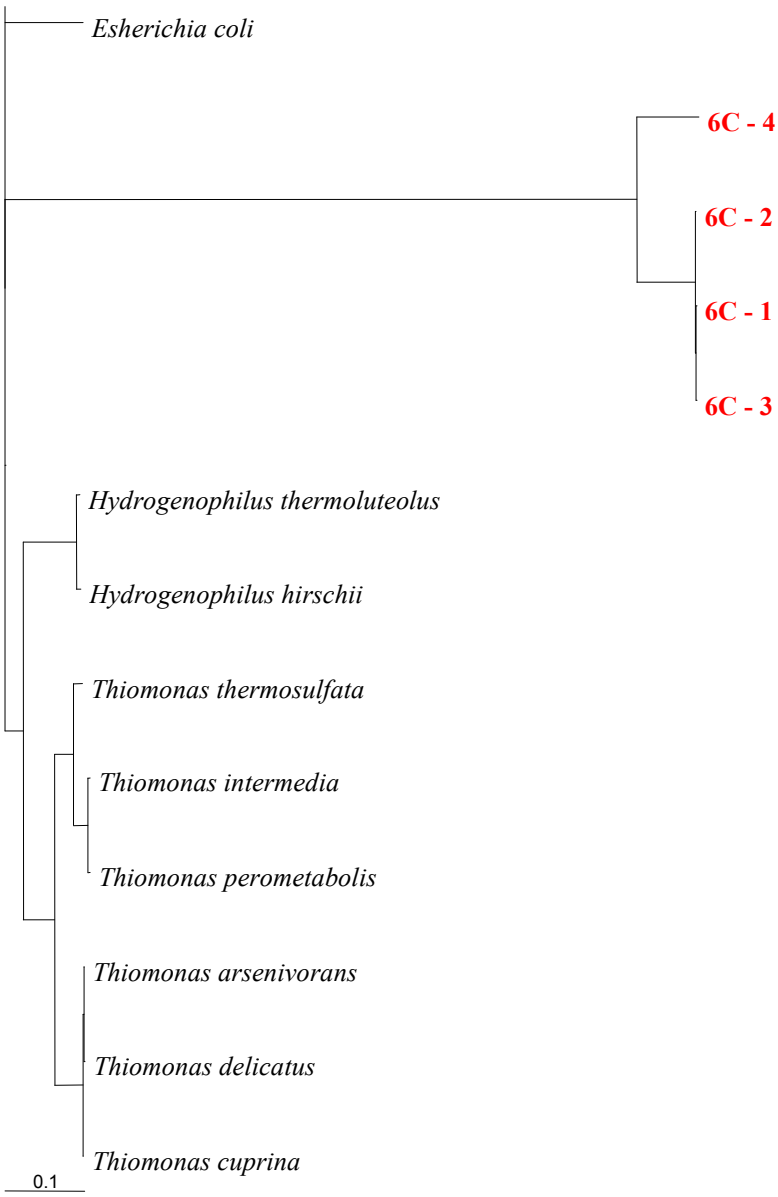


Figure 6. Phylogenetic tree showing relation of the four pure cultures isolated to Thiomonas thermosulfata (U27839), Tm. cuprina (AB331954), Tm. intermedia (AY455809),Tm. perometabolis (AY455808), Tm. arsenivorans (AY950676), Tm. delicata (AB245481), Hydrogenophilus thermoluteolus (AB009828) and Hydrogenophilus hirschii (AJ131694). E. coli (J01859) was used as outgroup bacteria

3.2.2 HOX and SOX metabolism

Thiomonas sp. have not been reported to oxidize hydrogen earlier. The genus originates from the genus *Thiobacillus* on the basis of the ability of the former to be able to grow mixotrophically (Moreira and Amils, 1997). *Thiobacillus ferrooxidans* is the only member of *Thiobacillus* that has been reported to grow on hydrogen (Drobner *et al*., 1990). Strain 6C originates from enrichment culture growing under HOX conditions.

Enrichment culture 6C showed uptake rate of 0.57 mmol H_2 L^{-1} h^{-1} and 13.35 mmol H_2 L^{-1} h^{-1} under batch and fed-batch conditions and biomass yield of 1.57 g mol^{-1} H_2^{-1} and 3.39 g mol^{-1} H_2^{-1} in batch and fed batch cultures, respectively. Pure culture of *Thiomonas* sp. showed uptake rate of 0.17 mmol H_2 L^{-1} h^{-1} and biomass yield of 1.80 g mol^{-1} H_2^{-1} under batch conditions (**II**).

Not surprisingly, strain 6C could utilize thiosulfate and produced sulfate when grown under SOX conditions (**II**). Under both HOX and SOX conditions similar growth spectrum was observed as under SOX conditions, i.e. similar amounts of sulfate were produced (14.9 mM) but only 25% of the hydrogen was utilized, compared to 65% under HOX conditions.

Preliminary experiments showed that the strain was able to grow in the presence of hydrogen sulfide and produce sulfate (data not shown).

3.2.3 Mixotrophic growth

Strain 6C was able to utilize few of the organic compounds tested (**II**). Growth was tested with and without yeast extract. Growth occurred on oxalate, acetate and pyruvate. In the presence of yeast extract, enhanced growth was observed on pyruvate and slight growth was observed on glutamate.

Studies of mixotrophic growth of strain 6C were done using pyruvate as a carbon source, with hydrogen and thiosulfate as electron donors (HOX, SOX and HOX + SOX culture conditions). The fastest growth rates were observed under mixotrophic conditions with thiosulfate as the electron donor resulting in a doubling time of 6.2 h (μ_{max} 0.11). Sulfate production was 15.1 mM. Degradation of pyruvate was not measured. During growth under HOX + SOX conditions hydrogen oxidation rates were very slow, 0.09 mmol H_2 L^{-1} h^{-1}, but sulfate production of 13.4 mM was measured. From these results it can be assumed that thiosulfate is preferred as electron donor rather than hydrogen under mixotrophic growth conditions.

3.3 Strain 16A

3.3.1 Purification of enrichments

After several HOX enrichments and end point dilutions from a sample from a hot spring (71°C, pH 6.75) the cultures were sent for 16S rRNA analysis. Enrichment culture 16A was analysed for 757 nucleotides revealing bacteria closely related to *Hydrogenobacter* sp. GV8L3A (97%). Before full 16S rRNA analysis was performed, the enrichment culture was re-inoculated several times to maintain growth. Full 16S rRNA analysis of enrichment culture 16A yielded 100% homology with *Hydrogenobacter hydrogenophilus* but results from DGGE analysis performed indicated heterotrophic contamination with *Geobacillus* sp. SB3.

After the DGGE analysis further end-point dilution series were done and repeated three times. No colonies were obtained from enrichment culture 16A since the solid media had tendency of melting down at 70°C. The final positive bottle in the last dilution series was submitted for partial 16S rRNA analysis. The results from the analysis showed 98% homology with *Hydrogenobacter hydrogenophilus.* Later, full 16S rRNA analysis was performed revealing that strain 16A showed 96% homology with *Hydrogenobacter hydrogenophilus.*

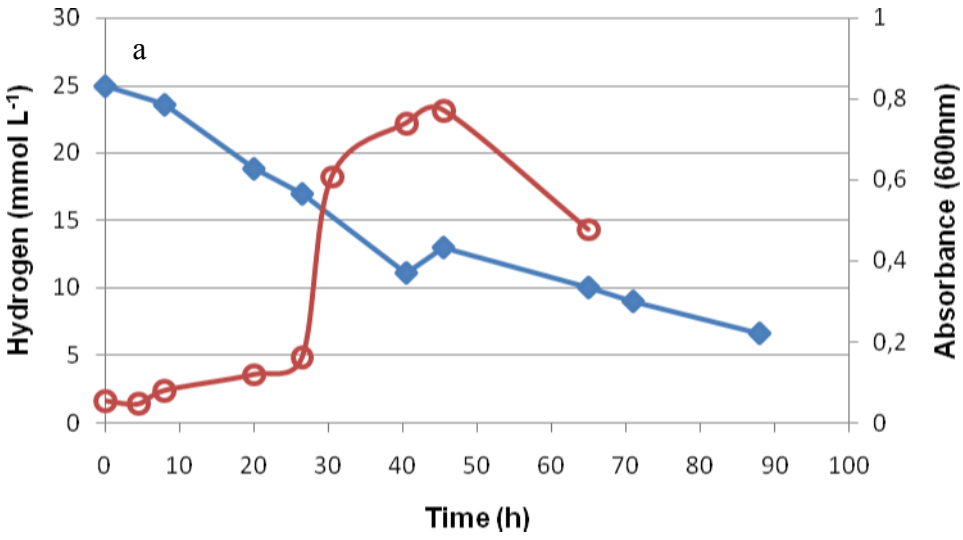
3.3.2 HOX and SOX metabolism

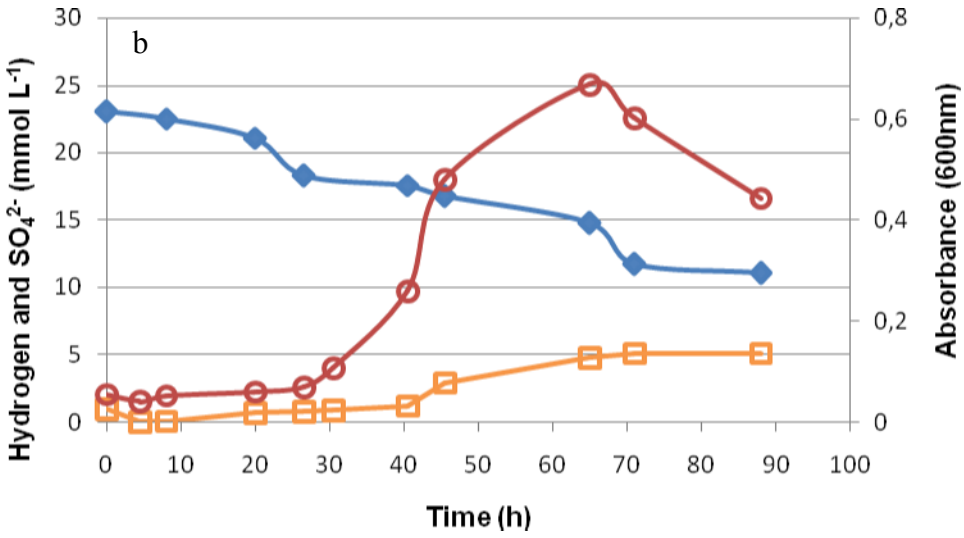
Hydrogen oxidizing capacity of *Hydrogenobacter* sp. has been well documented (Bonjour & Aragno, 1986; Shima & Suzuki, 1993; Kawasumi *et al*., 1984; Manelius *et al.,* 1997).

Strain 16A grew best at 70°C and pH 7. The strain was able to oxidize hydrogen at the rate of 0.21 mmol H_2 L^{-1} h^{-1} with biomass yield of 1.6 g mol^{-1} H_2^{-1} under HOX conditions and doubling time of 6.7 h (μ_{max} 0.13) (Fig. 7a). The enrichment culture, strain 16A was isolated from, showed hydrogen oxidation rates of 0.21 mmol H_2 L^{-1} h^{-1} and 8.32 mmol H_2 L^{-1} h^{-1} with biomass yield of 0.21 g mol^{-1} H_2^{-1} and 2.03 g mol^{-1} H_2^{-1} in batch and fed batch cultures, respectively. In the presence of thiosulfate and hydrogen (HOX + SOX conditions) the hydrogen oxidation rates were slower or 0.13 mmol H_2 L^{-1} h^{-1} and approximately 50% of the hydrogen initially added to the experimental bottles was oxidized. Less sulfate (5.1 mM) was produced under both

HOX and SOX culture conditions as compared to growth under SOX conditions and the pH was only slightly lowered (final pH was 65.9). Elemental sulfur was observed in very low concentration of 0.3 mM (data not shown). The doubling time of the strain under HOX + SOX conditions was 7.0 h (μ_{max} 0.1) (Fig. 7b).

Under SOX conditions strain 16A had a doubling time of 6.5 h (μ_{max} 0.11) and the final sulfate concentration was 11.5 mM (S° was 0.5 mM) (Fig. 7c). The amount of sulfate produced was considerable more under SOX conditions as compared to when both hydrogen and thiosulfate were used, resulting in slightly lower final pH at 5.3.





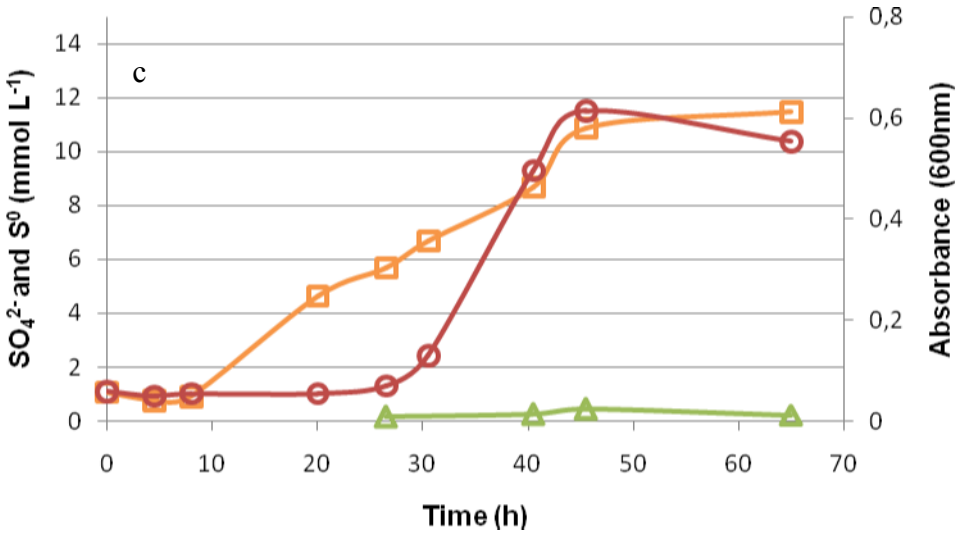


Figure 7. Growth of strain 16A under: (a) hydrogen oxidizing conditions, (b) hydrogen- and sulfur- oxidizing conditions, (c) sulfur oxidizing conditions
◆ *= hydrogen,* ○ *= growth,* □ *= sulfate ,* △ *= elemental sulfur*

Preliminary experiments showed that the strain was able to oxidize hydrogen and produce sulfate in the presence hydrogen sulfide (data not shown). Heterotrophic growth on various organic compounds was also tested. The strain was able to grow in the presence of fructose, mannose, glucose and crotonate.

3.4 Strain D10

3.4.1 Purification of enrichments

Enrichment culture D10 was obtained from a hot spring with temperature 63°C and pH 7.4. The first step in the isolation process of strain D10 were enrichments and end-point dilution series before partial 16S rRNA analysis. The culture was analysed for 521 nucleotides revealing bacteria closely related to *Aquificales* strain SRI-240 (99%) branching with sp. like *Sulfurihydrogenibium*. Full 16S rRNA analysis was not performed but DGGE analysis indicated presence of *Hydrogenobacter hydrogenophilus* as well as heterotrophic contaminant, *Geobacillus* sp. SB3, like in enrichment 16A. Further end-point dilution series were done and colonies were obtained on solid media on Petri dishes inoculated in boxes under HOX conditions. The results from partial 16S rRNA analysis on these two colonies indicated relationship to *Hydrogenobacter hydrogenophilus* (96%). On one of these colonies full 16S rRNA analysis was

performed revealing bacteria closely related (95%) to *Hydrogenobacter hydrogenophilus*.

3.4.1.1 HOX and SOX metabolism

Best growth rates of strain D10 were observed at 75°C and pH 7. The strain showed hydrogen uptake rates of 0.16 mmol H_2 L^{-1} h^{-1} under HOX conditions with a biomass yield of 3.8 g mol^{-1} H_2^{-1} (Fig. 8a). The enrichment culture showed uptake rates of 0.26 mmol H_2 L^{-1} h^{-1} and 1.46 mmol H_2 L^{-1} h^{-1} in batch and fed-batch cultures, respectively. Biomass yield in batch and fed-batch was 1.0 g mol^{-1} H_2^{-1} and 3.02 g mol^{-1} H_2^{-1}. The doubling time for strain D10 under HOX conditions was 7.4 h (μ_{max} 0.1).

In the presence of thiosulfate and hydrogen (HOX + SOX) the hydrogen oxidation rates were slightly slower as compared with HOX conditions, 0.13 mmol H_2 L^{-1} h^{-1} with doubling time of 8.5 h (μ_{max} 0.08) (Fig. 8b). Approximately 60% of the hydrogen initially added was oxidized. Sulfate accumulated in the cultures and the concentration was 10.1 mM at the end of the experimental time. Small amounts (0.4 mM) of sulfur were detected at the end of the experiment (data not shown). The production of sulfate caused the pH in the medium to drop from 6.8 to 6.1.

During growth under SOX conditions the doubling time was 8.2 h (μ_{max} 0.08) during exponential growth (Fig. 8c). During growth strain D10 produced both sulfate and elemental sulfur in the amount of 16.2 and 0.5 mM, respectively. More sulfate was produced during SOX conditions as compared with conditions were both hydrogen and thiosulfate were electron donors (HOX + SOX) and the pH dropped to 5.0.

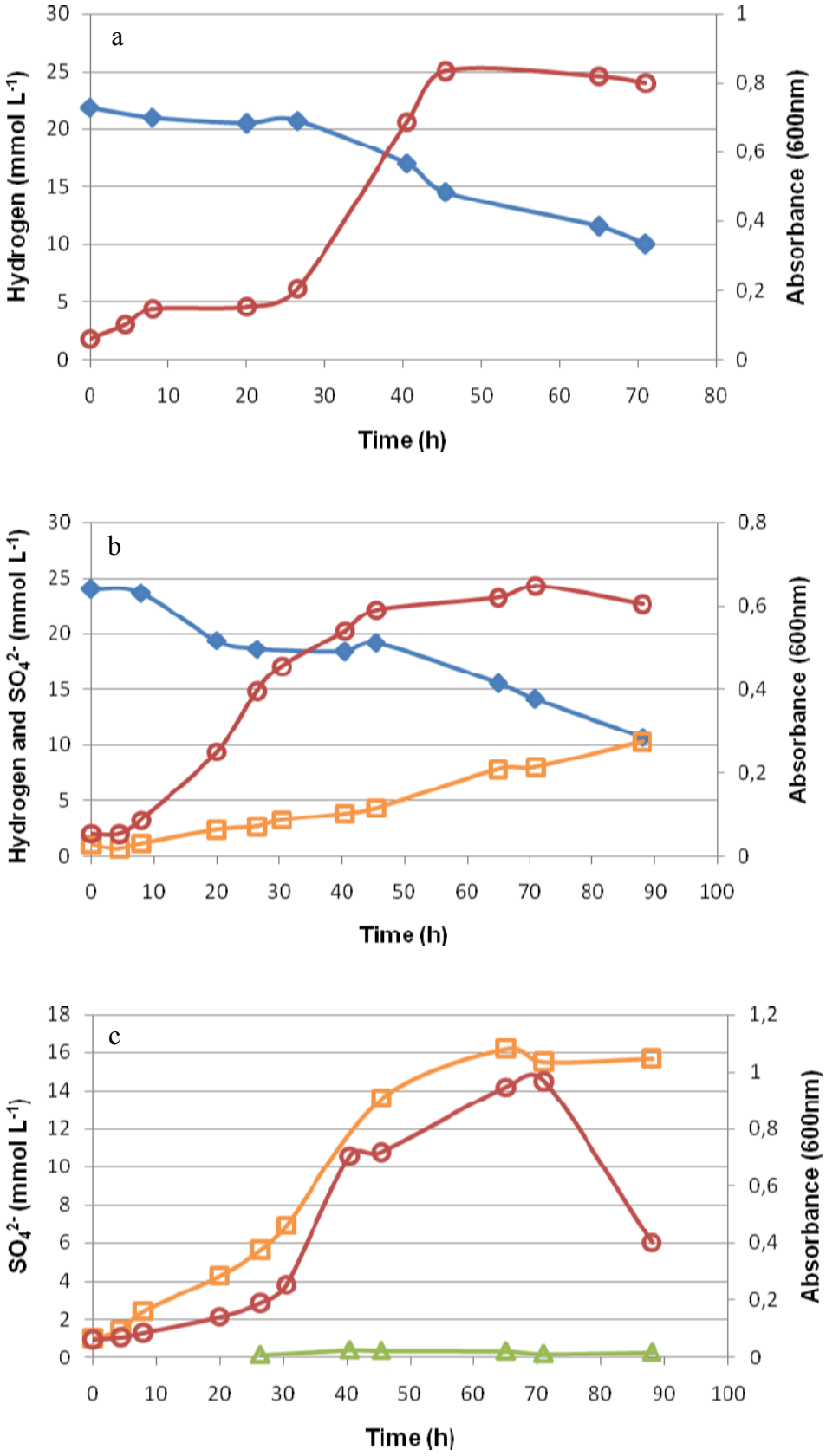


Figure 8. Growth of strain D10 under: (a) hydrogen oxidizing conditions, (b) hydrogen- and sulfur- oxidizing conditions, (c) sulfur oxidizing conditions
◆ = *hydrogen,* ○ = *growth,* □ = *sulfate,* △ = *elemental sulfur*

Preliminary experiments showed that strain D10 was able to oxidize hydrogen and produce sulfate in the presence hydrogen sulfide (data not shown). Heterotrophic growth on various organic compounds was also tested. The strain was able to grow in the presence of xylose, mannose and casamino acids.

3.5 Summary of metabolism of strains 16A and D10

Metabolic characteristics of strains 16A and D10 are summarized in table 1 as well as metabolic characteristics of the strain *Hydrogenobacter hydrogenophilus* retrieved from DSMZ (Deutshe Sammlung von Mikroorganismen und Zellkulturen) used as a comparison strain.

Table 3. Comparison of strains 16A, D10 and Hydrogenobacter hydrogenophilus under different culture conditions.

	Doubling time (h)	H_2 beginning (mM)	H_2 end (mM)	H_2 uptake rate (mM H_2 L^{-1} h^{-1})	SO_4 produced (mM)	S^0 end (mM)	pH end
Strain 16A							
HOX	6.7	24.9	6.6	0.21	nd	nd	6.4
HOX + SOX	7.0	23.0	11.1	0.13	5.1	0.3	5.9
SOX	6.5	nd	nd	nd	11.5	0.2	5.3
Strain D10							
HOX	7.4	21.8	8.4	0.16	nd	nd	6.4
HOX + SOX	8.5	23.9	10.7	0.13	10.1	0.4	6.1
SOX	8.2	nd	nd	nd	16.2	0.5	5.0
H. hydrogenophilus							
HOX	19.3	22.9	6.0	0.24	nd	nd	nd
HOX+SOX	18.5	26.7	8.5	0.24	5.3	0.2	4.4
SOX	19.5	nd	nd	nd	13.6	0.3	nd

nd not detected

Comparison of strains 16A and D10 shows that the former is more efficient in hydrogen metabolism and has faster growth and hydrogen uptake rates whereas strain D10 is more efficient in sulfur metabolism producing more sulfate and slightly more elemental sulfur. The reference strain *Hydrogenobacter hydrogenophilus* had however longer generation times than both strains under all conditions but faster hydrogen

uptake rates. D10 produced more sulfate and slightly more elemental sulfur but the pH for the reference strain was lower.

During growth of both strains under HOX and HOX + SOX conditions the hydrogen added was not oxidized completely. Under HOX + SOX condition less sulfate was produced by both strains as compared with pure SOX conditions. The most probable reason that the strains did not completely oxidize the hydrogen and for the lower amounts of sulfate produced is probably due to depletion of oxygen in the culture bottles. The depletion of oxygen was observed visibly by resazurin indicator but oxygen concentration can also be calculated from the equations:

$$S_2O_3^{2-} + H_2O + 2O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad (1)$$

$$H_2 + O_2 \rightarrow H_2O \quad (2)$$

Initially approximately 6.2 mmol L^{-1} of oxygen were present in the gas phase in the culture bottles. According to equation (2) all oxygen should be depleted when equimolar amounts (6.2 mmol L^{-1}) of hydrogen have been oxidized under HOX conditions. However, during growth of strain 16A, 18.3 mmol L^{-1} hydrogen were oxidized or approximately three times more than could theoretically be done by using the oxygen in the experimental bottles. During growth on strain D10 under HOX conditions 13.4 mmol L^{-1} hydrogen were oxidized, or approximately twice the amount that could theoretically be done from the by using the oxygen in the experimental bottles. The most reasonable explanation for the strains being able to use more hydrogen than expected is the presence of dissolved oxygen, originally in the liquid media but not in the gas phase.

According to equation (1) 5.1 mmol L^{-1} oxygen is needed to produce 5.1 mM sulfate produced by strain 16A under HOX and SOX conditions. In addition 12 mmol L^{-1} of hydrogen are oxidized causing clear depletion of oxygen in the culture bottles (total oxygen needed 17.1 mmol L^{-1}). The same applies to sulfate production and hydrogen oxidation of strain D10. It is clear that the amount of dissolved oxygen in the media is significant and thiosulfate (40 mM) was in excess since only 2.5 and 5.0 mM were utilized by strain 16A and D10, respectively, according to equation (1).

Under SOX conditions the concentration of oxygen was initially 12.5 mmol L^{-1}which should, according to equation (1), be sufficient to account for the production 12.5 mM sulfate. Sulfate production for strains 16A and D10 was 11.5 and 16.2 mM,

respectively. Like when the strains are grown under HOX and SOX conditions the thiosulfate added (40 mM) is also in excess under SOX conditions and only small fraction of the thiosulfate added is theoretically needed. Some part of the thiosulfate is oxidized to elemental sulfur. The rest of the thiosulfate could be oxidized to different intermediates such as polythionates in which the bacteria gain energy from. These intermediates were not measured in this study, except for the elemental sulfur, but this metabolism needs further studies.

Oxygen is obviously the limiting factor in the growth experiments. Since oxygen was not measured, neither in the gas phase or dissolved in the media, these values will always be a little speculative. The values given include only the oxygen in the gas phase, not the oxygen dissolved in the media which obviously effects the growth and sulfate production.

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5. Manuscript I

Hydrogenophilus islandicum sp.nov., a novel thermophilic hydrogen-oxidizing β-Proteobacterium isolated from Icelandic hot spring

Hydrogenophilus islandicum sp.nov., a novel thermophilic hydrogen-oxidizing β-Proteobacterium isolated from Icelandic hot spring

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Abstract

Strain 16C^T (T= type strain) was isolated from a hot spring in Grensdalur in the Hengill area in SW-Iceland. The cells of this organism were gram negative, rod shaped and motile. Cells are aerobic, capable of chemolithoautotrophic growth on hydrogen and carbon dioxide, heterotrophic growth on butyrate and several other organic compounds and mixotrophic growth on butyrate, hydrogen and carbon dioxide. Heterotrophic growth was most often enhanced in the presence of yeast extract. Autotrophic growth on hydrogen was observed at pH values between 5.0 and 10.0 and temperature between 37 and 60°C; optimum growth occurred at pH 8.0 and at 50°C. As determined by a 16S rRNA analysis strain 16C^T is a distinct species that belongs to the *β* subdivision of the Proteobacteria and is most closely related to *Hydrogenophilus thermoluteolus*. The GenBank accession number for the complete 16S rRNA gene sequence is EU625664. The name *Hydrogenophilus islandicum* is proposed for the new isolate.

Key words: Hydrogen oxidizing, moderately thermophilic, hot spring, Iceland, *Hydrogenophilus islandicum*.

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Introduction

The phylum *Proteobacteria* is presently the largest and phenotypically most diverse phylogenetic lineage of bacteria. The Proteobacteria account for over 40% of all validly published prokaryotic genera and show extreme metabolic diversity. The phylum *Proteobacteria* is divided to five major lineages alpha (α), beta (β), delta (Δ), gamma (γ) and epsilon (ε). Several representatives are of ecological importance and play key roles in the carbon, sulfur and nitrogen cycles. Most Proteobacteria are mesophilic but a few thermophilic species are known (Kersters *et al*., 2006). These are moderate thermophiles with a temperature optimum of 50-60°C. They are facultative chemolithoautotrophs and can grow heterotrophically on various organic substrates (Stöhr *et al*, 2001; Hayashi *et al*., 1999).

The origin of the genus *Hydrogenophilus* is from the work of Goto and co-workers (Goto *et al*., 1977; Goto *et al*., 1978). They isolated the first strains which were assigned to two different genera *Pseudomonas* and *Flavobacterium* (Goto *et al*., 1977; Goto *et al*., 1978). These bacteria have now been reassigned to the new genus, *Hydrogenophilus*, based on further phylogenetic studies (Hayashi *et al*., 1999). *Hydrogenophilus* sp. are straight rods, gram negative and non sporulating and use the calvin cycle to fix CO_2 to cell material (Aragno and Schlegel, 1992). The genus consist of two moderately thermophilic bacteria isolated from geothermal areas, *H. hirschii*, isolated from Yellowstone National Park (Stöhr, *et al*., 2001) and *H. thermoluteolus*, isolated from geothermal site in Japan (Goto *et al*., 1977; Hayashi *et al*., 1999). On databases there are however several *Hydrogenophilus* species unpublished, indicating that there may be more species emerging in near future.

There are more than 20 genera of both gram negative and gram positive mesophilic bacteria known today with the capability to oxidize hydrogen in the presence of oxygen for energy production (Friedrich & Schwarz, 1993). Most of these belong to the α- and β- subclasses of Proteobacteria and are often referred to as "knallgas bacteria". Within the "knallgas bacteria" facultative chemolithotrophs are common (Aragno, 1992). Most of them are also facultative autotrophs growing better on organic substrates than under

autotrophic conditions. Energy conservation under chemolithotrophic conditions is through hydrogen oxidation via an electron transport chain and to reduce carbon dioxide, usually through the ribulose biphosphate pathway (Calvin cycle) (Aragno & Schlegel, 1992). The best know bacteria within *Proteobacteria* with this ability are *Alcaligenes*, *Wautersia*, *Acidovorax*, *Paracoccus* and *Hydrogenophaga* (Aragno & Schlegel, 1992; Vaneechoutte *et al*., 2004). Among well known Gram-positive knallgas bacteria are *Mycobacterium, Amycolata, Arthrobacter, Bacillus* and *Nocardia* (Aragno & Schlegel, 1992; Friedrich & Schwarz, 1993). Among hydrogen oxidizing bacteria that thrive at high temperatures the best known are probably the hyperthermophiles *Aquifex* and *Hydrogenobacter* (Huber *et al*., 1992). Few hydrogen oxidizing bacteria have been isolated at moderate thermophilic temperatures. Present investigation led to the discovery of a new isolate; strain $16C^T$, a moderate thermophile, isolated from a hot-spring in Grensdalur in SW Iceland. The isolate is described as a novel hydrogen oxidizing species belonging to the genus *Hydrogenophilus* within the *β*-Proteobacteria.

Materials and methods

Isolation. Strain $16C^T$ was isolated from a hot spring (54°C, pH 6.75) in the Hengill area, Grensdalur, in SW of Iceland. The water/mud sample was taken from an off flow of a hot spring, approximately 2 m from the origin. The water in the spring was clear and the surroundings were rich in vegetation. Samples were collected by using an extended stick equipped with grip arms fixed at the end. Sterile bottles (120 ml) were fixed at the end, opened, filled with liquid samples and closed with butyl rubber stoppers and aluminium caps. The medium (18 mL) was inoculated with 2 mL (10% inoculation) of the sample. Positive samples (hydrogen uptake) were re-inoculated (10%) into fresh medium. This was repeated several times to get rid of heterotrophic contaminations. Thereafter, end point dilutions were performed and repeated three times. Each time the final positive sample (hydrogen uptake) was used for re-inoculation. The final positive sample from the third end point dilution series was inoculated on a solid media and incubated for three days. Colonies (pure cultures) were obtained and inoculated in liquid media and sent for partial 16S rRNA sequencing. One of these colonies was chosen and sent for full 16S rRNA sequencing.

Culture media. The media used for isolation and cultivation of strain $16C^T$ was DSM81, a mineral medium specific for chemolithotrophic growth (DSMZ, 2007a). The media consisted of the following stock solutions (in g L^{-1}): A: KH_2PO_4, 23; Na_2HPO_4, 23; NH_4Cl, 10; $NaHCO_3$, 5; B: $CaCl_2*2H_2O$, 0,1; C: $MgSO_4*7H_2O$, 5; D: Trace element solution, SL-6, according to medium 27: Rhodospirillaceae medium modified (DSMZ, 2007b); E: $FeNH_4$ citrate, 0,05 (20 mL distilled H_2O); F: Resazurin 0,1. Resazurin was added as an indicator of oxygen concentrations in the cultures. The medium was prepared by mixing solutions A and B (100 mL of each), solutions D and F (5 mL of each) and 670 mL of distilled water. The pH was adjusted at room temperature to 6.8 by using 1 M HCl or NaOH depending on the initial pH of the medium. The medium was autoclaved at 121°C for 15 min. The bottles had atmospheric air but were pressurized with 1 atm of hydrogen and carbon dioxide mixture (80/20). Thus, the final gas phase consisted of $H_2/CO_2/N_2/O_2$ (40/10/40/10). The gas was filter sterilized. Solid medium was prepared as described with addition of agar (15 g L^{-1}). After sterilization the media was poured on Petri dishes. The Petri dishes were inoculated in airtight boxes containing the same gas composition as before.

Physiological properties. Experiments to determine the optimum pH and temperature for growth were performed using medium DSM81 with hydrogen as the electron donor. The experimental bottles were prepared as before and supplemented with acid (HCl) or base (NaOH) to adjust pH accordingly. For the optimum pH determination the isolate was grown at 50°C. The initial pH was adjusted over the range of 3.0 – 11.0. For the temperature optimum determination the isolate was grown at pH 6.8 and temperature spectrum from 22-75°C.

Growth experiments under heterotrophic conditions were performed at 50°C using medium DSM81 supplemented with following organic compounds: (in 20 mM concentration) acetate, propionate, butyrate, formate, pyruvate, crotonate, oxalate, lactate, malate, aspartate, α-ketoglutarate, succinate, glutamate, alanine, glycine, serine, threonine, histidine, sorbitol, xylose, glucose, mannose, fructose, galactose (2 g L^{-1}) yeast extract, peptone, tryptone, beef extract and casamino acids. Growth was monitored under aerobic conditions (air). Mixotrophic growth experiments were performed using butyrate as carbon source and hydrogen as electron donor.

Growth experiments on thiosulfate were performed at 50°C using medium DSM81 supplemented with thiosulfate (40 mM). The gas phase consisted of $N_2/CO_2/O_2$ (60/20/20). Growth was followed by measuring absorbance and by analysing sulfur compounds formed in the culture medium. Growth experiments under hydrogen oxidizing conditions were performed as previously described. Growth experiments under both thiosulfate- and hydrogen oxidizing conditions were performed by using DSM81 (50°C) supplemented with thiosulfate (40 mM) and a gas phase consisting of $N_2/CO_2/H_2/O_2$ (40/10/40/10) under 2 atm pressure.

In all experiments growth was monitored by reading absorbancy at 600 nm (OD_{600}). Maximum (specific) growth rate (μ_{max}) for each growth experiment was derived from absorbance data (OD_{600}) using the equation: $\ln(x/x_0) = (\mu)(t)$, where x is the measurement of optical density of the culture, x_0 is the initial optical density of the culture, t is the elapsed time and μ denotes the maximum growth rate.

Analytical methods. Hydrogen was analysed using a Perkin Elmer gas chromatograph with a micro-thermo conductivity detector (TCD). Nitrogen was used as carrier gas at a rate of 15 mL min^{-1}, with another 5 mL min^{-1} as make-up gas in the detectors. The oven temperature was 80°C and the injector and detector temperatures were both kept at 220°C. The separation of gases was made on a Supelco 1010 Carboxen GC Plot Capillary Column.

Volatile fatty acid analysis (VFA) was performed by taking 1 mL of culture and centrifuge for 5 min at 13000 rpm. Of the supernatant 200 µL were added to 600 µL of distilled water. The sample was then acidified with 25% formic acid (100 µL) and crotonic acid (100 µL) was used as an internal standard. The analysis was made by using a Perkin Elmer gas chromatograph with a flame ionizing detector (FID). Nitrogen was used as carrier gas at a rate of 3 mL min^{-1}, with another 17 mL min^{-1} as make-up gas in the detectors. The separation was made on DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US).

Microscopy. Examination for purity of cultures and morphology were done using standard phase contrast microscope. Gram staining was performed using conventional methods (Madigan *et al*., 2003) and motility was determined using the standard "hanging drop" method in a phase contrast microscope.

G + C content analysis. Will be performed by DSMZ.

Phylogenetic analysis. 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir, et al, 2000), with PCR. The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, R1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analysed with 3730 DNA analyser from Applied Biosystems. The nucleotide sequence was displayed and analysed with Sequencer (Gene Code Corporation) (Skirnisdottir, et al., 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX were final alignments were done to generate phylogenetic trees. The program TreeView was used to view the trees. Diverse group of Proteobacteria were used as reference species and *Eserichia coli* was used as outgroup bacteria.

Results

Isolation of strain 16C^T. After repeated enrichment experiments and three end point dilution series, four pure cultures were obtained by transferring colonies from agar into liquid media. These four pure cultures were sent for partial 16S rRNA analysis and later one of them was sent for full 16S rRNA analysis (see later).

Morphological characteristics. A phase contrast microscopy (Fig. 1) shows the main characteristics of the cellular structure of strain 16C^T. Cells of 16C^T were Gram-negative, motile, straight rods, occurring singly or in pairs, 1.8 µm long and 0.9 µm wide.

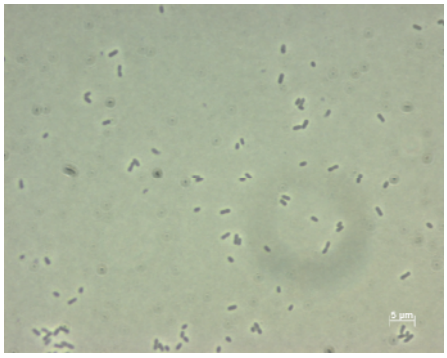


Figure 1. Phase contrast microscopy picture of strain 16C^T.

Physiological characterization. The strain grew well between 50 and 60°C with a doubling time of 1.8 h (μ_{max} 0.38) at 50°C. No growth was observed at room temperature and at 70°C (Fig. 2a). Growth of this strain occurred between pH 5.0 and 10.0 (Fig. 2b) with an optimum at pH 8.0.

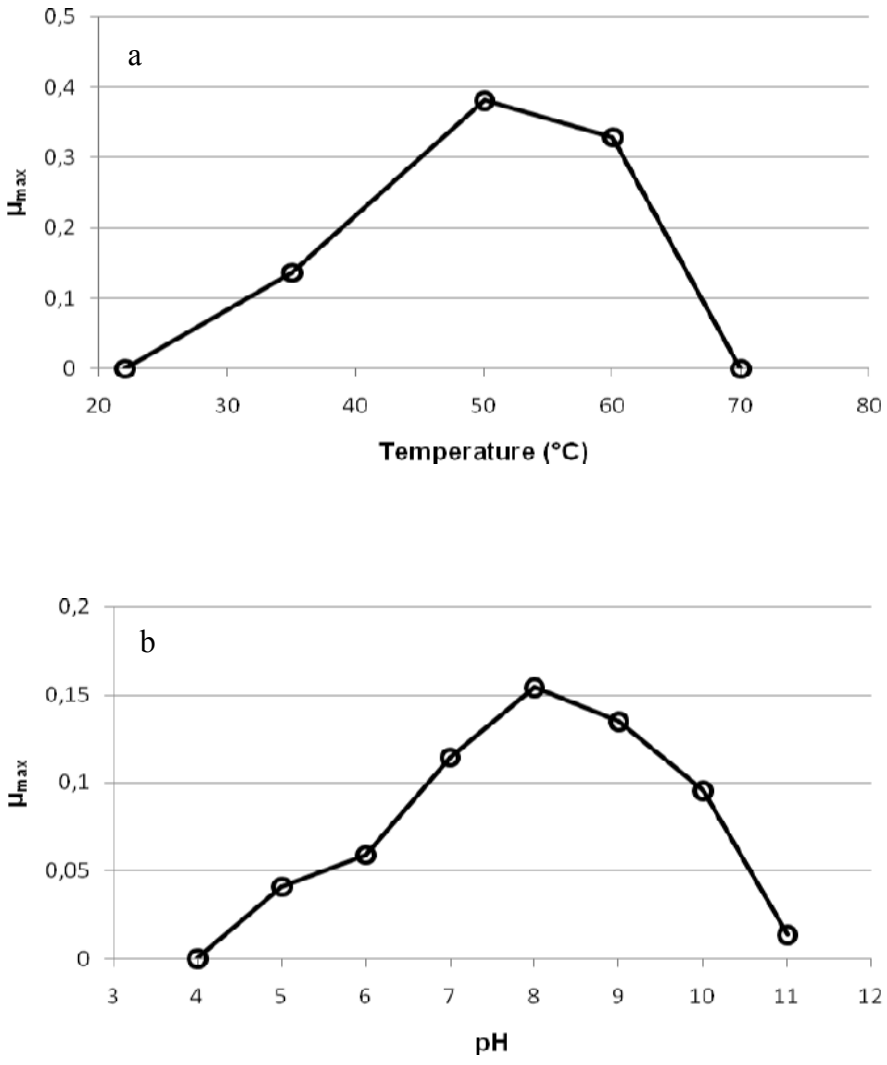
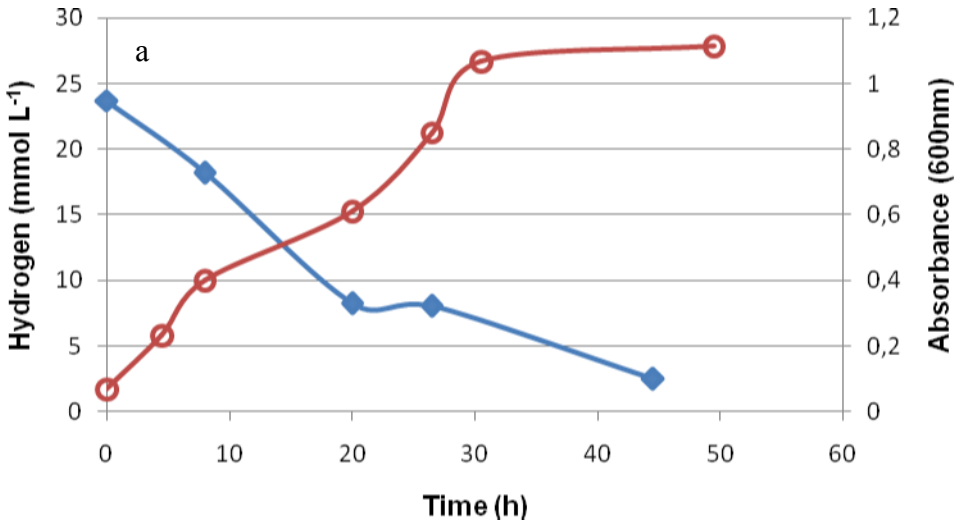


Figure 2. Optimum growth conditions of strain 16C (a) temperature, (b) pH.

Growth of strain 16C^T was followed under chemolithotrophic, heterotrophic and mixotrophic conditions. During growth under chemolithotrophic conditions with hydrogen as electron donor the hydrogen oxidation rate was 0.49 mmol H_2 L^{-1} h^{-1} and hydrogen concentrations dropped from 24.8 mM to 2.9 mM (Figure 3a). The doubling time was 10.1 h (μ_{max} 0.069). During heterotrophic growth on butyrate, the doubling time was faster than under chemolithotrophic conditions, or 6.6 h (μ_{max} 0.10) (Fig. 3b) and butyrate was degraded at a rate of 0.37 mM L^{-1} h^{-1}. Under mixotrophic conditions, hydrogen and butyrate were taken up at a rate of 0.15 mmol H_2 L^{-1} h^{-1} and 0.18 mmol butyrate L^{-1} h^{-1}, respectively (Fig. 3c). The doubling time under mixotrophic conditions was slower compared to chemolithotrophic and heterotrophic conditons, or 18.2 h (μ_{max} 0.039). The strain did not utilize thiosulfate.

The strain was tested for heterotrophic growth on various organic compounds in the absence (Table 1) and presence of yeast extract. Without the addition of yeast extract, the best growth was observed on butyrate. Other substrates that were positive were other complex substrates (casamino acids, beef extract, tryptone), propionate, lactate and pyruvate. Weak growth was observed on crotonate and peptone. In the presence of 2 g L^{-1} of yeast extract more profound growth was observed on most of the above mentioned substrates. Additionally, growth was observed on fructose, glucose and acetate where no growth occurred without yeast extract. Weak growth was also observed on malate.



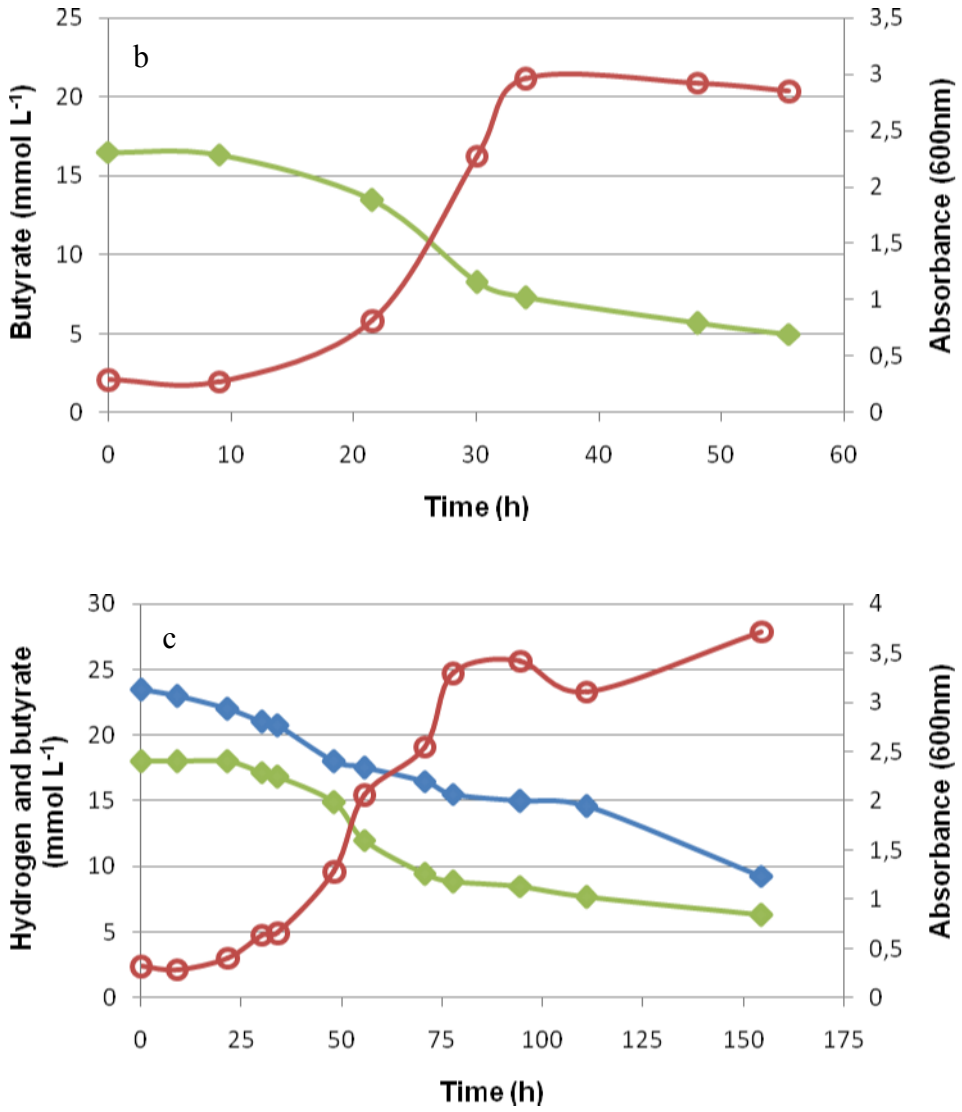


Figure 3. Growth of 16C[I] under various growth conditions: (a) hydrogen oxidizing conditions, (b) heterotrophic conditions on butyrate and (c) mixotrophic conditions on hydrogen and butyrate. ○ = growth, ◆ = hydrogen, ◆ = butyrate

Table 1. Utilization of various carbon sources by 16C^T, Hydrogenophilus hirschii and Hydrogenphilus thermoluteolus.
Data was obtained from this study, Stöhr *et al.,* (2001), Goto *et al*., (1978) and Hayashi *et al*., (1999).

	16C^T	*Hydrogenophilus hirschii*	*Hydrogenophilus thermoluteolus*
Optimum pH	8.0	6.5	7
Optimum temp (°C)	50	63	50-52
Motility	+	+	-
G+C content		61.0 mol%	63-65 mol%
Heterotrophic growth			
Acetate	+*	+	+
α-Ketoglutarate	-	-	+
Propionate	+**	na	+
Butyrate	+	-	-
Formate	-	-	-
Pyruvate	+**	+	+
Crotonate	+**	na	na
Oxalate	-	na	-
Lactate	+	+	+
Malate	(+)*	+	+
Succinate	-	+	+
Aspartate	-	na	na
Glutamate	-	+	na
Alanine	-	-	na
Glycine	-	-	na
Serine	-	na	na
Threonine	-	na	na
Histidine	-	-	na
Ribose	na	-	na
Arabinose	na	na	-
Xylose	-	na	na
Glucose	+*	-	-
Mannose	-	na	-
Fructose	+*	na	-
Sucrose	nd	-	-
Galactose	-	-	-
Maltose	nd	na	-
Yeast extract	+	+	na
Peptone	(+)	na	na
Tryptone	+	+	na
Beef extract	+	na	na
Casamino acids	+	na	na
Sorbitol	-	na	na
Chemolithotrophic growth			
H_2	+	+	+
Thiosulfate	-	na	+

( ) weak growth
* growth only in the presence of yeast extract
** growth enhanced in the presence of yeast extract
na not available
nd not determined

Phylogeny. The strain has been submitted to DSMZ and the DNA base composition will be determined.

From the 1492 bases of 16S rRNA analysed it is clear that strain 16C^T belongs to *β*-Proteobacteria. Phylogenetic analysis, including reference species from β-subclass and using *Escherichia coli* as the out-group organism, revealed that the isolate has the closest relation with *H. thermoluteolus* (95.6%) and *H. hirschii* (88.9%) (Figure 4).

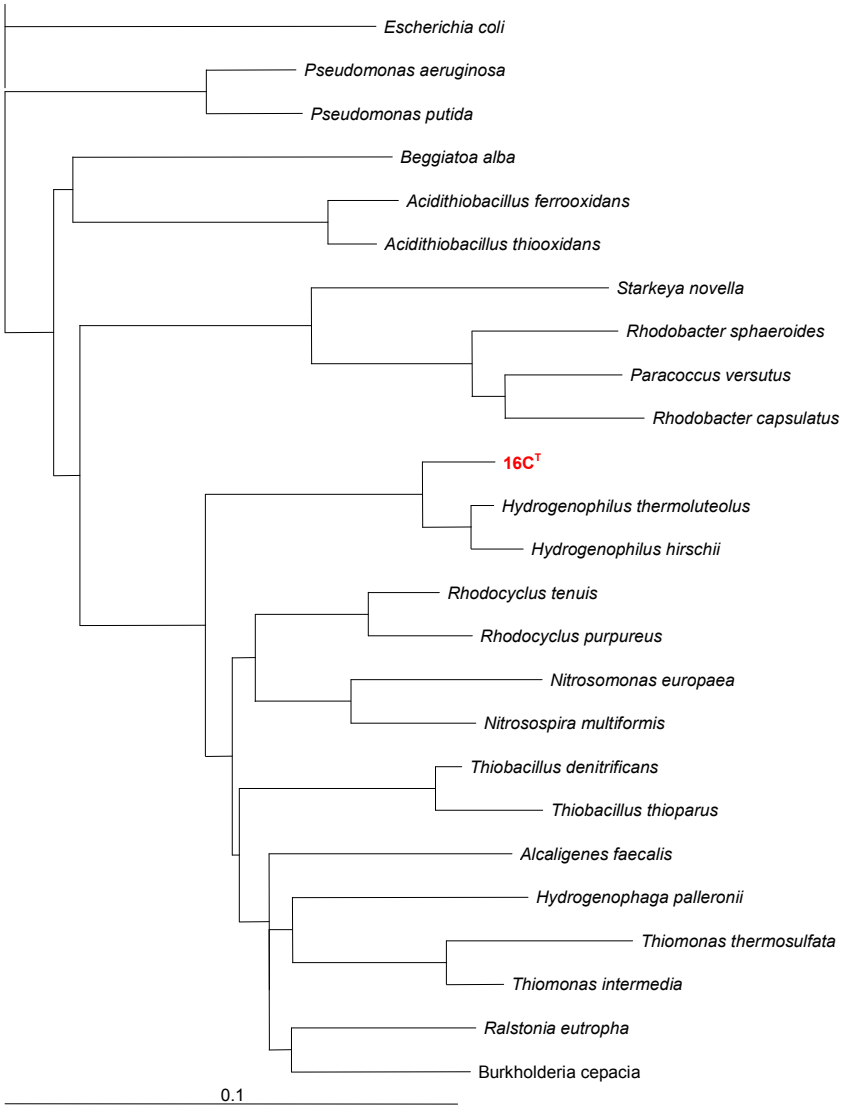


Figure 4. Phylogenetic dendogram based on 16S rRNA sequences showing the relationship of the novel isolate 16C^T to selected members of the proteobacteria. Confidence limits, expressed as percentages, were determined by bootstrap analysis with 1000 replicates. Strains and accession numbers used for calculation are: Alcaligenes faecalis EU567029, Acidithiobacillus thiooxidans EU084705, Acidithiobacillus ferrooxidans EU417841, Burkholderia cepecia EU567676, Eschericia coli J01859, Hydrogenophaga palleroni AF019073, Hydrogenophilus hirschii AJ131694, Hydrogenophilus thermoluteolus AB009829, Nitrosospira multiformis AY123807, Nitrosomonas europaea AY123795, Paracoccus versutus EU434456, Ralstonia eutropha AB007995, Rhodobacter sphaeroides EU263643, Rhodobacter capsulatus AM690347, Rhodocyclus tenuis D16209, Rhodocyclus purpureus M34132, Thiobacillus thioparus AF005628, Thiobacillus denitrificans EU546130, Thiomonas intermedia AY455809, Thiomonas thermosulfata U27839, Starkeya novella D32247, Beggiatoa alba AF110274, Pseudomonas putida EF051575 and Pseudomonas aeruginosa EU373311

Discussion

The novel isolate 16C^T represents a new moderate thermophilic bacterium within the phylum *Proteobacteria* and the genus *Hydrogenophilus*. Like many *β*-Proteobacteria, e.g. *Wautersia eutropha* (Vaneechoutte *et al*., 2004), *Hydrogenophaga* sp. (Willelms *et* al., 1989) and *Burholderia* sp. (Yabuuchi *et al*., 1992) strain 16C^T is a hydrogen oxidizer. Strain 16C^T shares many of the phenotypic features with these bacteria like facultative chemolithotrophy and the ability to utilize organic acids. The origin of the genus *Hydrogenophilus* is from the work of Goto and co-workers (Goto *et al*., 1977; Goto *et al*., 1978) who isolated the first strains from geothermal area that were later re-assigned to the genus *Hydrogenophilus* as *Hydrogenophilus thermoluteolus* (Hayashi *et al*., 1999). *Hydrogenophilus hirschii* was isolated from hot spring (Stöhr *et al*., 2001). Like the two *Hydrogenophilus* species strain 16C^T was isolated from hot spring.

Hydrogenophilus thermoluteolus has only 95.6% homology with strain 16C^T, and is thus clearly distinguished from the new isolate. Strain 16C^T should be assigned as a new species according to the 96% limits set for a new species (Stackenbrandt *et al*., 1999). The temperature optimum for 16C^T and *H. thermoluteolus* is similar (50-52°C) but the pH optimum for strain 16C^T is higher (8.0) compared to *H. thermoluteolus* (7.0). *H. thermoluteolus* has been reported to grow on complex substrates like, peptone, tryptone, meat extract and yeast extract and simple organic compounds like acetate, propionate, butyrate, succinate, DL-lactate, pyruvate and α-ketoglutarate (Hayashi *et al*., 1999). Strain 16C^T grows on the complex substrates (peptone, tryptone, meat extract or yeast extract), butyrate, propionate, lactate and pyruvate but no growth was observed on succinate and α-ketoglutarate. One physiological characteristic clearly distinguishes 16C^T from *H. thermoluteolus*, the motility, but 16C^T is motile but *H. thermoluteolus* is not. Clearly, some physiological characteristics between strain 16C^T and *H. thermoluteolus* are similar but other characteristics clearly distinguish between these two bacteria. *Hydrogenophilus hirschii* is far away from a phylogenetic point of view from strain 16C^T (88.9% homology). *H. hirschii* grows on complex substrates like, peptone, tryptone, meat extract and yeast extract (Stöhr *et al*., 2001) but not on any of the volatile and organic acids like both 16C^T and *H. thermoluteolus* do. Additionally, *H. hirschii* has higher temperature optimum as compared to strain 16C^T (and *H. thermoluteolus*) and lower pH optimum (6.5).

Relatively little data has been reported on hydrogen uptake rates of bacteria within the *β* subclass of Proteobacteria. In the few experiments that have been done very different culture conditions have been used and are thus not comparable to the data in the present study. However, in previous study at the University of Akureyri data on the hydrogen uptake rates of the strains *Hydrogenophilus hirschii, Wautersia eutropha, Hydrogenobacter hydrogenophilus* and *Sulfurihydrogenibium azorense* retrieved from DSMZ (Sammlung von Mikroorganismen und Zellkulturen) were investigated (data not shown). This study was performed under same culture conditions as in the present study. Strain $16C^T$ had high hydrogen uptake rates, 0.49 mmol H_2 L^{-1} h^{-1} under HOX conditions, compared to the strains from previous study which had hydrogen uptake rates ranging from 0.18 to 0.28 mmol H_2 L^{-1} h^{-1}. During growth of strain $16C^T$ the hydrogen concentration dropped from 23.7 to 4.7 mmol L^{-1}. The most reasonable explanation for the strain not to completely oxidize the hydrogen is a depletion of oxygen in the culture bottles which was observed visually by the rezasurin indicator. The same applies for mixotrophic growth on hydrogen and butyrate where the hydrogen oxidation rate was 0.15 mmol H_2 L^{-1} h^{-1} with hydrogen concentration dropping from 23.5 to 9.3 mmol L^{-1}. The highest absorbance value was obtained under mixotrophic conditions but fastest doubling time was during heterotrophic growth on butyrate, 6.6 h. That is in good consistence to the description of the members of *β*-Proteobacteria that are facultative autotrophs growing better on organic substrates than autotrophically (Aragno & Schlegel, 1992).

The potential of using HOX bacteria to produce biomass (single cell protein) has been investigated (Repaske & Mayer, 1976; Hreggvidsson *et al*., 1995). The most important factor regarding the potential of using HOX bacteria to produce biomass (single cell protein) is the biomass yield. Previous studies of HOX bacteria have been done under very various culture conditions and are hardly comparable to the present study. The assessment of biomass yield of strain $16C^T$ per mol hydrogen was related to the optical density (OD_{600}) where the measured value for increase of 1.0 in absorbance (OD_{600}) was 0.33 g DCW L^{-1}. The calculated dry cell weight (DCW) yield per mol hydrogen (g DCW mol^{-1} H^{-1}) was based on hydrogen oxidation of *Wautersia eutropha* (Takeshita & Ishizaki, 1996):

$21.36H_2 + 6.21O_2 + 4.09CO_2 + 0.7NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.39}N_{0.76} + 18H_2O$

Using this equation, the biomass yield for one mol of hydrogen would give approximately 5.4 g L^{-1} (Ívarsson, 2007). This value was used in following calculations. The biomass yield for strain $16C^T$ was 3.11 g mol^{-1} H_2^{-1} which is approximately 58% of the calculated value. Such a good yields are promising for further examinations of using strain $16C^T$ for single cell protein production. Other strains have been investigated in relation to biomass yield. Most studies have been done concerning *Wautersia eutropha* but the biomass yields are very different between studies, ranging from 1.65 to 25 g L^{-1}. This great difference is probably due to different culture conditions (Repaske & Mayer, 1976; Bongers, 1970; Amman *et al*., 1968). Other strains studied regarding to biomass yield are *Hydrogenophilus thermoluteolus*, yielding 2.3 g L^{-1} in batch culture (Goto *et al*., 1977), and *Hydrogenobacter thermophilus* with yields of 0.35 g L^{-1} in continuous culture (Manelius *et al*., 1997). In most cases more biomass yield is obtained in fed batch or continuous cultures but not in batch cultures. Strain $16C^T$ showed relatively good biomass yield in batch culture compared to some of the experiments with *Wautersia eutropha* and showed more biomass yield than *Hydrogenophilus thermoluteolus* and *Hydrogenobacter thermophilus.* Thus it would be interesting to cultivate strain $16C^T$ under continuous or fed-batch conditions. Thus, strain $16C^T$ might be a good candidate in single cell protein production considering good biomass yields and hydrogen uptake rates.

Description of *Hydrogenophilus islandicum* sp. nov.

Hydrogenophilus islandicum (island.i.cum. islandicum, isolated from Iceland).

Cells of *Hydrogenophilus islandicum* are moderately thermophilic, gram negative, motile straight rods, occurring as single cells or in pairs. Growth occurs under aerobic and microaerobic conditions. No growth occurred with thiosulfate as electron donor. Heterotrophic growth on yeast extract, casamino acids, tryptone, beef extract, peptone, propionate, butyrate lactate and pyruvate. The presence of yeast extract enhanced growth on most substrates and additionally growth was observed on fructose, acetate and malate. No growth was observed on glycine, serine, threonine, alanine, histidine,

glutamate, aspartate, oxalate, formate, sorbitol, glucose, mannose, xylose, succinate α-ketoglutarate and galactose. Growth factors are not required under HOX conditions. The optimum temperature for growth is 50°C. The maximum temperature is 60°C and no growth occurs below 37°C. The optimum pH is 8.0. Doubling times at 50°C on hydrogen oxidizing conditions were 10.1 h and on heterotrophic conditions on butyrate 6.6 h. The G + C content will be determined by the thermal denaturation method. The strain was isolated from water/mud sample taken from an off flow from a hot spring in Grensdalur in SW-Iceland. The strain belongs to the genus *Hydrogenophilus* within the *β*-Proteobacteria.

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6. Manuscript II

Isolation, phenotypic, physiological and phylogenetic characterization of a novel, mixotrophic, moderate thermophilic bacterium, *Thiomonas islandicum* sp.nov.

Isolation, phenotypic, physiological and phylogenetic characterization of a novel, mixotrophic, moderate thermophilic bacterium, *Thiomonas islandicum* sp.nov.

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Abstract

Strain 6C^T (T= type strain) was isolated from a hot-spring in Grensdalur in the Hengill area in SW-Iceland. The cells of this organism were gram negative, rod shaped and motile. Cells are aerobic, capable of chemolithotrophic growth on thiosulfate and hydrogen, heterotrophic growth on pyruvate, oxalate and acetate and on glutamate in the presence of yeast extract. Capable of mixotrophic growth on pyruvate with thiosulfate and/or hydrogen. Heterotrophic growth on pyruvate was greatly enhanced it the presence of yeast extract in the medium. During growth on thiosulfate, sulfuric acid was produced and the pH dropped from pH 6.8 to 2.7. Autotrophic growth on hydrogen was observed at pH values between 4.0 and 9.0 and temperature between 35 and 50°C; optimum growth occurred at pH 7.0 and at 50°C. As determined by 16S rRNA analysis strain 6C^T is a distinct species that belongs to the β subdivision of the Proteobacteria and is most closely related to *Thiomonas intermedia*. The GenBank accession number for the complete 16S rRNA gene sequence is EU625663. The name *Thiomonas islandicum* is proposed for the new isolate.

Key words: hydrogen oxidizing, sulfur oxidizing, thermophilic, hot spring, Iceland, *Thiomonas islandicum*

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Introduction

Formerly the genus *Thiobacillus* was defined by its ability to generate energy from inorganic sulfur compounds as well as carbon dioxide fixation and its rod shaped morphology. In addition to these characteristics many species within the former *Thiobacillus* genus can grow heterotrophically on various organic compounds. The lack of correlation between physiology and genetic groups within *Thiobacillus* lead to further genetic analysis. It revealed that species within the formerly *Thiobacillus* could be classified into three different subclasses of the Proteobacteria, α-, β- and γ-subclasses. Thus, the ability to oxidize sulfur and sulfur compounds is found in many groups of gram negative bacteria (Moreira & Amils, 1997; Robertson & Kuenen, 2006). Complicating the situation is that some photosynthetic and hydrogen oxidizing bacteria are also able to utilize sulfur compounds as energy source (Moreira & Amils, 1997).

The genus *Thiomonas* was proposed by Moreira & Amils (1997) to accommodate four former *Thiobacillus* species, *T. cuprinus, T. intermedius, T. perometabolis* and *T. thermosulfatus.* This classification was based on their ability to grow chemolithotrophically on reduced sulfur compounds but showing optimal growth in mixotrophic media containing both reduced sulfur compounds (or elemental sulfur) and various organic substrates (Moreira & Amils, 1997). Subsequently, another species was assigned to the genus, *Tm. delicata* (Katayama-Fujimura *et al.*, 2006) and recently *Tm. arsenivorans* (Battaglia-Brunet *et al*., 2005) has also been put forward as a new species but has at the moment not been accepted. Recently, Kelly and co-workers (Kelly *et al*., 2007) made a suggestion for the reassessment of the phylogenetic relationship of *Tm. cuprina* with other *Thiomonas* species. They suggest that in fact *Tm. cuprina*, *Tm. delicata* and *Tm. arsenivorans* differ only in three nucleotides from a full 16S rRNA analysis and are because of that indistinguishable but are still considered to be three species based on their physiological characteristics.

Several of the species within the genus *Thiomonas* have interesting physiological characteristics. The first *Thiomonas* species were isolated from soils (London, 1963; London and Rittenberg, 1967). These were originally isolated as *Thiobacillus* sp. but were later reassigned to the genus *Thiomonas* (Moreira and Amils, 1997). More recently isolations have been made from mining sites where metals are often present in

high concentrations (Dennison et al. 2001; Coupland et al. 2004). *Thiomonas cuprina* was originally isolated from a uranium mine, capable to utilize various sulfide ores (chalcopyrite, sphalerite, arseonpyrite and galena) as energy sources (Huber & Stetter, 1990). Some *Thiomonas* species are able to oxidize iron (Coupland et al. 2004; Johnson and Hallberg 2005) and some species have been demonstrated to oxidize arsenite (Battaglia-Brunet *et al*., 2002; Bruneel *et al*., 2003).
The ability of *Thiomonas* sp. to oxidize reduced sulfur compounds has been studied in relation to potential application in biological deodorisation (Chen *et al.*, 2004). Thus, species within *Thiomonas* seem to have a broad ability of biotechnological potential but until now, no *Thiomonas* sp. have been reported to be able to oxidize hydrogen but this has been shown to be a property of *Thiobacillus ferroxidans* (Drobner *et al*., 1990).

Here, the isolate $6C^T$ from a hot-spring in Grensdalur in SW Iceland is described as a novel species belonging to the genus *Thiomonas* within the *β* class of Proteobacteria.

Materials and methods

Isolation. Strain $6C^T$ was isolated from an off flow of a hot spring (45°C, pH 6.6) in the Hengill area, Grensdalur, in SW of Iceland. The water in the off flow was a little clayish and the surroundings were rich of vegetation. Samples were collected using an extendable stick with grip arms fixed at the end. Sterile bottles (120 mL) were fixed in the grip arms, opened, filled with liquid water-mud samples and closed with butyl rubber stoppers and aluminium caps. The medium (18 ml) was inoculated with 2 mL (10% inoculation) of the sample. Positive samples (hydrogen uptake) were re-inoculated (10%) into fresh medium. This was repetated seveal times to get rid of heterotrophic contaminations. Thereafter, end point dilutions were done and from the final positive sample (hydrogen uptake). This was repeated three times. The final positive sample from the third end point dilution series was then inoculated on solid agar media and icubated for three days. Colonies (pure cultures) were obtained and inouclated in liquid media and sent for partial 16S rRNA sequencing. One of these colonies was later sent for full 16S rRNA analysis.

Culture media. The media used for isolation and cultivation of strain 6C was DSM81, a mineral medium specific for chemolithotrophic growth (DSMZ, 2004a). The media consisted of the following stock solutions (in g L^{-1}): A: KH_2PO_4, 23; Na_2HPO_4, 23; NH_4Cl, 10; $NaHCO_3$, 5; B: $CaCl_2*2H_2O$, 0,1; C: $MgSO_4*7H_2O$, 5; D: Trace element solution, SL-6, according to medium 27: Rhodospirillaceae medium modified (DSMZ, 2004b); E: $FeNH_4$ citrate, 0,05 (20 mL distilled H_2O); F: Resazurin 0,1. Resazurin was added as an indicator of oxygen concentrations in the cultures. The medium was prepared by mixing solutions A and B (100 mL of each), solutions D and F (5 mL of each) and 670 mL of distilled water. The pH was adjusted at room temperature to 6.8 by using 1 M HCl or 1 M NaOH depending on the initial pH and autoclaved at 121°C for 15 min. The bottles had atmospheric air but were pressurized with 1 atm of hydrogen and carbon dioxide mixture (80/20). Thus, the final gas phase consisted of $H_2/CO_2/N_2/O_2$ (40/10/40/10). The gas was filter sterilized. . Solid medium was prepared as described with addition of agar (15 g L^{-1}). After sterilization the media was poured on Petri dishes. The Petri dishes were inoculated in airtight boxes containing the same gas composition as before.

Physiological properties. Experiments to determine the optimum pH and temperature for growth were performed using medium DSM81 with hydrogen as the electron donor. The experimental bottles were prepared as before and supplemented with acid (HCl) or base (NaOH) to adjust pH accordingly. For the pH optimum determination the isolate was grown at 50°C and the initial pH was adjusted over the range of 3.0 – 11.0. For the temperature optimum determination the isolate was grown at pH 6.8 and at a temperature spectrum from 22-75°C.

Growth experiments under heterotrophic conditions were performed at 50°C using medium DSM81 supplemented with following organic compounds (in 20 mM concentration): acetate, propionate, butyrate, formate, pyruvate, crotonate, oxalate, lactate, malate, aspartate, glutamate, alanine, glycine, serine, threonine, histidine, xylose, glucose, mannose, fructose, galactose, sorbitol, (2 g L^{-1}) yeast extract, peptone, tryptone, beef extract and casamino acids. Growth was monitored under aerobic conditions (air).

Growth experiments on thiosulfate were performed at 50°C using medium DSM81 supplemented with thiosulfate (40 mM). The gas phase consisted of $N_2/CO_2/O_2$ (60/20/20). Growth was followed by measuring absorbance, by analysing sulfur compounds formed and decrease in pH in the medium. Growth experiments under hydrogen oxidizing conditions were performed as previously described. Growth experiments under both sulfur- and hydrogen oxidizing conditions were performed by using DSM81 (50°C) supplemented with thiosulfate (40 mM) and a gas phase consisting of $N_2/CO_2/H_2/O_2$ (40/10/40/10) under 2 atm pressure.
Growth experiments under mixotrophic conditions were done at 50°C in DSM81 using hydrogen as energy source and organic compound as carbon source. The culture was grown under hydrogen oxidizing conditions and the medium was supplemented with pyruvate (20 mM). When grown under sulfur oxidizing conditions and on organic compounds the culture was grown as previously described (thiosulfate; 40 mM and $N_2/CO_2/O_2$) with addition of pyruvate (20 mM).
In all experiments growth was monitored by reading absorbancy at 600 nm (OD_{600}). Maximum (specific) growth rate (μ_{max}) for each growth experiment was derived from the absorbance data (OD_{600}) using the equation: $\ln(x/x_0) = (\mu)(t)$, where x is the measurement of optical density of the culture, x_0 is the initial optical density of the culture, t is the elapsed time and μ denotes the maximum growth rate.

Analytical methods. Sulfur was determined according to Sorbö (1957) modified by Kelly *et al*., (1969). Absorbance was read at 460 nm and the amount of sulfur evaluated with standard curve previously generated.

Sulfate was determined according to Tabatabai (1974). Samples were filtered through a 0.45 µl Whatman filters and diluted ten- (0.5 mL of sample) or hundred- (0.05 mL of sample) times, depending on the amount of sulfate in the medium, and filled up to 5.0 mL with distilled water. Then 0.5 mL of 0.5 M HCl was added and thereafter 0.25 mL of barium-chloride gelatine reagent. The solution was incubated for 30 minutes at room temperature. Before measurement the flasks were swirled and absorbance measured at 400 nm (OD_{400}). Standards were made from standard sulfate solution containing 1.0 g L^{-1} sulfate.

Hydrogen was analysed using a Perkin Elmer gas chromatograph with a micro-thermo conductivity detector. Nitrogen was used as carrier gas at a rate of 15 mL min^{-1}, with another 5 mL min^{-1} as make-up gas in the detectors. The oven temperature was 80°C and the injector and detector temperatures were both kept at 220°C. The separation of gases was made on a Supelco 1010 Carboxen GC Plot Capillary Column (Carboxen 1010).

Microscopy. Examination for purity of cultures and morphology were done using standard phase contrast microscope. Gram staining was performed using conventional methods (Madigan *et al.*, 2003) and motility was determined using the standard „hanging drop“ method in a phase contrast microscope.

G + C content will be analysed.

Phylogenetic analysis. For 16S rRNA analysis 1.376 unambiguous bases were used. 16S rRNA genes were amplified from DNA with primers F9 and R1544, specific for bacterial genes (Skirnisdottir, et al, 2000), with PCR. The PCR products were sequenced with universal 16S rRNA primers: F9, F515, F1392, R357, R1195 and R1544 by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently the DNA was analysed with 3730 DNA analyser from Applied Biosystems. The nucleotide sequence was displayed and analysed with Sequencer (Gene Code Corporation) (Skirnisdottir *et al*., 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn). Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the results from the sequencing in the program BioEdit and ClustalX where final alignments were done to generate phylogenetic trees. The program TreeView was used to view the trees. Diverse group of Proteobacteria were used as reference species and *Eserichia coli* was used as outgroup bacteria.

Results

Isolation. After repeated enrichment experiments and three end point dilution series, four pure cultures were obtained by transferring colonies from agar into liquid media. These four pure cultures were sent for partial 16S rRNA analysis and later one was sent for full 16S rRNA analysis (see later).

Morphology. A phase contrast microscopy (Fig. 1) shows the main characteristics of the cellular structure of strain $6C^T$. Cells of $6C^T$ were gram negative, motile, slightly bended rods, occurring singly, 1.5 – 2.0 µm wide and 5.0 – 7.0 µm long.

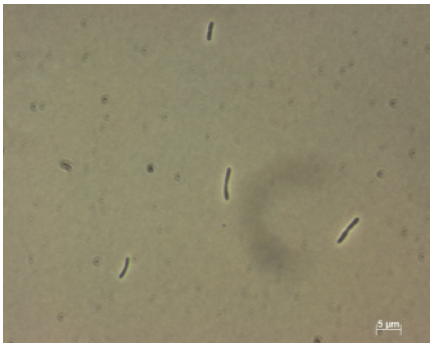
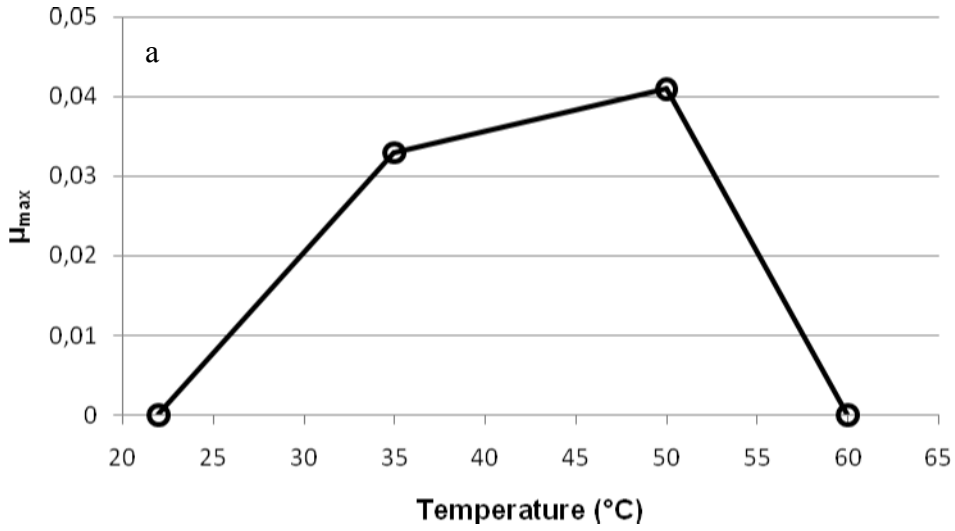


Figure 1. Phase contrast microscopy picture of strain $16C^T$

Physiological characterization. Strain $6C^T$ grew best at 50°C. No growth was observed at room temperature and at 60°C (Fig. 2a). The highest growth rates were observed at pH 7.0. No growth was observed at pH 3.0 or at pH 10.0 or higher (Fig. 2b).



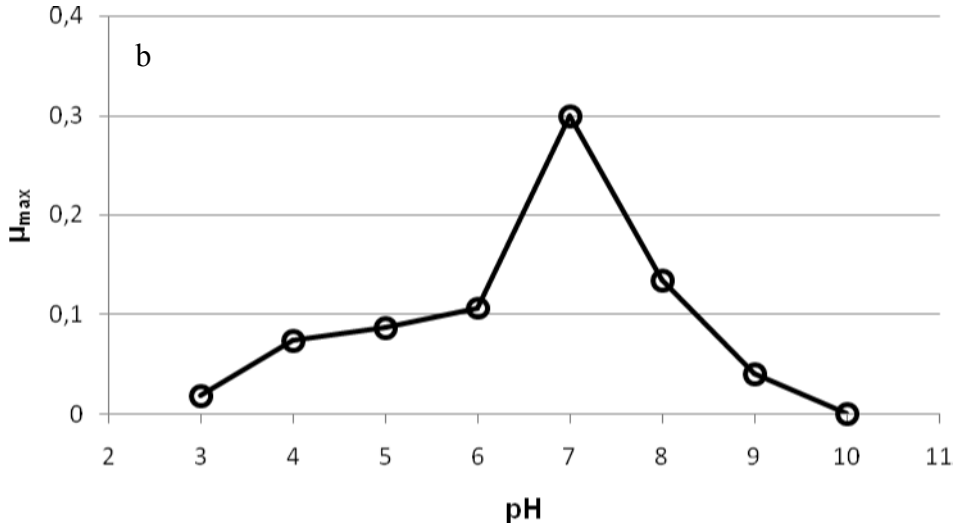
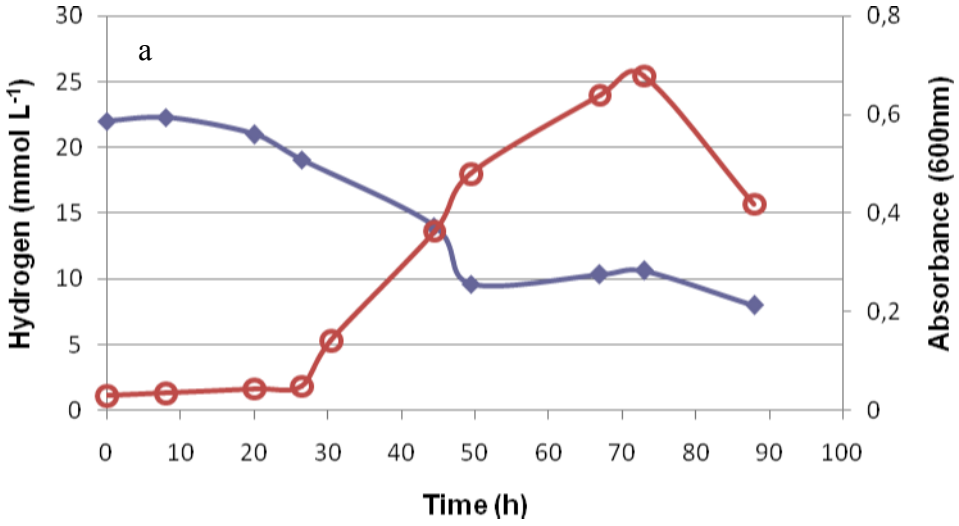


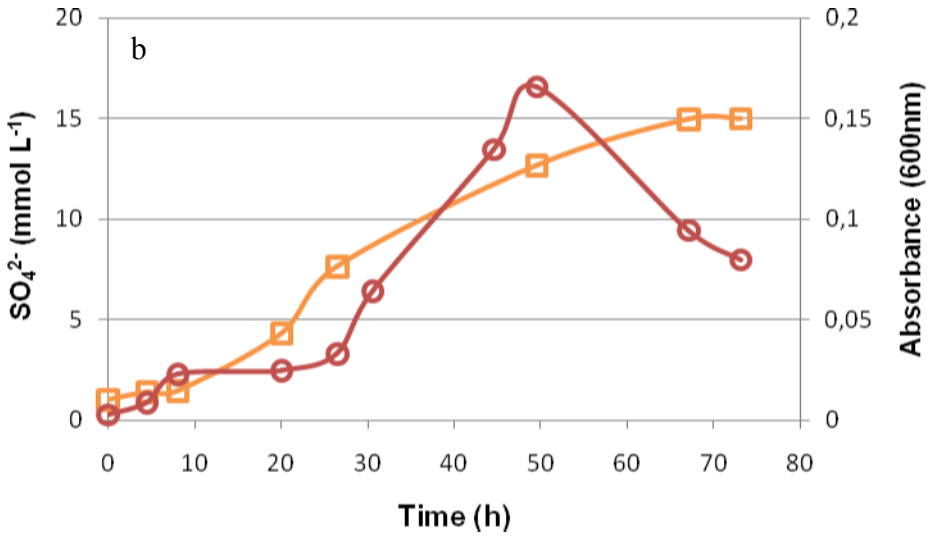
Figure 2. Optimum growth conditions of strain 6C (a) temperature, (b) pH.

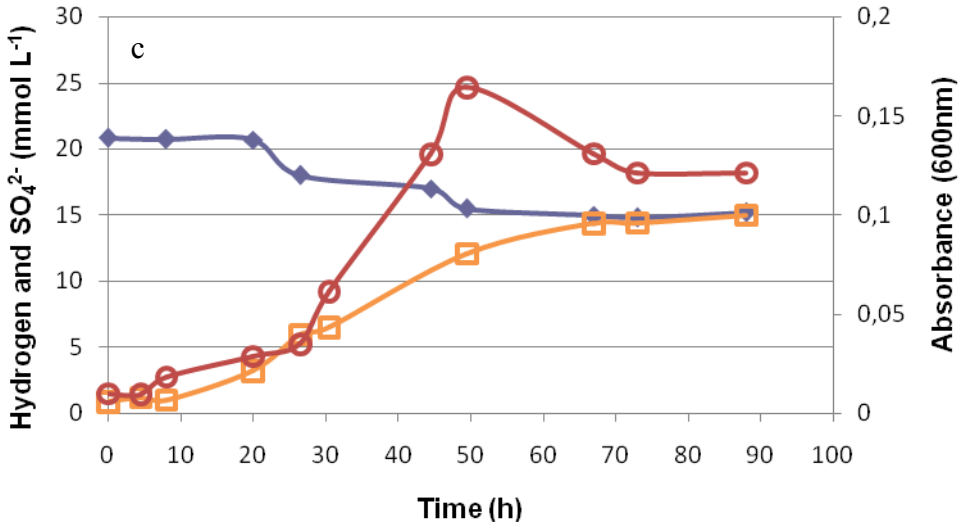
Growth of strain $6C^T$ was followed under chemolithotrophic, heterotrophic and mixotrophic conditions. After a lag-phase of more than 20 h hydrogen uptake and growth was observed in the hydrogen oxidizing (HOX) experimental bottles (Fig. 3a). During growth phase the strain utilized hydrogen at a rate of 0.17 mmol H_2 L^{-1} h^{-1} and the concentration of hydrogen dropped from 22.3 mM to 8.0 mM. The doubling time was 6.5 h (μ_{max} 0.107). During growth under autotrophic conditions with thiosulfate as electron donor (SOX) a similar lag-phase was observed as under HOX conditions (Fig. 3b). Growth was observed but the increase in biomass (OD) was only around 25% of the HOX value. The doubling time was 12.6 h (μ_{max} 0.057). End product from thiosulfate oxidation was sulfate which accumulated to 14.6 mM resulting in a pH of 2.7 at the end of the culture. When both hydrogen and thiosulfate were electron donors (HOX + SOX) very similar growth spectrum was observed as under SOX conditons (Fig. 3c). Although hydrogen decreased a little it was much less than compared to HOX conditions and similar biomass was produced as under SOX conditions. The doubling time during exponential growth phase was 11.2 h (μ_{max} 0.062) but the hydrogen uptake rate was only 0.033 mmol H_2 L^{-1} h^{-1}, or more than ten times slower than compared to the rates under HOX conditions. The final sulfate concentration was 14.6 mM. The drop in pH was similar to the experiment under SOX conditions, resulting in pH of 2.6.

Strain $6C^T$ is also capable of growing under heterotrophic conditions (Table 1). It grows on oxalate, acetate and pyruvate. Additonally, in the presence of yeast extract, enhanced growth was observed on pyruvate and slight growth was observed on the amino acid, glutamate.

Studies of mixotrophic growth of strain $6C^T$ were done using pyruvate as a carbon source and with hydrogen and thiosulfate as electron donors (HOX, SOX and HOX + SOX). During growth on pyruvate with hydrogen as electron donor a 20 h lag phase was observed (Fig 3d). During exponential growth the doubling time was 12.1 h (μ_{max} 0.059) and the hydrogen uptake rate was 0.19 mmol H_2 $L^{-1}h^{-1}$. The maximum optical density measured was 0.92, higher than compared to growth on only hydrogen as the energy source (OD = 0.68). Growth on pyruvate and thiosulfate under mixotrophic conditions (Fig 3e) showed a slightly shorter lag-phase (10 h). A similar growth spectrum was observed as compared to pure SOX conditons without the pyruvate with the exception that the culture reached higher OD-values (0.37). The doubling time was 6.2 h (μ_{max} 0.111) and the final concentration of sulfate was 15.1 mM. During growth on pyruvate with both hydrogen and thiosulfate as electron donors a very similar spectrum was observed as compared to growth on SOX condtions, either with hydrogen (Fig. 3c) or with pyruvate (Fig 3e). Hydrogen oxidation rate was very slow or 0.09 mmol H_2 L^{-1} h^{-1} and doubling time was 10.5 h (μ_{max} 0.067) (Fig. 3g). Final sulfate conentration was similar as observed before or 13.4 mM at the end of the experiment.


a
Hydrogen (mmol L-1)
Absorbance (600nm)
Time (h)
0
5
10
15
20
25
30
0
0,2
0,4
0,6
0,8
0
10
20
30
40
50
60
70
80
90
100


b
SO4 2- (mmol L-1)
Absorbance (600nm)
Time (h)
0
5
10
15
20
0
0,05
0,1
0,15
0,2
0
10
20
30
40
50
60
70
80


c
Hydrogen and SO4 2- (mmol L-1)
Absorbance (600nm)
Time (h)
0
5
10
15
20
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10
20
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90
100

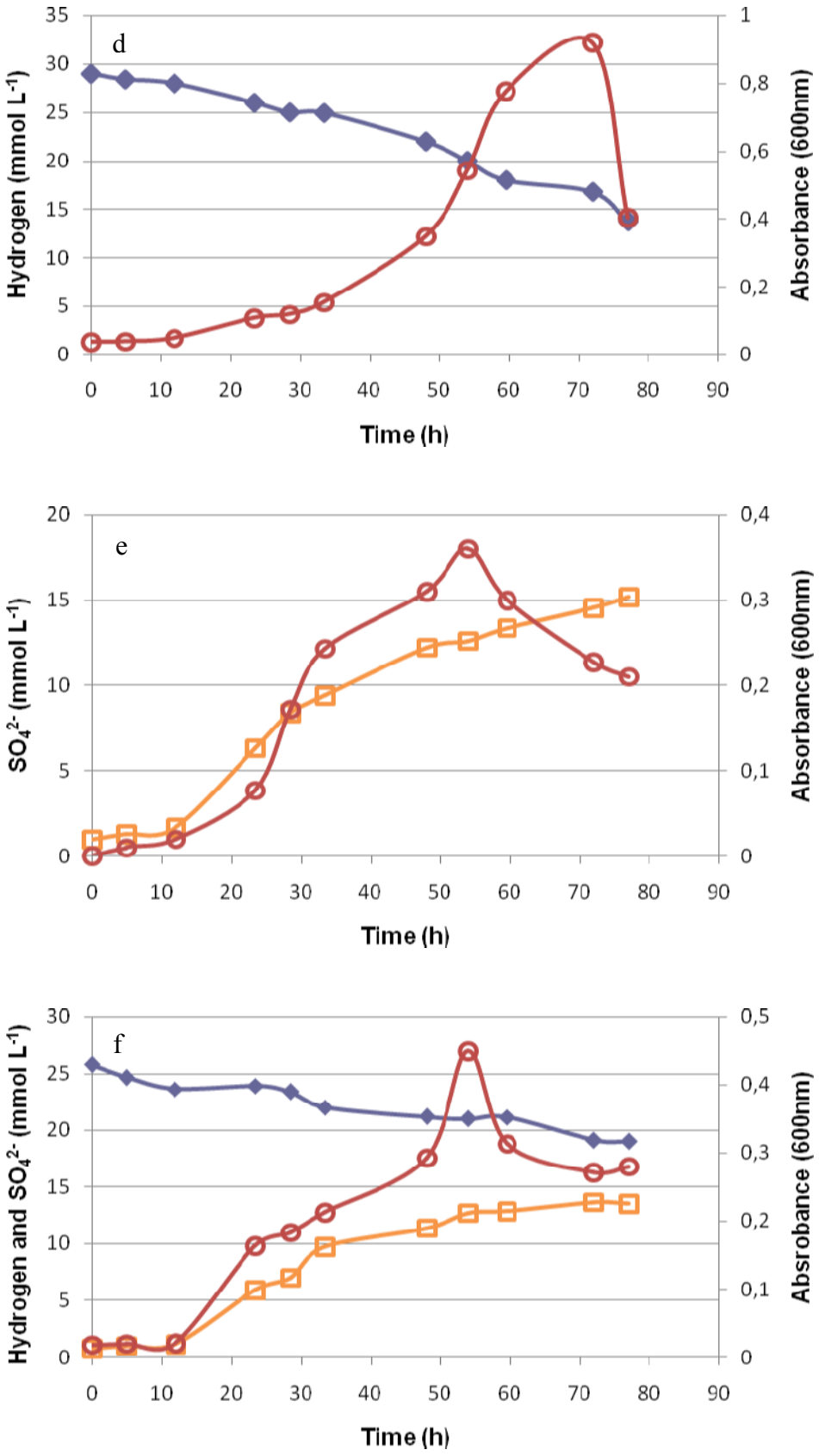

Figure 3. Growth of strain 6C[I] under various conditions: (a) HOX conditions, (b) SOX conditions, (c) HOX and SOX conditions, (d) mixotrophic conditions on hydrogen and pyruvate, (e) mixotrophic conditions on pyruvate and thiosulfate, (f) mixotrophic on hydrogen, thiosulfate and pyruvate. ◆ = hydrogen, ○ = growth, □ = sulfate

Table 1. Characteristics of strain 6C^T compared with other known Thiomonas sp.

Data was obtained from this study, Battaglia-Brunet *et al.* (2005), Katayama-Fujimura *et al*. (1984), Moreira & Amils, (1997) and Huber & Stetter, (1990).

	6C	***Tm. arsenivorans***	***Tm. perometabolis***	***Tm. intermedia***	***Tm. thermosulfata***	***Tm. cuprina***	***Tm. delicata***
Optimum pH	7.0	4-7.5	5.0-7.0	5.0-7.0	5.0-6.0	3.0-4.0	5.5-6
Optimum temp (°C)	50	20-30	30-37	30-37	50	30-36	30-35
Motility	+	+	+	+	+	+	-
G+C content		65	65-66 (65,0)	65-67	61	66-69	66
Heterotrophic growth							
Acetate	+	-	-	-	-	-	-
Propionate	-	nd	-	-	nd	nd	-
Butyrate	-	nd	-	-	nd	nd	-
Formate	-	-	-	-	-	-	-
Pyruvate	+	+	-	-	-	w	-
Crotonate	-	nd	nd	nd	nd	nd	nd
Oxalate	+	nd	-	w	nd	nd	w
Lactate	-	nd	-	+	nd	-	w
Malate	-	nd	+	+	nd	-	+
Succinate	nd	+	+	+	+	-	nd
Aspartate	nd	+	+	-	-	nd	+
Alanine	-	nd	+	+	nd	nd	+
Glycine	-	nd	nd	nd	nd	-	nd
Serine	-	nd	+	+	nd	-	-
Threonine	-	nd	nd	nd	nd	nd	nd
Histidine	-	nd	w	w	nd	nd	w
Ribose	nd	nd	-	-	-	-	-
Arabinose	nd	nd	-	-	-	-	-
Xylose	-	nd	nd	nd	-	nd	nd
Glucose	-	+	+	-	-	-	w
Mannose	-	nd	nd	nd	-	-	nd
Fructose	-	nd	nd	nd	-	-	w
Sucrose	nd	+	-	-	-	-	-
Galactose	-	nd	-	-	nd	-	-
Yeast extract	-	+	+	+	+	+	+
Peptone	-	nd	nd	nd	nd	+	nd
Bacto Tryptone	-	nd	nd	nd	nd	nd	nd
Beef extract	-	nd	nd	nd	nd	+	nd
Casamino acids	-	nd	nd	nd	nd	+	nd
Glutamate	+*	+	+	-	+	-	+
Sorbitol	-					nd	
Chemolithotrophic growth							
H_2	+	nd	nd	nd	nd	-	nd
Sulfur	+	+	+	+	+	+	+
Thiosulfate	+	+	+	+	+	-	+

\+ growth; +* growth in the presence of yeast extract
\- no growth
w weak growth
nd not determined

DNA base composition. The DNA G + C contents of strain 6C^T will determined by the thermal denaturation method.

Phylogenetic analysis. The result of the analysis of the complete sequence of 6S rRNA are presented as a similarity matrix (Table 2) and as phylogenetic dendogram (Fig. 4) showing the position of strain 6C^T within the genus *Thiomonas* and other closely related species. Strain 6C^T togeter with *Tm. thermosulfatus, Tm. perometabolis* and *Tm. intermedia* form a subcluster within the genus that branches off above the other speceis of *Thiomonas (Tm. aresenivorans, Tm. cuprina, Tm. delicata)*. Intracluster 16S rRNA similarity values for strain 6C^T within the *Thiomonas* ranged between 94.7 – 97.3%.

Table 2. Evolutionary distance matrix determined from a comparison of the 16S rRNA sequences of Thiomonas species used for phylogenetic analysis[a].

	% Sequence similarity						
Organism	*Escherichia coli*	6C^T	*Thiomonas thermosulfata*	*Thiomonas delicata*	*Thiomonas arsenivorans*	*Thiomonas curprina*	*Thiomonas perometabolis*
6C^T	80.4						
Thiomonas thermosulfata U27839	77.8	94.4					
Thiomonas delicata AB245481	80.1	94.6	90.3				
Thiomonas arsenivorans AY950676	80.2	94.7	90.5	99.9			
Thiomonas cuprina AB331954	80.1	94.8	90.6	99.8	99.8		
Thiomonas perometabolis AY455808	80.4	97.2	94.0	93.6	93.4	99.7	
Thiomonas intermedia AY455809	80.4	97.3	94.0	93.4	93.4	93.6	99.7

[a] The sequences used in this analysis were obtained from the NCBI GenBank database. 1.376 unambiguous bases were used. See materials and methods.

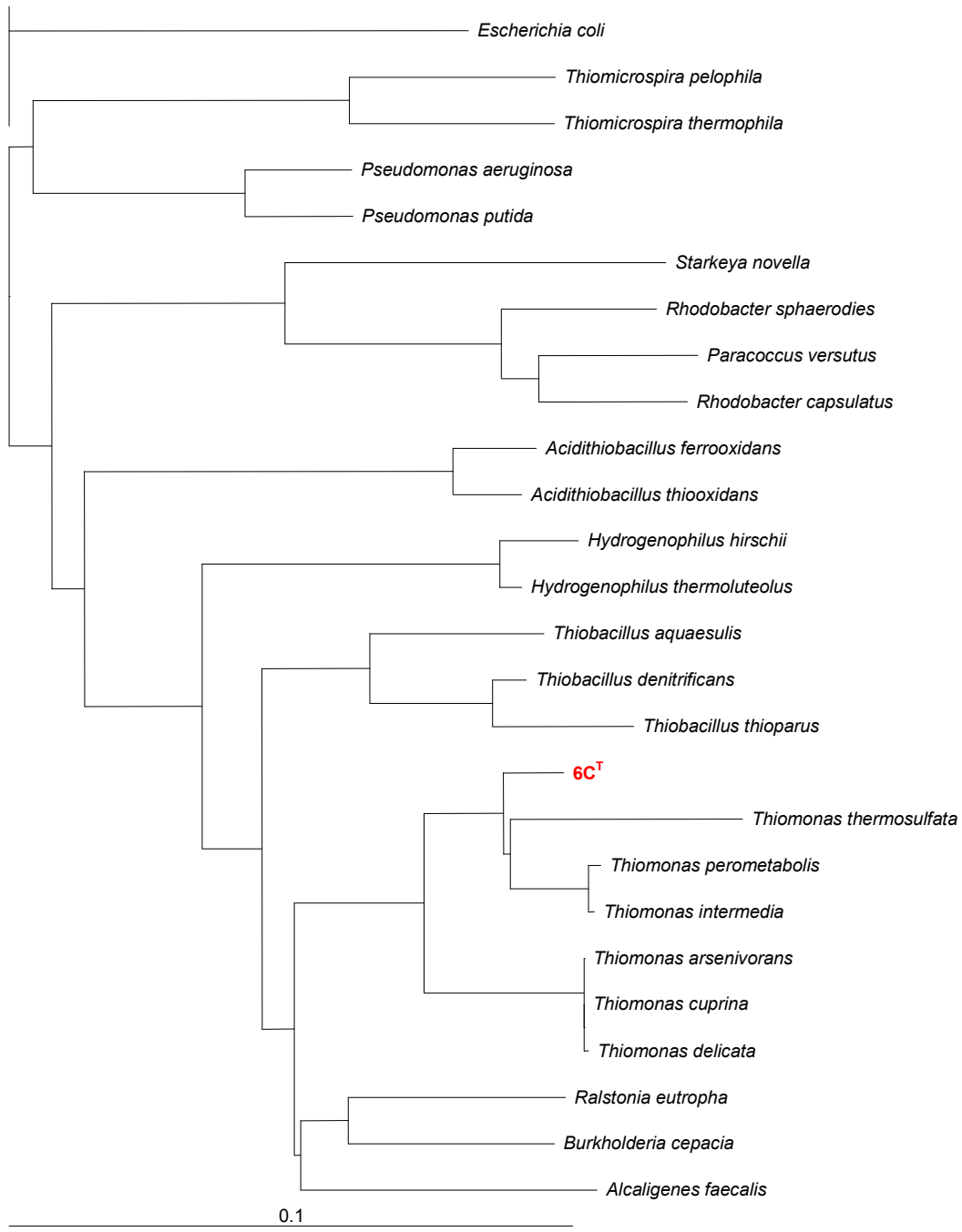

Figure 4. Phylogenetic dendogram based on 16S rRNA sequences showing the relationship of the novel isolate 16C to selected members of the proteobacteria. Confidence limits, expressed as percentages, were determined by bootstrap analysis with 1000 replicates. Strains and accession numbers used for calculation are: Alcaligenes faecalis EU567029, Acidithiobacillus thiooxidans EU84705, Acidithiobacillus ferrooxidans EU417841, Burkholderia cepacia EU567676, Eschericia coli J01859, Hydrogenophaga palleronii AF019073, Hydrogenophilus hirschi AJ131694, Hydrogenophilus thermoluteolus AB009829, Paracoccus versutus EU434456, Ralstonia eutropha AB007995, Rhodobacter sphaeroides EU263643, Rhodobacter capsulatus AM690347, Rhodocyclus tenuis D16209, Thiobacillus aquaesulis U58019, Thiobacillus thioparus AF005628, Thiobacillus denitrificans EU546130, Thiomicrospira pelophila L40809, Thiomicrospira thermophila AB166731, Thiomonas delicata AB245481, Thiomonas arsenivorans AY950676, Thiomonas intermedia AY455809, Thiomonas thermosulfata U27839, Thiomonas cuprina AB331954, Thiomonas perometabolis AY455808, Starkeya novella D32247, Pseudomonas putida EF051575 and Pseudomonas aeruginosa EU373311.

Discussion

The novel isolate 6C^T represents new moderately thermophilic hydrogen- and sulfur oxidizing bacteria within the genus *Thiomonas.* The isolate is phenotypically different from all other *Thiomonas* species isolated. The major difference is the ability of strain 6C^T to use hydrogen as electron donor and energy source. No other *Thiomonas* sp., until now, have been shown to have this ability but the former *Thiobacillus* species, *T. ferrooxidans* (now *Acidithiobacillus ferrooxidans*) (Kelly & Wood, 2000) is able to oxidize hydrogen but the oxidation rates have not been determined (Drobner *et al*., 1990). It is though well known that many sulfur oxidizing bacteria are capable of oxidizing hydrogen (Aragno, 1992).

Examination of the complete 16S rRNA sequence revealed that strain 6C^T belongs to the genus *Thiomonas* and is most closely related to *Tm. intermedia* (97.3% homology) and *Tm. perometabolis* (97.2% homology). Strain 6C^T differs from these two species in temperature optimum which is relatively higher, or 50°C compared to 30-35°C (Katayama-Fujimura *et al.,* 1984). The pH optimum for the two species is slightly lower, 5.5 – 6.0 of both species (Katayama-Fujimura *et al*., 1984) when the optimum for 6C^T is 7.0. *Tm. intermedia* and *Tm. perometabolis* were able to utilize more carbon sources than strain 6C^T which could only grow on the organic acids acetate, pyruvate and oxalate and in addition in the presence of yeast extract growth was detected on glutamate. *Tm. intermedia* and *Tm. perometabolis* were able to utilize few organic acids, serine and yeast extract and *Tm. perometabolis* could also grow on glucose (Katayama-Fujimura *et al.*, 1983). The G+C content in strain 6C^T will be determined but *Tm. intermedia* has 64.9 mol% and *Tm. perometabolis* 65.0 mol% (Katayama-Fujimura *et al*., 1984).

Relatively little data has been reported on hydrogen uptake rates of bacteria within the β subclass of Proteobacteria. Few experiments have been reported but the culture conditions were very different and the experiments are thus not comparable to the data in this study. However, in previous study at the University of Akureyri data on hydrogen uptake rates of the strains *Wautersia eutropha, Hydrogenophilus hirschii, Hydrogenobacter hydrogenophilus* and *Sulfurihydrogenibium azorense* retrieved from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) were

investigated (data not shown). In this study the same culture conditions were used as in the present study. Strain $6C^T$ had lower hydrogen oxidation rates, 0.17 mmol L^{-1} h^{-1}, compared to the known HOX bacteria from the previous study which had hydrogen uptake rates ranging from 0.18 to 0.28 mmol L^{-1} h^{-1}. During growth of $6C^T$ under HOX conditions the hydrogen concentration dropped from 22.3 to 8.0 mmol L^{-1}. Under hydrogen- and sulfur oxidizing conditions the hydrogen uptake rate was 0.033 mmol L^{-1} h^{-1} and the hydrogen concentration dropped from 20.8 to 15.2 mmol L^{-1}. During the period of growth, oxygen was depleted from the bottle as observed visibly by the rezasurin indicator. The depletion of oxygen as well as drop in pH to 2.7 when grown under HOX and SOX conditions are the most reasonable explanations of the strain not completely utilizing the hydrogen added to the bottle. The same applies for mixotrophic growth with hydrogen and pyruvate. Under mixotrophic growth conditions the hydrogen uptake rate was 0.19 mmol L^{-1} h^{-1}. The highest absorbance value was obtained under mixotrophic conditions with hydrogen and pyruvate (0.92) but the fastest doubling time was under mixotrophic conditions with thiosulfate and pyruvate, 6.2 h. That is in good consistence to the description of the members of *β*-Proteobacteria that are facultative autotrophs growing better on organic substrates than autotrophically (Aragno & Schlegel, 1992).

The sulfur oxidation rates were not measured in this study but when compared to growth under both hydrogen- and sulfur- oxidizing conditions the strain grows almost two times faster with hydrogen as the sole electron donor (maximum specific growth rates with hydrogen as electron donor was 0.107 but with thiosulfate is was 0.057). Thus, it would be interesting to see whether the hydrogen metabolism is indeed an important physiological property of other *Thiomonas* species.

All *Thiomonas* species except for *Tm. cuprina* are reported to be able to oxidize thiosulfate to sulfate. The oxidation of thiosulfate can occur in two pathways, one involving polythionates as intermediates (S4I pathway) and other not involving polythionates (PSO pathway). The S4I pathway is common in obligately chemolithotrophic thiobacilli, like *Thiomonas* sp. (Kelly *et al*., 1997). The sulfur oxidation follows the route of first oxidizing thiosulfate to elemental sulfur and further to sulfuric acid. This is in good correlation with the metabolism of strain $6C^T$. Very

common way for sulfur oxidizing bacteria is to store elemental sulfur inside the cell (Masau *et al*., 2001) which could have been the case for $6C^T$ since no sulfur was detected in the media. Sulfate was clearly accumulated as a result of the oxidation resulting in drop in pH in the culture media. From one mol of thiosulfate and two moles of oxygen it is theoretically possible to gain two moles of sulfate by the equation:

$S_2O3^{2-} + H_2O + 2O_2 \rightarrow 2SO_4^{2-} + 2H^+$

According to the equation 15 mM oxygen and 7.5 mM of thiosulfate are needed for the 15 mM of sulfate produced by $6C^T$. In the gas phase added when the strain is grown under SOX condition ($CO_2/N_2/O_2$ – 60/20/20) the average amount of oxygen is 15 mM which is in good relation to the amount of sulfate produced in the medium. Thus it is clearly the oxygen that is the limiting factor since only approximately 20% of the thiosulfate added, is theoretically needed. The thiosulfate could though be partially oxidized to different intermediates like polythionates and intracellular sulfur particle. This needs further examinations.

Thus it is clear that strain $6C^T$ differs from the *Thiomonas* sp., in both phylogenetic and physiological properties and could be proposed as new species.

Description of *Thiomonas islandicum* sp. nov.

Thiomonas islandicum (island.i.cum. islandicum, isolated from Iceland).

Cells of *Thiomonas islandicum* are straight rods, 1.5 – 2.0 µm wide and 5.0 – 9.0 µm long, occurring singly, motile and gram negative. Growth occurs under aerobic and conditions but not without oxygen. Small, greyish, rounded colonies are formed on agar containing thiosulfate. The optimum temperature for growth is 50°C. No growth occurs below 37°C or at 60°C. The optimum pH under hydrogen-oxidizing conditions is 7.0. Doubling times at 50°C at pH 6.8 under HOX, SOX and HOX + SOX conditions were 6.5, 12.6 and 11.2 h, respectively. The strain shows heterotrophic, chemolithotrophic and mixotrophic growth properties. Heterotrophic growth on acetate, oxalate and pyruvate, Growth was detected on the amino acid glutamate in the presence of yeast extract. No growth occurred propionate, butyrate, formate, crotonate, lactate, malate, alanine, glycine, serine, threonine, histidine, xylose, glucose, mannose, fructose,

galactose, yeast extract, peptone, tryptone, beef extract, casamino acids or sorbitol. The G + C content will be determined by the thermal denaturation method. The strain is isolated from water/mud sample of a hot spring in Grensdalur in SW-Iceland. No growth factors are required. Moderately thermophilic. The strain belongs to the genus *Thiomonas* within the *β*-Proteobacteria.

Acknowledgements

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7. Conclusion

In this study four enrichment cultures from previous study were chosen on the basis of phylogenetic analysis and their metabolic potential. Two of the isolates from the enrichment cultures (16C and 6C) were moderately thermophilic and two (16A and D10) were true thermophiles. Phylogenetic analysis had revealed that heterotrophic contamination was persistent in the enrichment cultures. After several dilution series and obtained colonies, 16S rRNA analysis revealed pure cultures of hydrogen and sulfur oxidizing bacteria. The results presented show that the two moderately thermophilic strains are new species within the genuses *Hydrogenophilus* and *Thiomonas*. These two strains are presented in the form of manuscripts of scientific papers. The other two strains are within the genus *Hydrogenobacter*. All of the isolates were able to oxidize hydrogen and three of them (6C, 16A and D10) were sulfur oxidizing.

Chemolithotrophic (HOX and SOX) metabolism was described for all isolates and in addition mixotrophic and heterotrophic metabolism were studied on the new species, 16C and 6C. Hydrogen oxidation rates and biomass yield was then compared to results from the previous study.

Strain 16C was identified as new species, able to oxidize hydrogen but not thiosulfate.

With hydrogen as energy source high hydrogen oxidation rates were observed as well as good biomass yield. Under mixotrophic conditions even higher OD values were obtained. Compared to strains investigated in a previous study at the University of Akureyri, the hydrogen oxidation rates of strain 16C are very fast. Considering the fast hydrogen uptake rates and good biomass yields, strain 16C could be considered as a good candidate for single cell protein production with hydrogen and oxygen as the only energy source and carbon dioxide as the carbon source or under mixotrophic growth conditions with butyrate.

Strain 6C was also identified as new species capable of growing under both hydrogen- and sulfur-oxidizing conditions. The strain was able to utilize hydrogen as the only energy source which has not been reported before for species within the genus *Thiomonas*. Compared to strain 16C the strain is not competitive in single cell protein production because of low hydrogen uptake rates and biomass yield. On the other hand

the strain is capable of oxidizing thiosulfate. Preliminary results have also shown that the strain is also capable of growing in the presence of hydrogen sulfide and has a potential of being used in bioremediation, removing hydrogen sulfide from gases. These abilities have to be further investigated.

Strains 16A and D10 were true thermophiles and have higher temperature optimum than strains 16C and 6C. They able to grow under both hydrogen- and sulfur-oxidizing conditions. Strain 16A showed better hydrogen uptake rates while strain D10 had a very good biomass yield, better than strain 16C, and should be considered to be a candidate for single cell protein production. When grown on thiosulfate, both of the strains produced sulfate and elemental sulfur. Preliminary results show that strains D10 and 16A are also capable of growing in the presence of hydrogen sulfide.

The interesting results from preliminary investigation on growth in the presence of hydrogen sulfide indicate that the sulfur oxidizing strains might be able to contribute to bioremediation, e.g. in decreasing gas emission from geothermal power plants. To test these abilities further as well as biomass production from hydrogen the culture conditions should be changed to fed-batch or continuous cultures to get a better image on the growth characteristics of the strains in larger cultures. This will be done in the near future.