Dissertation der Fakultät für Biologie der
Ludwig-Maximilians-Universität München

Autotrophy in Groundwater Ecosystems

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades

vorgelegt von
Claudia Sabine Kellermann
aus München

München
im November 2008

1. Gutachter: Prof. Dr. Anton Hartmann, LMU München

2. Gutachter: Prof. Dr. Dirk Schüler, LMU München

Tag der Abgabe: 06.11.2008
Tag des Promotionskolloquiums: 15.07.2009

Publications originating from this Thesis

Chapter 2

Kellermann, C & Griebler, C (2008) *Thiobacillus thiophilus* D24TN^T sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments. International Journal of Systematic and Evolutionary Microbiology (IJSEM), 59: 583-588

Chapter 3

Kellermann, C, Selesi, D, Hartmann, A, Lee, N, Hügler, M, Esperschütz, J, & Griebler, C (2008) Chemolithoautotrophy in an organically polluted aquifer – Potential for CO_2 fixation and in situ bacterial autotrophic activity. (in preparation)

Contributions

Chapter 3

Enzyme assays were performed in cooperation with Dr. Michael Hügler at the IFM-GEOMAR, Kiel, Germany.

Chapter 4

FISH-MAR analysis was performed in cooperation with Prof. Dr. Natuschka Lee at the Technical University Munich, Germany. Enzyme assays were performed in cooperation with Dr. Michael Hügler at the IFM-GEOMAR, Kiel, Germany. PLFA analysis was performed by Dr. Jürgen Esperschütz at the Institute of Soil Ecology, Helmholtz Center Munich, Germany.

I hereby confirm the above statements

Claudia Kellermann Prof. Dr. Anton Hartmann

Autotrophy in Groundwater Ecosystems

Claudia Kellermann

Abstract: The major role in global net CO_2 fixation plays photosynthesis of green plants, algae and cyanobacteria, but other microorganisms are also important concerning autotrophy; *i.e.* autotrophic microorganisms can be found in most bacterial groups (Eubacteria) and there are even numerous representatives within the Archaea. CO_2 fixation is not only one of the world's most important biogeochemical processes and responsible for the buildup of organic compounds which are needed for biological functions (*e.g.* cell growth or nutrition of heterotrophic organisms); ultimately all ecosystems are based on inputs of carbon and energy provided by autotrophic organisms which can be found in almost all environments. While the importance of CO_2 fixation on the surface is known, there is almost no information about autotrophic processes in the subsurface. The widespread opinion is that subsurface communities are dominated by heterotrophic microorganisms, but it is unlikely that all subsurface biomass depends on the limited amounts of organic carbon imported from the surface or on pollution dumping. Groundwater systems comply with all requirements for autotrophic growth processes (electron donors *e.g.* H_2, $S_2O_3^{2-}$ and electron acceptors *e.g.* NO_3^-, O_2 are available as well as plenty of inorganic carbon), so autotrophic microorganisms could significantly contribute to the carbon flux in at least some of those systems. In summary, the existence and the role of chemolithoautotrophic CO_2 fixation in the terrestrial subsurface is hardly known.

To date, five CO_2 fixation pathways are described, *i.e.* the Calvin-Benson-Bassham cycle (Calvin cycle), the reductive tricarboxylic acid cycle, the reductive acetyl CoA pathway, the 3-hydroxypropionate cycle and the 3-hydroxypropionate/4-hydroxybutyrate CO_2 fixation pathway, with the Calvin cycle being the most intensively studied and probably the most abundant one. A sixth fixation pathway was just recently discovered.

Objective of this thesis was to prove the CO_2 fixation potential within the microbial communities in different groundwater ecosystems by means of functional gene analysis (*cbbL, cbbM* and *acl* genes) and to link this potential with *in situ* autotrophic activities as evaluated by different isotope and fatty acid approaches (FISH-MAR and PLFA analysis). Furthermore enrichment cultures under obligate chemolithoautotrophic conditions were started to get an idea about the diversity of those communities.

The detection of the *cbb* genes in a contaminated and a pristine aquifer proved the occurrence of CO_2 fixation potential being present in the bacterial communities of those ecosystems. Concerning the tar-oil contaminated aquifer, the majority of all retrieved *cbb* sequences was closely related to the *cbbL* and *cbbM* sequences belonging to the genus *Thiobacillus*, indicating that this genus might be of importance in groundwater ecosystems. This hypothesis is further supported by the results retrieved in the investigation at the organically poor site, the Testfield Scheyern. Here, most *cbbM* sequences detected were also closely related to the *cbb* sequences of *Thiobacillus ssp.*. The successful labelling of bacterial cells deriving from the tar-oil contaminated aquifer via fluorescent *in situ* hybridization (FISH) indicated considerable bacterial activity in this aquifer, but the detection of radiolabeled cells failed. ^{13}C-labelled $CaCO_3$ was exposed together with sterile sediment in the same aquifer. Cell counts suggested a successful colonization of the exposed sediments, but PFLA concentration was low. However, the incorporation of ^{13}C-carbon into two of the detected fatty acids was a direct hint for bacterial CO_2-uptake. Successful enrichment cultures out of both investigated aquifers proved the actual occurrence of autotrophs in those ecosystems. In total four new chemolithoautotrophic bacterial strains could be isolated, one of them, belonging to the genus *Thiobacillus*, was further characterized. It was an obligate chemolithoautotrophic strain, using the Calvin cycle for CO_2 fixation. It was described as a new species, *Thiobacillus thiophilus* D24TN sp. nov..

Autotrophie in Grundwasserökosystemen

Claudia Kellermann

Zusammenfassung: Den größten Anteil der globalen Netto-CO_2-Fixierung macht die Photosynthese der grünen Pflanzen, Algen und Cyanobakterien aus, aber andere Mikroorganismen spielen in Bezug auf Autotrophie ebenfalls eine wichtige Rolle. Autotrophe Mikroorganismen können in fast allen Bakteriengruppen (Eubacteria) gefunden werden und auch innerhalb der Archaea sind einige autotrophe Vertreter bekannt.

CO_2-Fixierung ist nicht nur einer der der wichtigsten biogeochemischen Prozesse weltweit und verantwortlich für den Aufbau organischer Verbindungen, welche für biologische Funktionen benötigt werden (z.B. für Zellwachstum oder für die Ernährung heterotropher Organismen), letztendlich basieren alle Ökosysteme auf dem durch autotrophe Organismen geleisteten Eintrag organischer Kohlstoffverbindungen und Energie. Während die Wichtigkeit der CO_2-Fixierung auf der Erdoberfläche anerkannt ist, ist über die Bedeutung autotropher Prozesse und ihre Rolle in Bezug auf Stoff- und Energieumsatz in unterirdischen Systemen fast nichts bekannt. Die weit verbreitete Meinung ist, dass unterirdische Gemeinschaften von heterotrophen Mikroorganismen dominiert werden, allerdings ist es unwahrscheinlich, dass die gesamte unterirdische Biomasse von den geringen Mengen organischen Kohlenstoffs, der von der Erdoberfläche eingetragen wird oder von Verschmutzungen stammt, abhängig ist. Grundwassersysteme bieten meist alle Voraussetzungen für autotrophes Wachstum (Elektronendonoren wie z.B. H_2, $S_2O_3^{2-}$ und auch Elektronenakzeptoren wie z.B. NO_3^-, O_2 sind ebenso vorhanden wie ausreichend anorganischer Kohlenstoff), was bedeutet, dass Autotrophe zumindest in einigen solcher Systeme signifikant zum Kohlenstoffkreislauf beitragen könnten. Insgesamt ist aber über die Existenz und die Bedeutung chemolithoautotropher CO_2-Fixierung in terrestrischen unterirdischen Systemen wenig bekannt.

Bisher sind fünf verschiedene CO_2-Fixierungswege bekannt, der Calvin-Zyklus, der reduktive Citrat-Zyklus, der reduktive Acetyl-CoA-Weg, der 3-Hydroxypropionat-Zyklus und der 3-Hydroxypropionat/ 4-Hydroxybutyrat-Weg. Der Calvin-Zyklus ist nicht nur der von allen am besten untersuchte, sondern wahrscheinlich auch der am häufigsten vorkommende CO_2-Fixierungsweg. Ein sechster CO_2-Fixierungsweg wurde erst vor kurzem entdeckt.

Ziel dieser Arbeit war es, das CO_2-Fixierungspotential der mikrobiellen Gemeinschaften in verschiedenen Grundwasserökosystemen anhand funktioneller Genanalyse (*cbbL*, *cbbM* und

acl Gene) nachzuweisen und dieses Potential mit tatsächlicher *in situ* autotropher Aktivität unter Verwendung verschiedener Methoden (FISH-MAR und PLFA-Analyse) zu verbinden. Anhand von Anreicherungskulturen unter obligat chemolithoautotrophen Bedingungen sollte ein Einblick in die Diversität dieser Gemeinschaften gewonnen werden.

Durch den Nachweis der *cbb* Gene konnte das Vorhandensein des CO_2-Fixierungspotentials sowohl in einem kontaminierten als auch in einem unkontaminierten Grundwasserleiter bewiesen werden. Die Mehrheit aller aus dem kontaminierten Aquifer erhaltenen *cbb* Sequenzen zeigte eine nahe Verwandtschaft zu *cbbL* und *cbbM* Sequenzen der Gattung *Thiobacillus*, was ein Hinweis darauf sein könnte, dass diese Gattung eine wichtige Rolle in Grundwasserökosystemen spielt. Diese These wird zusätzlich durch die Untersuchungsergebnisse des unkontaminierten Standorts unterstützt. Auch hier waren die meisten der erhaltenen *cbbM* Sequenzen nah verwandt zu den *cbbM* Sequenzen der Gattung *Thiobacillus*.

Das erfolgreiche Markieren von Bakterienzellen aus dem kontaminierten Aquifer unter Verwendung von fluoreszierender *in situ* Hybridisierung (FISH) lässt auf eine hinreichende Aktivität der Mikroorganismen schließen, der Nachweis ^{14}C-markierter Zellen war allerdings nicht möglich. ^{13}C-markiertes Kalziumkarbonat wurde zusammen mit sterilem Sediment in dem gleichen Grundwasserleiter inkubiert. Zellzahlbestimmungen ließen darauf schließen, dass das Sediment erfolgreich von Bakterien besiedelt wurde, trotzdem konnten nur wenige Fettsäuren nachgewiesen werden. Allerdings war der Einbau von ^{13}C-markiertem Kohlenstoff in zwei der gemessenen Fettsäuren ein direkter Hinweis auf bakterielle CO_2-Fixierung. Das tatsächliche Vorkommen Autotropher in den untersuchten Grundwasserleitern konnte anhand erfolgreicher Anreicherungen bewiesen werden. Insgesamt konnten aus den Anreicherungen vier neue, bisher unbekannte chemolithoautotrophe Bakterienstämme isoliert werden, einer davon, zur Gattung der *Thiobacillen* gehörend, wurde genauer charakterisiert. Er wurde als neue *Thiobacillus*-Art beschrieben, *Thiobacillus thiophilus* D24TN^T sp. nov., ein obligat chemolithoautotropher, über den Calvin-Zyklus CO_2-fixierender Stamm.

Table of Contents

Table of contents

1. General introduction

1.1 Autotrophy

CO_2 fixation is without a doubt one of the most important biogeochemical processes worldwide. Ultimately all ecosystems depend directly or indirectly on organic carbon provided by autotrophic CO_2 fixation. This way, every year about 120 billion tons of inorganic carbon are transferred into organic compounds (Kinkle and Kane, 2000; Hügler, 2003; Thauer, 2007), most of it by oxygenic photosynthesis of green plants, algae and cyanobacteria. However, chemolithoautotrophic bacteria and *Archaea* also contribute to global CO_2 fixation. Compared to eukaryotic phototrophs, which exhibit a relatively similar photosynthesis machinery, chemolithoautotrophs distribute across the archaeal and bacterial domains and show a high phylogenetic, metabolic and ecological diversity (Kinkle and Kane, 2000). These microorganisms, including sulfide-, sulfur-, metal-, ammonium-, and nitrite-oxidizing and aerobic hydrogen- and CO-oxidizing bacteria gain their energy for CO_2 fixation from the oxidation of inorganic substances instead from light (Tolli and King, 2005).

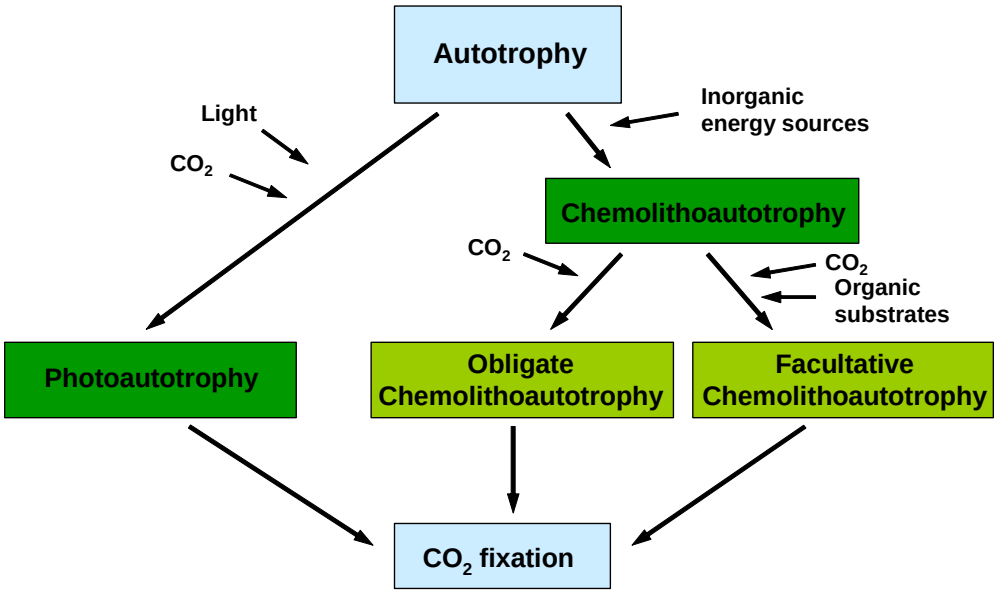

Figure 1.1: Autotrophic processes

The chemolithoautotrophs may be divided into two groups, *i.e.* (i) the obligate chemolithoautotrophs, which can use only inorganic carbon and inorganic compounds as carbon and electron donors for growth, and (ii) the facultative chemolithoautotrophs, which can, besides inorganic carbon and inorganic compounds, use organic compounds as carbon

and/or energy sources (Kusian and Bowien, 1997; Shively *et al.*, 1998; Badger and Bek, 2008). For a long time, chemolithoautotrophic microorganisms have been considered relatively unimportant with respect to the global carbon cycle (Fredrickson *et al.*, 1989; Kinkle and Kane, 2000). Nowadays it is known that chemolithotrophs may play a major role in elemental cycles (Fenchel and Finlay, 1995; McCollom and Amend, 2005). Bacterial chemolithoautotrophic communities have been investigated in various environments, *e.g.* Tolli and King (2005) found a high diversity among terrestrial facultative lithotrophs in pine forest and agroecosystem soils. Elsaied and Naganuma (2001) investigated the phylogenetic diversity concerning the *cbbL* genes at deep-sea seeps and hydrothermal vents, Selesi *et al.* (2005) dealt with the diversity of *cbbL* types in agricultural soil bacteria; in both studies the detected phylogenetic diversity of the RubisCO genes was quite high.

1.2 CO_2 fixation pathways

There are six CO_2 fixation pathways known so far, the sixth one was only recently discovered in the hyperthermophilic archaeum *Ignicoccus hospitalis* (Thauer, 2007; Huber *et al.*, 2008). The distribution of the pathways within different groups of organisms is not only depending on the phylogeny of the individual autotrophic organisms, but also determined by different characteristics, *e.g.* the demand of energy, the availability of reducing compounds, the requirements for metals, usage of coenzymes and the oxygen sensitivity of enzymes (Berg *et al.*, 2007).

The reductive tricarboxylic acid cycle (Reductive TCA cycle)

The reductive Tricarboxylic Acid cycle was already proposed in 1966 by Evans *et al.* (Evans *et al.*, 1966), but it took until 1990 for the details of this cycle to be worked out (Thauer, 2007). It basically is the citric acid cycle in reverse, a cycle which is used in heterotrophic metabolisms to assimilate acetyl-CoA. To enable the reversed cycle, three enzymes, catalyzing irreversible reactions in the citric acid cycle, have to be replaced. The ATP citrate lyase, which is one of the key enzymes of the reductive TCA cycle, catalyzing the ATP-dependent cleavage of citrate in oxalacetate and acetyl-CoA, replaces the citrate synthase. Another key enzyme of the reductive TCA cycle, the 2-oxoglutarate: ferredoxin oxidoreductase, which catalyzes the reductive carboxylation of succinyl-Coa to α-ketoglutarate, replaces the 2-oxoglutarate dehydrogenase. The third enzyme of the citric acid cycle which is replaced is the succinate dehydrogenase; instead the fumarate reductase can be found, which is reducing fumarate to succinate (Fig. 1.2). The presence of these enzymes

active in autotrophically grown bacteria and Archaea is indicative of a functioning reductive TCA cycle (Hügler *et al.*, 2005). As this cycle involves enzymes that are sensitive to oxygen, it only occurs in anaerobes or microaerophiles. So far, it has been detected in *Hydrogenobacter*, *Aquifex*, some sulfate-reducers and several thermophilic archaeal strains (Thauer, 2007) and is the only confirmed autotrophic pathway in ε-proteobacteria (Hügler *et al.*, 2005).

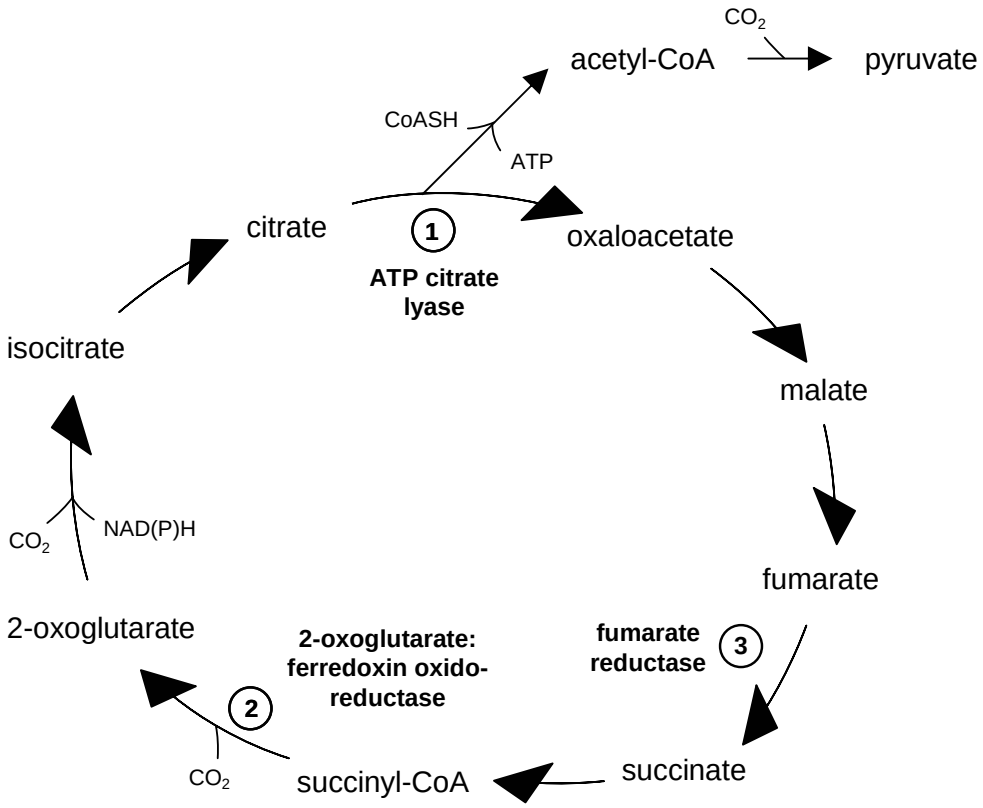

Figure 1.2: Reductive tricarboxylic acid cycle (reductive TCA cycle). The three key enzymes are (1) ATP citrate lyase, (2) 2-oxoglutarate:ferredoxin oxidoreductase and (3) fumarate reductase.

The reductive acetyl CoA pathway

This pathway was discovered at the beginning of the 1980s by Wood *et al.* (1986). In this pathway one CO_2 molecule is reduced to CO and one to methanol, subsequently forming acetyl-CoA from those (Wood *et al.*, 1986; Kinkle and Kane, 2000; Thauer, 2007). The CO_2 fixing key enzyme of this pathway is the CO dehydrogenase/acetyl-CoA synthase complex (Fig. 1.3). The reductive Acetyl CoA pathway is the only non-cyclic CO_2 fixation pathway known so far. As the CO dehydrogenase/acetyl-CoA synthase is very oxygen-sensitive it can only be found in strict anaerobes (Thauer, 2007). In those strict anaerobic bacteria CO_2 plays a dual role in metabolism, it can be used as an electron acceptor but it can also serve as the sole cell carbon source (Fuchs, 1986). The pathway can be found in most acetogenic and sulfate-reducing bacteria known so far, in autotrophic microorganisms that produce acetate and hydrogen sulfide respectively and in several archaeal autotrophs (Kinkle and Kane, 2000).

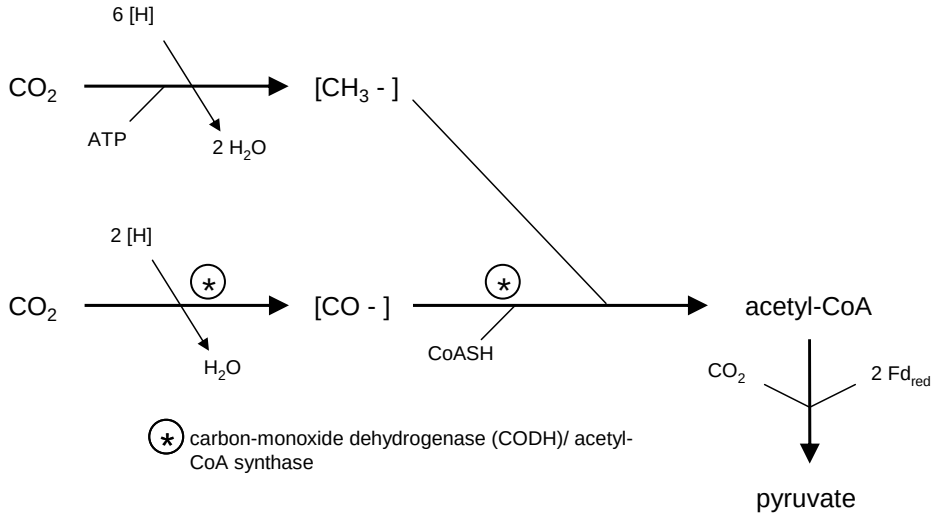

Figure 1.3: Reductive acetyl-CoA pathway. The key enzyme is the (*) CO dehydrogenase (CODH)/acetyl-CoA synthase complex

The 3-hydroxypropionate cycle

The 3-Hydroxypropionate cycle is an unidirectional pathway, reducing acetyl-CoA via 3-hydroxypropionate and propionyl-CoA to succinyl-CoA (Hügler *et al.*, 2003; Ishii *et al.*, 2004). The formation of 3-hydroxypropionate is characteristic for the cycle and this metabolite is excreted even when cell growth becomes limited (Holo, 1989; Alber and Fuchs, 2002). The cycle was originally discovered in the phototrophic bacterium *Chloroflexus aurantiacus* by Holo and Sirevåg (1986) and later was additionally detected in some chemotrophic *Archaea* (Alber and Fuchs, 2002; Hügler *et al.*, 2003; Berg *et al.*, 2007). The key enzymes are malonyl-CoA reductase and propionyl-CoA synthase (Fig. 1.4) (Hügler *et al.*, 2003). Interestingly, none of this cycle's enzymes is inherently oxygen sensitive (Thauer, 2007).

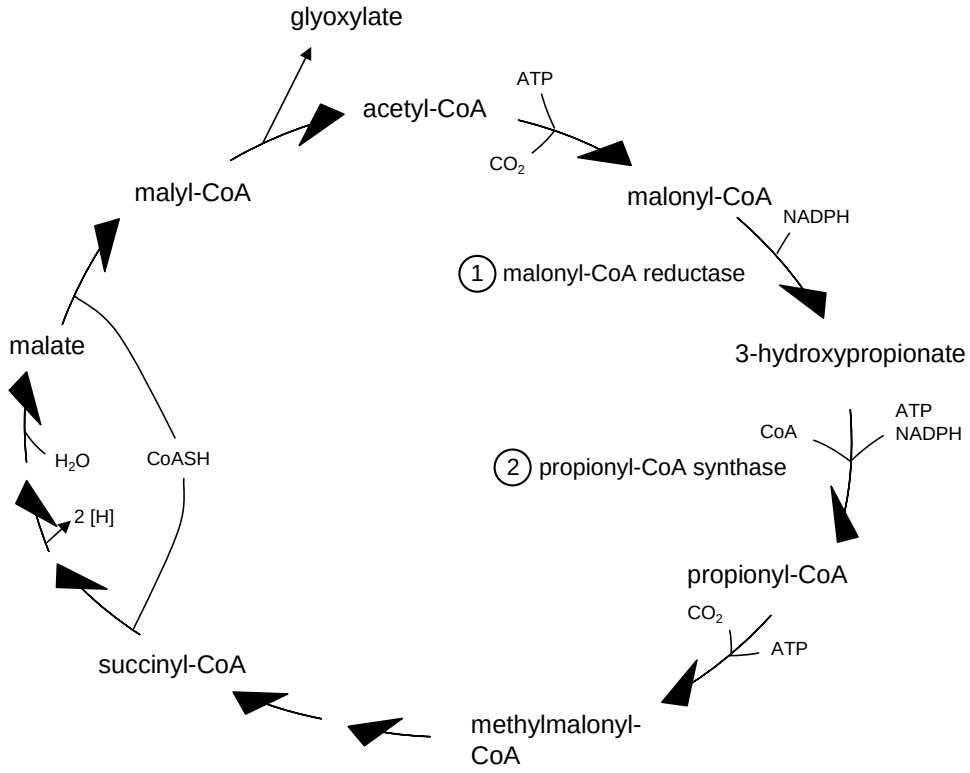

Figure 1.4: 3-hydroxypropionate cycle. The two key enzymes are (1) malonyl-CoA reductase and (2) propionyl-CoA synthase.

The 3-hydroxypropionate/ 4-hydroxybutyrate pathway

This CO_2 fixation pathway was described by Berg *et al.* (2007). It was first found to be operative in *Metallosphaera sedula*, microorganisms growing on H_2 and O_2 as the energy source (Thauer, 2007). Since then, the key genes of the pathway were also found in *Sulfolobus*, *Archaeoglobus* and *Cenarchaeum* species. It has some intermediates in common with the 3-Hydroxypropionate cycle, also forming succinyl-CoA from acetate and two CO_2 molecules via 3-hydroxypropionate. From succinyl-CoA on, the two pathways show considerable differences (Thauer, 2007).

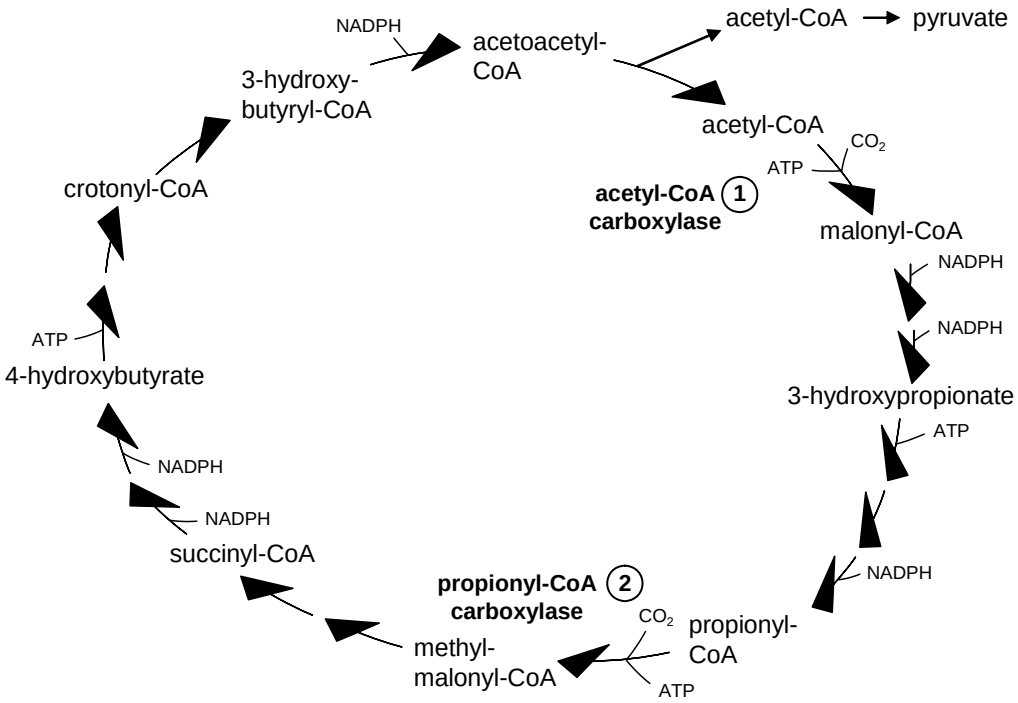

Figure 1.5: 3-hydroxypropionate/4-hydroxybutyrate pathway. The two enzymes responsible for CO_2 fixation are (1) acetyl-CoA carboxylase and (2) propionyl-CoA carboxylase.

The dicarboxylate/4-hydroxybutyrate cycle

This novel CO_2 fixation cycle was discovered in *Ignicoccus hospitalis*, an anaerobic, obligate autotrophic, hyperthermophilic archaeum and so far seems to be restricted to a small number of *Crenarchaeota* (Huber *et al.*, 2008).

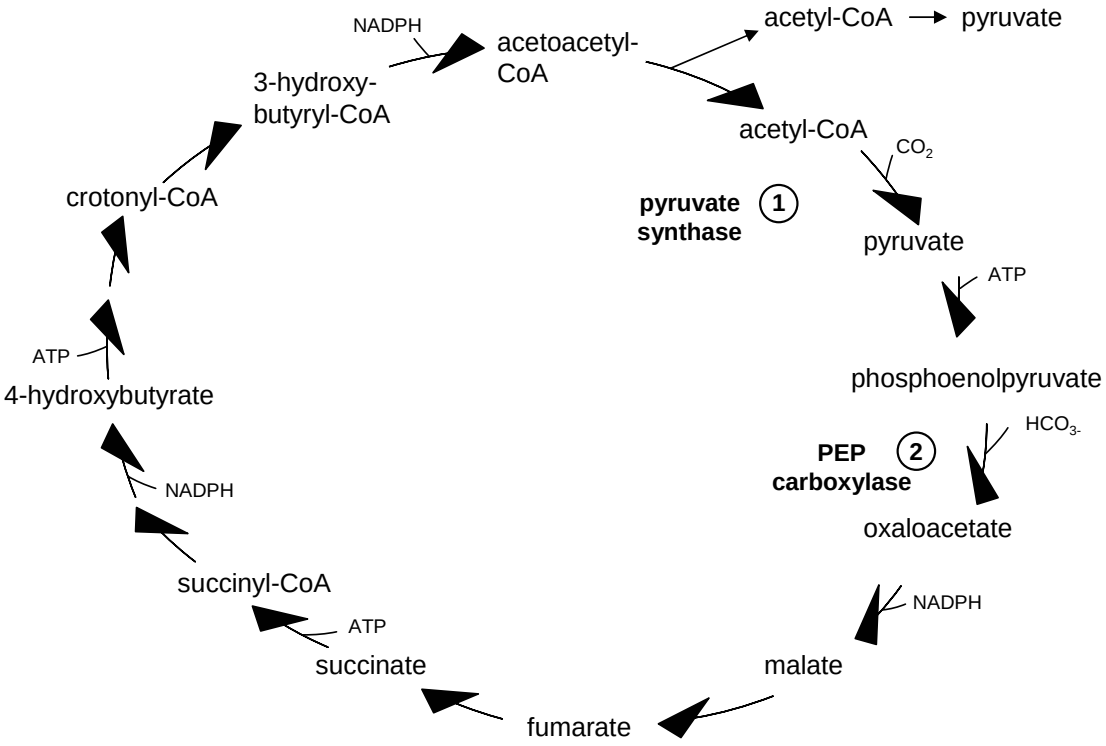

Figure 1.6: Proposed dicarboxylate/4-hydroxybutyrate cycle. The two enzymes responsible for CO_2 fixation are (1) pyruvate synthase and (2) phosphoenolpyruvate carboxylase.

Calvin-Benson-Bassham cycle

Of all CO_2 fixation pathways, the Calvin cycle seems to be the most abundant and important one (Tabita, 1999; Tolli and King, 2005; Badger and Bek, 2008). The cycle is characterized by two unique enzymatic activities: phosphoribulokinase and ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the most abundant enzyme in the world (Ellis, 1979). In the Calvin cycle RubisCO is the only enzyme responsible for the actual CO_2 fixation. It incorporates a molecule of CO_2 into ribulose 1,5-bisphosphate, forming two molecules of 3-phosphoglycerate and leading eventually to fructose-6-phosphate, which can be assimilated into biomass. The other enzymes of the cycle serve ribulose 1,5-bisphosphate regeneration (Fig. 1.6) (Shively *et al.*, 1998; Kinkle and Kane, 2000; Atomi, 2002).

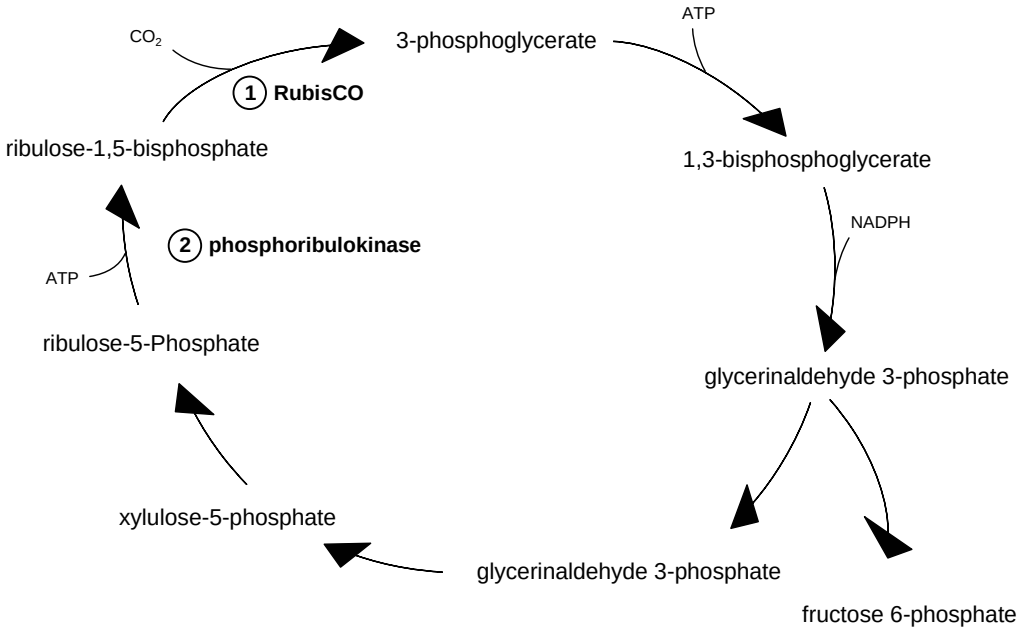

Figure 1.7: Calvin-Benson-Bassham cycle (Calvin cycle). The two key enzymes are (1) ribulose 1,5-bisphosphate carboxylase/ oxygenase (RubisCO) and (2) phosphoribulokinase.

It can be distinguished between four forms of RubisCO, which differ in structure, catalytic property and oxygen sensitivity (Tabita, 1988). Form I is the dominant type of RubisCO (*cbbL*), occurring in plants as well as in photo- and chemoautotrophic bacteria (Atomi, 2002; Selesi *et al.*, 2007). It consists of eight large and eight small subunits, encoding the large subunit of form I RubisCO. With a size of 1400 bp it is large enough to be used for significant phylogenetic analyses, therefore being ideally qualified to be used as functional marker (Watson and Tabita, 1997). There is a discrepancy between phylogenies based on the *cbbL* gene and those based on other genes. According to Delwiche and Palmer (1996) it is very

likely that several events of lateral gene transfer and/or gene duplications and differential loss are responsible for this; a good example for lateral gene transfer is the presence of the green-like RubisCO in *Rhodobacter capsulatus*, whereas *R. sphaeroides* has a red-like protein. In addition, both species contain a closely related form II RubisCO (Shively *et al.*, 1998). Another explanation for this discrepancy is the possession of two RubisCO operons in ancestral proteobacteria and cyanobacteria (Delwiche and Palmer, 1996). Form I can be divided into two groups: green-like and red-like. The green-like group can be further subdivided into Type IA, present in α-, β- and γ-*proteobacteria* and cyanobacteria and into Type IB, found in cyanobacteria and in plastids of plants and green algae. According to Badger and Bek (2008), Type IA enzymes are even further divided into two distinct types, IAc and IAq, based on distinct types of small subunits and gene arrangements. Type IB enzymes can be subclassified into IB and IBc to indicate the Type IBc in cyanobacteria which is associated with carboxysomes. The red-like group can also be divided into two groups. One detected in various chemoautotrophic bacteria and some photoautotrophic proteobacteria (Type IC), the other one present in marine non-green algae (Type ID) (Shively *et al.*, 1998; Horken and Tabita, 1999). Form II RubisCO has a comparable simple structure; it consists of only large subunits. It has poor catalytic characteristics, only functioning well at low oxygen and high CO_2 concentrations, conditions that reflect the ancient earth atmosphere. Those characteristics suggest that the more complex form I derives from form II (Watson and Tabita, 1997; Shively *et al.*, 1998). Form II occurs in some chemolithotrophs and phototrophs (Tolli and King, 2005). Some bacteria possess form I as well as form II *e.g. Thiobacillus denitrificans*. Form III can be found in *Archaea*, containing catalytic active amino acid residue, that are necessary for carboxylation as well as oxygenation (Horken and Tabita, 1999; Selesi *et al.*, 2005). Form IV has been discovered in *Bacillus subtilis*, *Chlorobium tepidum* and *Archaeoglobus fulgidus* (Selesi *et al.*, 2005). It is considered not to be involved in the Calvin cycle due to the lack of several of the required amino acid residues for the catalytic activity of RubisCO (Hanson and Tabita, 2001) and is therefore termed 'RubisCO-like' (Badger and Bek, 2008). The Calvin cycle is energetically very expensive. In total, it expends nine molecules of ATP and six of NADH for the formation of one molecule of triose phosphate from three molecules of CO_2.

1.3 Groundwater

As the investigations introduced in this thesis mainly concentrates on microorganisms living in groundwater and aquifers, it may help the reader to get a brief definition of these two terms. What exactly is groundwater? Following the traditional definition of hydrologists and hydrogeologists, groundwater is the subsurface water contained in the zone of saturation that can move freely and can easily be extracted (Pfannkuch, 1969; Freeze and Cherry, 1979). From the microbiologist's point of view, the definition often includes all the subsurface water found in the unsaturated sediments below soil horizons and in the zone of saturation (Madsen and Ghiorse, 1993). Groundwater ecologists generally refer to water in the saturated zone, however, including *e.g.* water existing within the superficial riverbed sediments (Rouch and Danielopol, 1997). The geological unit (*e.g.* porous sediments, karstic or granitic rock) filled or partly filled with groundwater we call an aquifer. More important than those definitions is the fact that groundwater constitutes the largest reservoir of freshwater in the world, accounting for over 97% of all freshwater available on earth (excluding glaciers and ice caps) and is an important component of the global hydrological cycle (Gibert, 2001; Danielopol *et al.*, 2003). With beginning of civilization humans started to exploit groundwater resources, but massive resource usage was mainly restricted to the last 50 years (Foster and Chilton, 2003). For a long time groundwater was thought to be naturally protected from pollution and an inexhaustible resource. Due to this wrongful assumption many aquifers were depleted and/or polluted with toxic chemicals, impossible to regenerate their good status in the near future. For a long time, only little information was available on organisms and processes in groundwater ecosystems, but due to those soaring problems an increasing number of studies investigating the subsurface biological aspects was initiated (Ghiorse and Wilson, 1988; Ghiorse, 1997; Danielopol *et al.*, 2003). However, compared to aquatic and near-surface terrestrial environments, our knowledge on the ecology of the subsurface is still a relatively recent development (Chandler *et al.*, 1998).

1.4 Microorganisms in groundwater ecosystems

The very first general idea of the subsurface was that from a mainly sterile or only scarcely inhabited one (Fredrickson and Onstott, 1996). Early investigators observed low bacterial numbers in soils decreasing with depth, suggesting sparse microbial populations in subsurface environments and their activity being restricted to the uppermost layer of the earth's crust (Phelps *et al.*, 1989). Nowadays it is estimated that life can exist to a depth of at least 3.5 km. Colonization limits are temperature, water availability, extremely acidic or alkaline pH and to

some extent pore size of sediment and rock. Considering the huge volumes of subsurface sediment and rock that might be colonized, the extent of subsurface biomass could potentially approach or exceed levels observed on the surface (Krumholz, 2000; Kieft *et al.*, 2005; Griebler and Lueders, 2008). Whitman *et al.* (1998) stated as a minimum estimate, that the biomass of groundwater dwelling prokaryotes in the unconsolidated subsurface domain accounts for about 6-40% of the earth's total microbial biomass. Bacterial cell numbers vary in different groundwater ecosystems, from as low as 10^2 to 10^6 cells per cm^3 in water and between 10^4 and 10^8 cells per cm^3 of sediment (Griebler and Lueders, 2008). Subsurface microorganisms and their influence on geochemical transformations have mostly been neglected until it was shown that those organisms are active and relevant for biochemical processes (Fredrickson *et al.*, 1989; Bachofen *et al.*, 1998; Griebler and Lueders, 2008). They are involved in weathering and formation of minerals and store important quantities of carbon, nitrogen and phosphorus in their biomass, therefore influencing groundwater chemistry and being responsible for the major turnover of energy and matter in the subsurface (Chapelle *et al.*, 1987; Fliermans and Balkwill, 1989; Phelps *et al.*, 1989; Chapelle and Lovley, 1990; Phelps *et al.*, 1994; Danielopol *et al.*, 2003).

1.5 Autotrophic microorganisms in groundwater ecosystems

Coming back to autotrophy in subsurface ecosystems, for a long time, subsurface communities have thought to be exclusively heterotrophic, dependent on organic matter once deposited with the formation sediments or on organic matter that continuously is imported from the surface. Organisms were therefore suggested to exist mostly in a kind of starvation-survival mode in these typically oligotrophic environments (Kinkle and Kane, 2000; Krumholz, 2000; Morita, 2000). But even though most pristine groundwater ecosystems are oligotrophic with no or only very little organic carbon available, they most often constitute all necessary conditions for chemolithoautotrophic growth, *i.e.* electron donors as well as electron acceptors are available, together with plenty of inorganic carbon (Labrenz *et al.*, 2005). By increasing the carbon content in those systems, they even can support heterotrophic bacteria with organic compounds (Fredrickson *et al.*, 1989; McCollom and Amend, 2005). Over the last years interest in subsurface chemolithoautotrophic communities increased and to date there exist quite a few studies concerning autotrophic microorganisms in different types of groundwater ecosystems, highlighting the occurrence of autotrophic methanogens, acetogens, nitrifiers and sulfur-oxidizers among others (Fredrickson *et al.*, 1989; Stevens and McKinley, 1995; Pedersen, 1997; Kotelnikova and Pedersen, 1998; Krumholz, 2000;

Chapelle *et al.*, 2002). Also in organically contaminated aquifers autotrophic microorganisms may be suggested to play a significant role, having some advantages over chemotrophs. Organically polluted aquifers often are overloaded with complex mixtures of persistent organic compounds. Those compounds, if not toxic, are non degradable for most microorganisms, leaving them in the same nutrient-poor situation as microorganisms in pristine aquifers (Alfreider *et al.*, 2003).

1.6 Aim of this thesis

Most biogeochemical transformations in groundwater ecosystems are mediated by bacteria. The widespread opinion is that subsurface bacterial communities are dominated by heterotrophic microorganisms. Surveys of the abundance, activity and diversity of heterotrophs exist, but there is still only little knowledge about autotrophic processes in the subsurface.

The aim of the present thesis is to gain new insights into the role of microbial primary production in different subsurface systems, focussing on the following questions:

- Can CO_2 fixation potential in pristine and contaminated aquifers be detected?
- How diverse are those autotrophic communities?
- Can CO_2 fixation potential be connected with actual autotrophic activity?

The detection of CO_2 fixation potential in groundwater and aquifer sediment samples mainly concentrates on the analysis of functional marker genes encoding for key enzymes of the Calvin cycle and the reductive Tricarboxylic Acid cycle. Besides this, microorganisms have been enriched and isolated under chemolithoautotrophic conditions in artificial groundwater medium. A new chemolithoautotrophic nitrate-reducing and thiosulfate-oxidizing bacterium, *Thiobacillus thiophilus* D24TN^T sp. nov., could be described. Bacterial CO_2 uptake was measured by *in situ* ^{13}C incubation and incorporation of ^{13}C-label into the phospholipid fatty acids (PLFAs) and by fluorescence *in situ* hybridization combined with microautoradiography (FISH-MAR).

1.7 References

Alber, B.E., and Fuchs, G. (2002) Propionyl-coenzyme A synthase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO_2 fixation. *J Biol Chem* **277**: 12137-12143.

Alfreider, A., Vogt, C., Hoffmann, D., and Babel, W. (2003) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecol* **45**: 317-328.

Atomi, H. (2002) Microbial enzymes involved in carbon dioxide fixation. *J Biosci Bioeng* **94**: 497-505.

Bachofen, R., Ferloni, P., and Flynn, I. (1998) Microorganisms in the subsurface. *Microbiol Res* **153**: 1-22.

Badger, M.R., and Bek, E.J. (2008) Multiple RubisCO forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J Exp Bot* **59**: 1525-1541.

Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in *Archaea*. *Science* **318**: 1782 - 1786.

Chandler, D.P., Wagnon, C.A., and Bolton, H. (1998) Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. *Appl Environ Microbiol* **64**: 669-677.

Chapelle, F.H., and Lovley, D.R. (1990) Rates of microbial metabolism in deep coastal plain aquifers. *Appl Environ Microbiol* **56**: 1865-1874.

Chapelle, F.H., Zelibor, J.L., Grimes Jr., D.J., and Knobel, L.L. (1987) Bacteria in deep coastal plain sediments of Maryland - a possible source of CO_2 to ground water. *Water Resour Res* **23**: 1625-1632.

Chapelle, F.H., O'Neill, K., Bradley, P.M., Methe, B.A., Ciufo, S.A., Knobel, L.L., and Lovley, D.R. (2002) A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* **415**: 312-315.

Danielopol, D.L., Griebler, C., Gunatilaka, A., and Notenboom, J. (2003) Present state and future prospects for groundwater ecosystems. *Environ Conserv* **30**: 104–130.

Delwiche, C.F., and Palmer, J.D. (1996) Rampant horizontal transfer and duplication of RubisCO genes in eubacteria and plastids. *Mol Biol Evol* **13**: 873-882.

Ellis, R.J. (1979) The most abundant protein in the world. *Trends Biochem Sci* **4**: 241-244.

Elsaied, H., and Naganuma, T. (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**: 1751-1765.

Evans, M.C., Buchanan, B.B., and Arnon, D.I. (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. *P Natl Acad Sci USA* **55**: 928-934.

Fenchel, T., and Finlay, B.J. (1995) *Ecology and Evolution in Anoxic Worlds*: Oxford University Press.

Fliermans, C.B., and Balkwill, D. (1989) Microbial life in deep terrestrial subsurfaces. *BioScience* **39**: 370-377.

Foster, S.S.D., and Chilton, P.J. (2003) Groundwater: the processes and global significance of aquifer degradation. *Philos T Roy Soc B* **358**: 1957-1972.

Fredrickson, J.K., and Onstott, T.C. (1996) Microbes deep inside the earth. *Sci Am* **275**: 68-73.

Fredrickson, J.K., Garland, T.R., Hicks, R.J., Thomas, J.M., Li, S.W., and McFadden, K.M. (1989) Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. *Geomicrobiol J* **7**: 53-66.

Freeze, R.A., and Cherry, J.A. (1979) *Groundwater*. Englewood Cliffs, New Jersey: Prentice-Hall.

Fuchs, G. (1986) CO_2 fixation in acetogenic bacteria: variations on a theme. *FEMS Microbiol Rev* **39**: 181-213.

Ghiorse, W.C. (1997) Subterranean life. *Science* **275**: 789-790.

Ghiorse, W.C., and Wilson, J.T. (1988) Microbial ecology of the terrestrial subsurface. *Adv Appl Microbiol* **33**: 107-172.

Gibert, J. (2001) Basic attributes of groundwater ecosystems. In *Groundwater ecology A tool for management of water resources. EC Conference proceedings.* Griebler, C., Danielopol, D.L., Gibert, J., Nachtnebel, H.-P., and Notenboom, J. (eds). Luxembourg: European Commission; Environment and climate programme and Austrian Academy of Sciences, pp. 39-52.

Griebler, C., and Lueders, T. (2008) Microbial biodiversity in groundwater ecosystems. *Freshwater Biol* **early online doi:10.1111/j.1365-2427.2008.02013.x**.

Hanson, T.E., and Tabita, F.R. (2001) A ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. *Proc Natl Acad Sci USA* **98**: 4397-4402.

Holo, H. (1989) *Chloroflexus aurantiacus* secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO_2 and acetate. *Arch Microbiol* **151**: 252-256.

Holo, H., and Sirevåg, R. (1986) Autotrophic growth and CO_2 fixation of *Chloroflexus aurantiacus*. *Arch Microbiol* **145**: 173-180.

Horken, K.M., and Tabita, F.R. (1999) Closely related form I ribulose bisphosphate carboxylase/oxygenase molecules that possess different CO_2/O_2 substrate specificities. *Arch Biochem Biophys* **361**: 183-194.

Huber, H., Gallenberger, M., Jahn, U., Eylert, E., Berg, I.A., Kockelkorn, D. *et al.* (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum *Ignicoccus hospitalis*. *Proc Natl Acad Sci USA* **105**: 7851-7856.

Hügler, M. (2003) Autotrophe CO_2-Fixierung in thermophilen Mikroorganismen. Freiburg: Albert-Ludwigs-Universität Freiburg i. Br., pp. 127

Hügler, M., Huber, H., Stetter, K.O., and Fuchs, G. (2003) Autotrophic CO_2 fixation pathways in archaea (Crenarchaeota). *Arch Microbiol* **179**: 160-173.

Hügler, M., Wirsen, C.O., Fuchs, G., Taylor, C.D., and Sievert, S.M. (2005) Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria. *J Bacteriol* **187**: 3020-3027.

Ishii, M., Chuakrut, S., Arai, H., and Igarashi, Y. (2004) Occurrence, biochemistry and possible biotechnological application of the 3-hydroxypropionate cycle. *Appl Microbiol Biot* **64**: 605-610.

Kieft, T.L., McCuddy, S.M., Onstott, T.C., Davidson, M., Lin, L.-H., Mislowac, B. *et al.* (2005) Geochemically generated, energy-rich substrates and indigenous microorganisms in deep, ancient groundwater. *Geomicrobiol J* **22**: 325-335.

Kinkle, B.K., and Kane, T.C. (2000) Chemolithoautotrophic micro-organisms and their potential role in subsurface environments. In *Ecosystems of the World*. Wilkens, H., Culven, D.C., and Humphreys, W.F. (eds). Amsterdam: Elsevier, pp. 309-318.

Kotelnikova, S., and Pedersen, K. (1998) Distribution and activity of methanogens and homoacetogens in deep granitic aquifers at Aspo Hard Rock Laboratory, Sweden. *FEMS Microbiol Ecol* **26**: 121-134.

Krumholz, L.R. (2000) Microbial communities in the deep subsurface. *Hydrogeol J* **8**: 4-10.

Kusian, B., and Bowien, B. (1997) Organization and regulation of cbb CO_2 assimilation genes in autotrophic bacteria. *FEMS Microbiol Rev* **21**: 135-155.

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005) Impact of different *in vitro* electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ Microbiol* **71**: 6664-6672.

Madsen, E., and Ghiorse, W.C. (1993) Groundwater microbiology: subsurface ecosystem processes. In *Aquatic Microbiology: an ecological approach*. Ford, T.E. (ed). Boston: Blackwell Scientific Publications, pp. 167-213.

McCollom, T.M., and Amend, J.P. (2005) A thermodynamic assessment of energy requirements for biomass synthesis by chemolithoautotrophic micro-organisms in oxic and anoxic environments. *Geobiology* **3**: 135-144.

Morita, R.Y. (2000) Is H_2 the universal energy source for long-term survival? *Microb Ecol* **38**: 307-320.

Pedersen, K. (1997) Microbial life in deep granitic rock. *FEMS Microbiol Rev* **20**: 399-414.

Pfannkuch, H.O. (1969) *Elsevier's Dictionary of Hydrobiology*. Amsterdam: Elsevier.

Phelps, T.J., Murphy, E.M., Pfiffner, S., and White, D.C. (1994) Comparison between geochemical and biological estimates of subsurface microbial activity. *Microb Ecol* **28**: 335-349.

Phelps, T.J., Fliermans, C.B., Garland, T.R., Pfiffner, S., and White, D.C. (1989) Methods for recovery of deep terrestrial subsurface sediments for microbiological studies. *J Microbiol Meth* **9**: 267-279.

Rouch, R., and Danielopol, D.L. (1997) Species richness of microcrustacea in subterranean freshwater habitats. Comparative analysis and approximate evaluation. *Int Rev Ges Hydrobio* **82**: 121-145.

Selesi, D., Schmid, M., and Hartmann, A. (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71**: 175-184.

Selesi, D., Pattis, I., Schmid, M., Kandeler, E., and Hartmann, A. (2007) Quantification of bacterial RubisCO genes in soils by *cbbL* targeted real-time PCR. *J Microbiol Meth* **69**: 497-503.

Shively, J.M., van Keulen, G., and Meijer, W.G. (1998) Something from almost nothing: Carbon dioxide fixation in chemoautotrophs. *Annu Rev Microbiol* **52**: 191-230.

Stevens, T.O., and McKinley, J.P. (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270**: 450-454.

Tabita, F.R. (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol Rev* **52**: 155–189.

Tabita, F.R. (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective. *Photosynthesis Research* **60**: 1-28.

Thauer, R.K. (2007) A Fifth Pathway of Carbon Fixation. *Science* **318**: 1732-1733.

Tolli, J., and King, G.M. (2005) Diversity and structure of bacterial chemolithotrophic communities in pine forest and agroecosystem soils. *Appl Environ Microbiol* **71**: 8411-8418.

Watson, G.M.F., and Tabita, F.R. (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* **146**: 13-22.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA* **95**: 6578-6583.

Wood, H.G., Ragsdale, S.W., and Pezacka, E. (1986) CO_2 fixation into acetyl-CoA: a new pathway of autotrophic growth. *Trends Biochem Sci* **11**: 14-18.

2. *Thiobacillus thiophilus* D24TN^T sp. nov. , a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments

2.1 Introduction

CO_2 fixation is one of the world's most important biogeochemical processes. While the importance of CO_2 fixation on the terrestrial surface is known, there is little information about autotrophic processes in the subsurface (Kinkle and Kane, 2000). Most biogeochemical transformations in groundwater ecosystems are mediated by bacteria and the subsurface does comply with all requirements for chemolithoautotrophic processes. At numerous sites electron donors (*e.g.* NH_4^+, HS^-, H_2, $S_2O_3^{2-}$) and electron acceptors (*e.g.* Fe(III), Mn(IV), NO_3^-, O_2) are available in appropriate combination and sufficient amounts together with plenty of inorganic carbon (Madsen and Ghiorse, 1993; Labrenz *et al.*, 2005). When looking at the organic carbon in groundwater ecosystems, two extremes are often met, either pristine systems are depleted in dissolved organic carbon (DOC) or polluted aquifers are overloaded with complex mixtures of organic compounds sometimes toxic and degradable only by individual microorganisms (Alfreider *et al.*, 2003). Both situations may support argumentation towards a significant role of chemolithoautotrophic members in the microbial community. It may be the limited availability of DOC in the first case, and the necessity to get rid of electrons in the latter case.

In the course of field studies on the importance of autotrophic microorganisms in groundwater ecosystems a new thiosulfate-oxidizing strain was isolated under chemolithoautotrophic conditions from a tar oil contaminated aquifer. This strain belongs to the genus *Thiobacillus* within the *β-Proteobacteria* and is phylogenetically most closely related to *Thiobacillus denitrificans* (97.6% sequence similarity).

2.2 Material and Methods

2.2.1 Sampling site and sediment sample collection

The sediments from the quaternary homogenous sandy aquifer were collected during a well drilling campaign in June 2005. The aquifer is situated on a former gasworks site in the river Rhine valley in Duesseldorf-Flingern, Germany. Here, during the first half of the 20th century large amounts of tar oil phase have been released into the subsurface resulting in a contaminant plume with BTEX (Benzene, Toluene, Ethylbenzene, and Xylene isomers) concentrations >100 mg L^{-1} and PAH (polycyclic aromatic hydrocarbon) concentrations >10 mg L^{-1}. Since 1996 the major part of the oil phases in soil and groundwater has been removed due to several remediation activities. Residual BTEX concentrations are still at about 20-50 mg L^{-1} in the plume centre, while the average PAH concentrations are still about 10 mg L^{-1}. Further detail concerning the field site can be found with Anneser *et al.* (2008). Strain D24TN^T was isolated from anoxic sediment originating 11.2 m below soil horizon and 4.9 m below groundwater table. To protect the samples from contact with oxygen, sediment liners were removed from the borehole under argon atmosphere and put into a box flushed with argon gas for further processing. Sediment subsamples were then taken with an autoclaved spoon, immediately transferred into sterile Schott flasks filled with anoxic groundwater from the aquifer and stored at 4°C in darkness.

2.2.2 Enrichment conditions

Enrichment cultures for chemolithoautotrophic growth were prepared using diluted Widdel freshwater medium (Widdel and Bak, 1992) (dilution 1:10; pH 7.3, anoxic), with sodium thiosulfate (10 mM) as electron donor and sodium nitrate (10 mM) as electron acceptor (both, nitrate (Anneser *et al.*, 2008) as well as thiosulfate (Einsiedl, unpublished data) could be detected in sufficient amounts in the aquifer). The redox indicator resazurin (1 mg L^{-1}) was used to confirm anoxic conditions during incubation. 10 g sediment (wet-weight) was filled into 120 mL serum bottles containing 50 mL enrichment medium. The headspace was replaced by N_2/CO_2 gas (80:20 v/v) and bottles were sealed with butyl stoppers (Ochs). Primary enrichment cultures were incubated at 16°C (*in situ* aquifer temperature) in the dark for 3-4 months and transferred to fresh medium when visibly turbid. For purification of the most abundant cell type in the enrichment culture two dilution series at appropriate dilutions (highest dilution 10^{-9}; enrichment culture cell abundance was $1.4*10^8$) under the same culturing conditions were performed.

2.2.3 Purity assessment

To confirm its purity, strain D24TN^T was checked by phase-contrast microscopy. Pure cultures were frequently transferred every 2-3 weeks; cell growth was controlled by measuring optical density at 580 nm (OD_{580}; Varian), occasionally cell counts and microscopy (Zeiss).

2.2.3.1 Cell morphology

To determine cell size and shape D24TN^T was observed at 1000-fold magnification and transmission electron micrographs were taken. For the electron micrographs 200 mL of a culture grown for a week were centrifuged. After transfer to a plastic capsule, the pellet was covered with 2.5% glutaraldehyde in cacodylat buffer (Science Services). After three washing steps with cacodylat buffer (each 20 minutes), the pellet was incubated in a chrome osmium acid mixture as a second fixation step. The mixture contained one part buffer solution (100 mL 5% aqueous $K_2Cr_20_7$ solution set to pH 7.2 with 12 mL 2.5 n KOH), one part salt solution (3.4% NaCl) and two parts 2% OsO_4 solution. After three times rinsing with double distilled water five dehydration steps (30%, 50%, 70%, 90% and 96% ethanol; each 15 to 20 minutes) followed. First a 1:1 mixture of EPON 812 solution (Shell Chemical Corp) and propylenoxyde and after incubation for 1 hour, pure Epon solution was added. After 12 hours the EPON was replaced by fresh EPON solution, followed by the polymerisation step (at 60°C for 24-48 hours). The ultrathin sections (60-70 nm), cut in an ultramicrotome (Reichert-Jung), were covered for contrastation with 0.5% uranylacetate and 3% leadcitrate and afterwards investigated by transmission electron microscopy (EM 10 CR; Zeiss).

2.2.3.2 Growth characteristics

Unless otherwise described, routine cultivation and growth tests were performed in 120 mL serum bottles containing 50-60 mL Widdel freshwater medium (dilution 1:2, pH 7.3, oxic) with sodium thiosulfate (10 mM) as electron donor and sodium nitrate (10 mM) or oxygen as electron acceptor in at least duplicate incubations. Strain D24TN^T was isolated under anoxic conditions; additionally it was tested for the ability of aerobic growth. Therefore the same medium as described above but with oxygen as electron acceptor instead of nitrate was used.

Growth tests on agar plates were performed, under oxic as well as under anoxic conditions. Agar plates containing different media were used: the usual D24TN medium, M832 (*Thiobacillus denitrificans* medium; published by German Collection of Microorganisms and Cell Cultures GmbH) and a standard nutrient medium (meat extract and peptone). To test if

strain D24TN^T was able to grow in a medium with higher salt concentrations, a 1:2 dilution instead of a 1:10 dilution of the Widdel freshwater medium was used. To check if the strain is an obligate or a facultative chemolithoautotroph, chemoorganoheterotrophic growth was tested in complex medium (nutrient broth) and Widdel freshwater medium (without thiosulfate as electron source) supplemented with either glucose (10 mM), fructose (10 mM), sodium acetate (10 mM) or formate (10 mM) under aerobic conditions. A similar test series was run to test for the capacity of chemolithoheterotrophic growth under aerobic conditions with thiosulfate (10 mM) as electron source. Here, growth was monitored via optical density measurements. Additionally in case of sodium acetate consumption growth was followed by means of ion chromatography (DX-100 Ion Chroamtograph; Dionex).

Temperature range and optimum were determined via growth experiments at different temperatures ranging from -2°C to 37°C. The pH range and the optimal pH for growth were determined checking 13 different pH values ranging from 6.0 to 8.9. The initial medium pH was set using 1 M HCl or 0.5 M Na_2CO_3. Since D24TN^T continuously lowered the pH during growth, pH in the medium had to be adjusted every second day. Growth rates at different pH values were determined by measuring optical density at 580 nm (OD_{580}; Varian). The ability of D24TN^T to utilize different electron donors was tested applying H_2 (30 mL H_2 in headspace of serum bottle), NH_4^+ (5 mM), S^{2-} (5 mM), FeS (3 mM), $S_4O_6^{2-}$ (10 mM) and S^0 (0.5 g L^{-1}) using oxygen and/ or nitrate (5 mM) as electron acceptor. The utilization of electron acceptors alternative to oxygen and nitrate, was investigated testing SO_4^{2-} (5 mM) and Fe(III) (ferrihydrite 40 mM). D24TN^T was further tested for its ability to grow with different salt concentrations (0.5%, 1%, 2%, 3%, 4%, 5% and 8% NaCl (w/v)). Gram staining was performed using a gram-staining Kit from Sigma-Aldrich according to the manufacturer's instructions, with *Bacillus subtilis* and *Pseudomonas putida* strain F1 as positive and negative controls, respectively. Spore formation was tested applying pasteurization. Therefore cultures were heated 10 min at 80°C, transferred in fresh medium and incubated at 30°C for several weeks. Catalase activity was determined placing a 3% hydrogen peroxide solution on a cell pellet of a freshly grown culture. For testing oxidase activity the oxidase test of Fluka (Fluka/Sigma Aldrich) was performed according to the manufacturer's instructions.

2.2.3.3 G + C content determination

Determination of the G + C content was performed at the German Collection of Microorganisms and Cell Cultures (DSMZ). It was calculated from the ratio of deoxyguanosine (Badger and Bek, 2008) and thymidine (dT) according to the method described by Mesbah *et al.* (1989) by means of HPLC analysis (Shimadzu).

2.2.3.4 DNA-DNA hybridization

To determine the genomic relatedness between strain D24TN^T and its closest relative *Thiobacillus denitrificans*, DNA-DNA hybridization was performed. Therefore from each of the two species 3 g cell material was produced, isolated by centrifugation and resuspended in isopropanol/ H_2O_{dest} 1:1 (v/v). All other steps were carried out by the DSMZ as described by Huß *et al.* (1983) and De Ley *et al.* (1970), with the modifications of Cashion *et al.* (1977).

2.2.3.5 16S rDNA analysis

Extraction of genomic DNA from liquid cultures (50 mL) was performed using a modified protocol from Lueders *et al.* (2004) and Gabor *et al.* (2003). PCR components (Fermentas) consisted of 5 µL 10x buffer, 3 µL 25 mM $MgCl_2$, 0.5 µL each deoxynucleoside triphosphate at 10 mM, 0.5 µL bovine serum albumin (0.25 mg mL^{-1}), 0.5 µL each primer at 50 µM (MWG), 1 µL template DNA, 0.2 µL (0.2 units) Taq Polymerase and 38.8 µL GIBCO™ Water (Invitrogen). PCR was performed in an Eppendorf Mastercycler (Eppendorf) in a total volume of 50 µL per reaction. For amplification of the 16S rRNA gene the universal 16S primer 27-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R (5'-CGGYTACCTTGTTA CGACTT-3') (Weisburg *et al.*, 1991) were used. Cycling parameters were as follows: an initial denaturation for 90 s at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 52°C and 90 s at 70°C, and a terminal extension for 5 min at 70°C. Amplification products were analyzed electrophoretically on a 2% agarose gel to ensure correct size (16S rRNA 1.4 kb). PCR products were purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions. For sequencing the Big Dye™ Terminator labelling kit (Applera) with an ABI PRISM™ 3730 DNA analyser (Applied Biosystems) was used. Sequence data were analysed with the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were calculated by maximum-likelihood (Olsen *et al.*, 1994) and neighbour-joining (Saitou and Nei, 1987) analysis.

2.2.3.6 CO_2 fixation via the Calvin-Benson-Bassham cycle (Calvin cycle)

Since the Calvin cycle is the most prominent CO_2 fixation pathway in aerobes and facultative anaerobes and due to its close relatedness to *Thiobacillus denitrificans*, D24TN^T was tested for the corresponding marker genes, *i.e.* the *cbb* genes, encoding ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme of the Calvin cycle. The primer sets for form I RubisCO (*cbb*LG and *cbbL*R) *cbbL*G1-F (5'-GGCAACGTGTTCGGSTTCAA-3') and *cbbL*G1-G (5'-TTGATCTCTTTCCACGTTTCC-3'), *cbbL*R1-F (5'-AAGGAYGACGAGAA

CATC-3') and *cbbL*R1-R (5'-TCGGTCGGSGTGTAGTTGAA-3') (Selesi *et al.*, 2005) and the primer set for form II RubisCO (*cbbM*) *cbbM*-f (5'-GGCACCATCATC AAGCCCAAG-3') and *cbbM*-r (5'-TCTTGCCGTAGCCCATGGTGC-3') (Alfreider *et al.*, 2003) were used to amplify the RubisCO genes, applying following cycling parameters: an initial denaturation for 4 min at 94°C followed by 32 cycles of 1 min at 94°C, 1 min at 62°C (*cbbL*G) or 57°C (*cbbM*) and 1 min at 70°C, concluding with a 10 min extension at 70°C. Amplification products were analyzed electrophoretically on a 2% agarose gel to ensure correct size (*cbbL*G 1.1 kp, *cbbL*R 0.8 kb, *cbbM* 0.5 kb). PCR products were processed for sequencing the same way as described before for the 16S PCR product. Sequence data were analysed with the ARB software package (Ludwig *et al.*, 2004) as described before. Phylogenetic trees were also calculated by maximum-likelihood (Olsen *et al.*, 1994) and neighbour-joining (Saitou and Nei, 1987) analysis. To investigate whether D24TN^T is actually using the Calvin cycle for CO_2 fixation, RT-PCR was used to detect transcription of the *cbb* genes. For total RNA isolation 50 mL densely aerobically and anaerobically grown culture was centrifuged (4.000 rpm; 15 min). Preparation was performed as described by Schmitt *et al.* (1990). To obtain pure RNA without DNA, 25 µL DNA/RNA extract were digested with DNaseI (20 U; Fermentas) at 37°C for 45 min. AccessQuick™ RT-PCR System (Promega) was used to monitor the transcription of RubisCO form I and II genes using the same primers as described above. Reactions were carried out in 50 µL volumes according manufacturer's instructions. RT-PCR parameters were 30 min at 45°C and 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57 and 62°C (depending on the specific primer pair), and 1 min at 68°C, followed by one cycle of 5 min at 68°C. Amplification products were separated electrophoretically on 2% agarose gels in 1x TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA), stained with ethidium bromide, and visualized under UV light. For each RT-PCR, a negative control PCR without AMV reverse transcriptase was performed to rule out DNA contamination. Additionally, enzyme activity tests were performed, measuring RubisCO activity in cell extracts of aerobically grown cells as well as in extracts of anaerobically grown cells of strain D24TN^T. The enzyme assays were performed as described by Hügler *et al.* (2003).

2.3 Results and Discussion

2.3.1 Isolation of *Thiobacillus thiophilus* D24TN

Thiobacillus thiophilus D24TN^T was isolated out of an enrichment culture deriving from sediments of a tar oil contaminated aquifer under thiosulfate-oxidizing, obligate chemolithoautotrophic conditions.

2.3.2 Cell morphology

The thiosulfate-oxidizing strain is rod shaped with an average cell size of 1.8-2.5 µm in length and 0.5-0.8 µm in diameter (Fig. 2.1a and 2.1b). Aggregate formation could not be observed in any form. Cells were gram-negative upon staining.

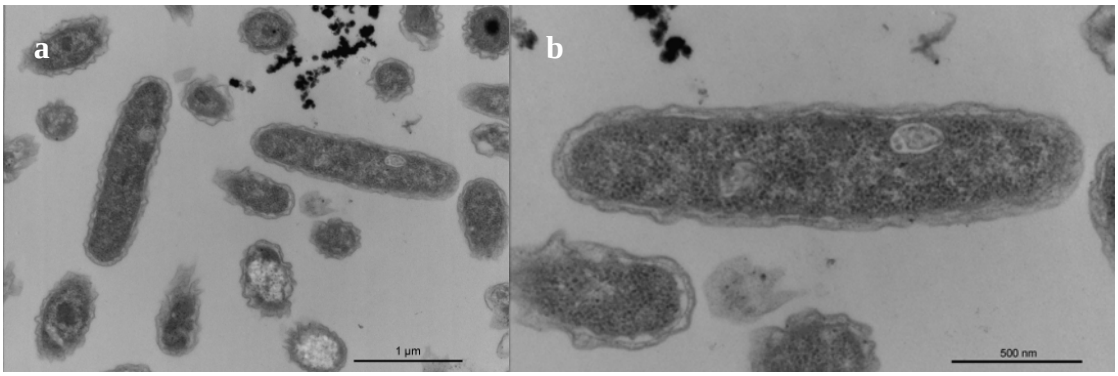

Figure 2.1: Transmission electron micrographs of strain *Thiobacillus thiophilus sp. nov*. D24TN^T. In (a) transverse ultrathin sections and longitudinal ultrathin sections of several single cells are shown. Bar represents 1 µm. In (b) the magnified transverse ultrathin section of a single cell is depicted. Bar represents 500 nm.

2.3.3 Physiological properties

Growth occurred under oxic and anoxic conditions, exhibiting faster growth in aerobic medium (μmax = 0.069 h^{-1}) than under anoxic conditions (μmax = 0.051 h^{-1}) (Fig. 2.2). Strain D24TN^T thus can be characterized as a facultative anaerobe.

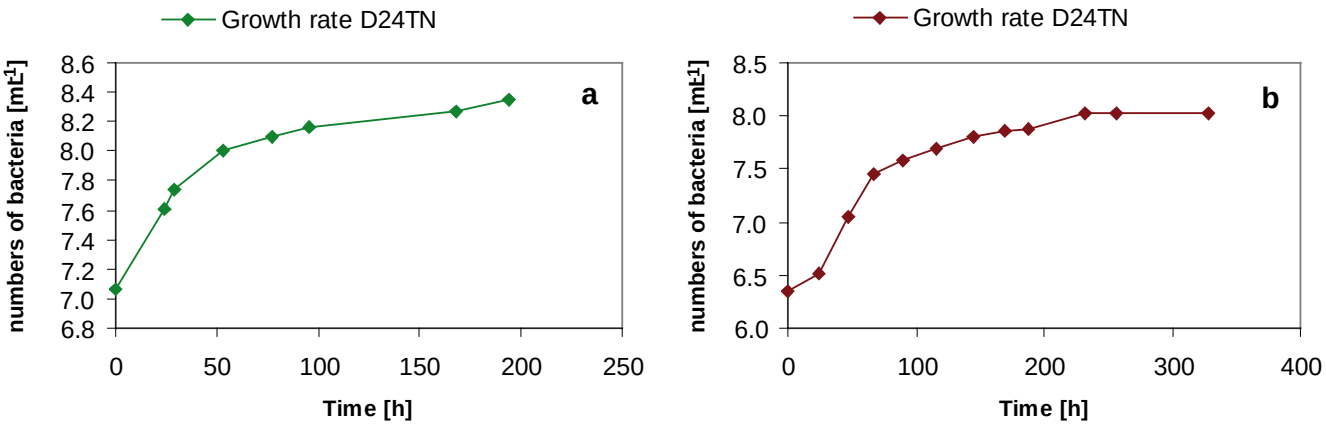

Figure 2.2: Growth rates of D24TN^T under (a) oxic and (b) anoxic conditions

It showed faster growth in the less diluted medium (μmax$_{[WS\ 1:10]}$ = 0.054 h^{-1}; μmax$_{[WS\ 1:2]}$ = 0.069 h^{-1}). The ability of D24TN^T to utilize different electron donors was tested applying H_2 (30 mL H_2 in headspace of serum bottle), NH_4^+ (5 mM), S^{2-} (5 mM), FeS (3 mM), $S_4O_6^{2-}$ (10 mM) and S^0 (0.5 g L^{-1}) using oxygen and/or nitrate (5 mM) as electron acceptor. Growth only occured with thiosulfate, along with the production of sulfate and small amounts of elemental sulfur, and with tetrathionate as electron donors.

The utilization of electron acceptors alternative to oxygen and nitrate was investigated testing SO_4^{2-} (5 mM) and Fe(III) (ferrihydrite 40 mM). Strain D24TN^T exhibited growth only in medium containing O_2 or NO_3^-. Aerobically grown cells completely converted thiosulfate to sulfate (electron balance 101-103%) with only small amounts of elemental sulfur precipitated, while in anaerobically grown cells only 60-75% of the thiosulfate was converted into sulfate (Fig. 2.3) with a visibly higher amount of S^0 precipitation formed. At the same time, part of the nitrate which disappeared in the aerobic incubations showed up as nitrite (32-41%), while in anaerobically grown bottles 69-72% of the nitrate consumed went into nitrite (Fig. 2.3), which may suggested an intermediate total nitrate reduction.

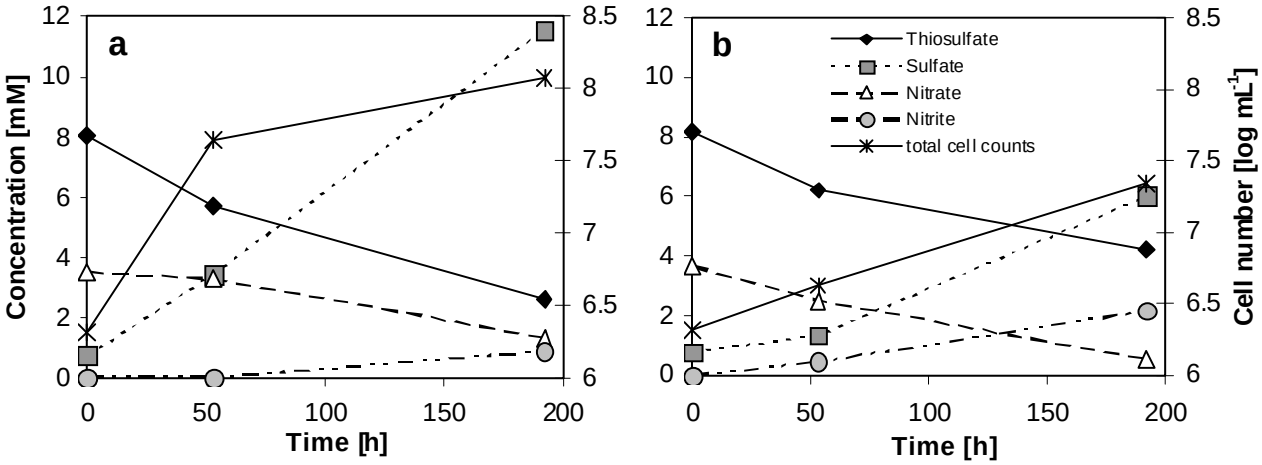

Figure 2.3: Growth curves of aerobically (a) and anaerobically (b) grown cells of strain D24TN^T showing consumption of the electron donor thiosulfate and the electron acceptor nitrate, as well as the reaction products sulfate and nitrite. Cells were grown in a 1:2 diluted WS medium (for expalantion see text).

Growth occurred at -2 to 30°C (over 1-2 weeks, slower growth at -2, 0 and 4°C) with an optimum between 25-30°C (Fig. 2.4). Optimum pH was 7.5 to 8.3, no growth occurred at pH values lower than pH 6.3 or higher than pH 8.7 (Fig. 2.5).

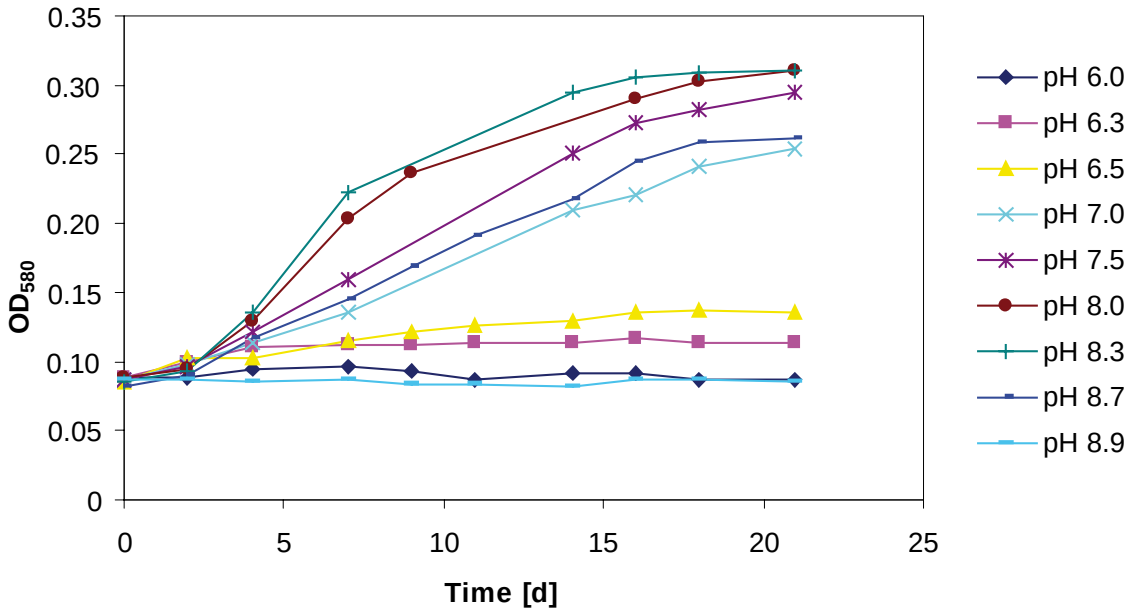

Figure 2.4: Growth of the thiosulfate oxidizing strain D24TN^T at various pH values. The electron acceptor was oxygen, and the electron donor was thiosulfate.

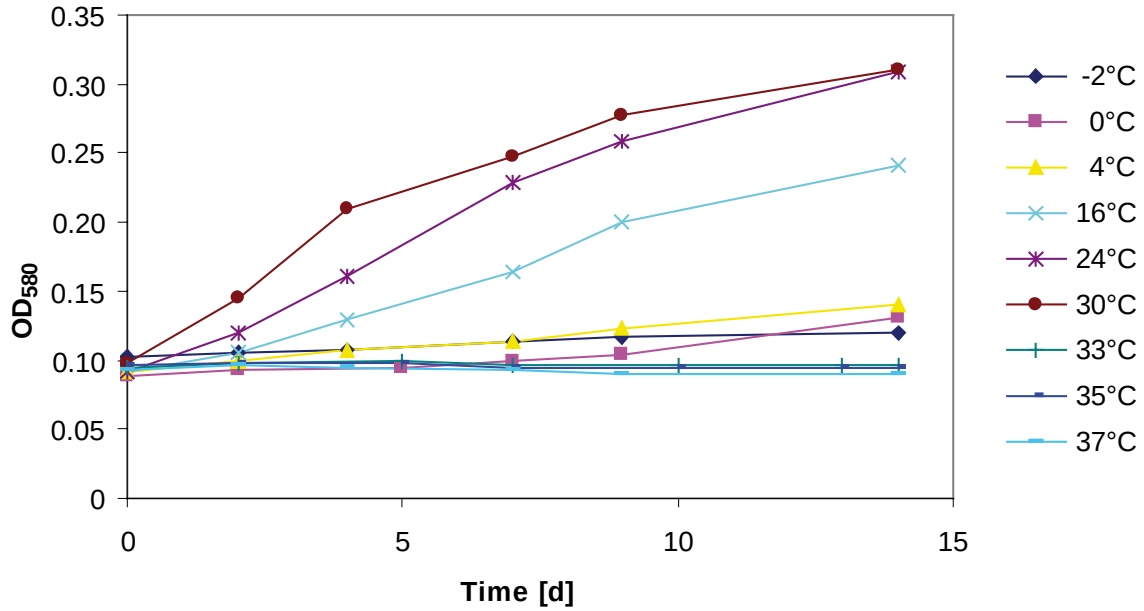

Figure 2.5: Growth of the thiosulfate-oxidizing strain D24TN^T at various temperatures. The electron acceptor was oxygen.

At salinities of 0.5%, 1% and 2% the isolate showed growth after two days incubation, no growth occurred at 3% NaCl or above. The strain tested positive for both, catalase and oxidase activity. Under the given conditions, strain D24TN^T did not form spores.

It did not grow on agar under oxic conditions, neither on its usual medium containing agar nor on nutrient agar plates (meat extract, peptone and agar) or on *Thiobacillus denitrificans* medium (M832; published by German Collection of Microorganisms and Cell Cultures GmbH). Growth on agar under anoxic conditions could only be observed on medium M832, not on agar plates of its usual medium or on nutrient agar plates.

Under chemoorganoheterotrophic conditions no growth could be observed; also tests for chemolithoheterotrophic growth under oxic conditions with thiosulfate (10 mM) as electron source were negative; organic substrates could not be used as electron or carbon source.

2.3.4 Phylogeny, G + C content and DNA-DNA hybridization

The new isolate is a rod shaped gram negative bacterium with a G + C content of 61.5%. It is an obligate chemolithoautotrophic microorganism, growing under oxic and anoxic conditions. Based on phylogenetic analysis of the 16S rRNA gene sequence, strain D24TN^T is closely affiliated with the genus *Thiobacillus* (Fig. 2.6) with the closest relatives being *Thiobacillus denitrificans* (97.6% sequence similarity) and *Thiobacillus thioparus* (97.5% sequence similarity) (Table 2.1). According to Wayne *et al.* (1987) strains with a greater DNA-DNA

relatedness than 70% belong to the same species. DNA-DNA hybridization analysis between the isolate and *Thiobacillus denitrificans* showed a relatedness value of 24.8 %, thus D24TN^T is clearly distinct from *Thiobacillus denitrificans*.

Table 2.1: Characters distinguishing *Thiobacillus thiophilus* D24TN^T from its closest relatives *Thiobacillus denitrificans* and *Thiobacillus thioparus*; (a) data from Robertson and Kuenen (2006); (b) data from Kelly and Wood (2000); (c) data from Beller *et al.* (2006); (d) data from Vlasceanu *et al.* (1997); nd = no data available

Characteristics	*Thiobacillus thiophilus* D24TN^T	*Thiobacillus thioparus*	*Thiobacillus denitrificans*
Morphology	Rods	Short rods	Rods
Cell length (μm)	1.8-2.5	1.0-2.0	1.0-3.0 [b]
Relation to O_2	Facultative anaerobe	Aerobe [b]	Facultative anaerobe [b]
Sporulation	-	-	nd
Temperature optimum (°C)	25-30	25-30 [a]	28-32 [b]
Temperature limits for growth (°C)	-2-30	nd	nd
pH optimum	7.5-8.3	6.0-8.0 [a]	6.8-7.4 [b]
pH limits for growth	6.3-8.7	5.0-9.0 [d]	nd
Catalase	+	nd	+
Oxidase	+	+	+
Growth on/ oxidation of			
Thiosulfate	+	+	+
Hydrogen	-	nd	nd
Sulfur	-	- [d]	+ [b]
Ammonium	-	nd	nd
Sulfide	-	+ [d]	+ [b]
FeS	-	nd	+ [c]
Tetrathionate	+	+ [b]	+ [b]
Thiocyanate	-	+ [b]	+ [b]
Chemoorganotrophic growth	-	- [b]	- [b]
DNA G + C content (mol %)	61.5	61.0-66.0 [a]	63.0-68.0 [a]
16S rRNA sequence similarity with *Thiobacillus thiophilus* D24TN^T (%)	(100)	97.5	97.6

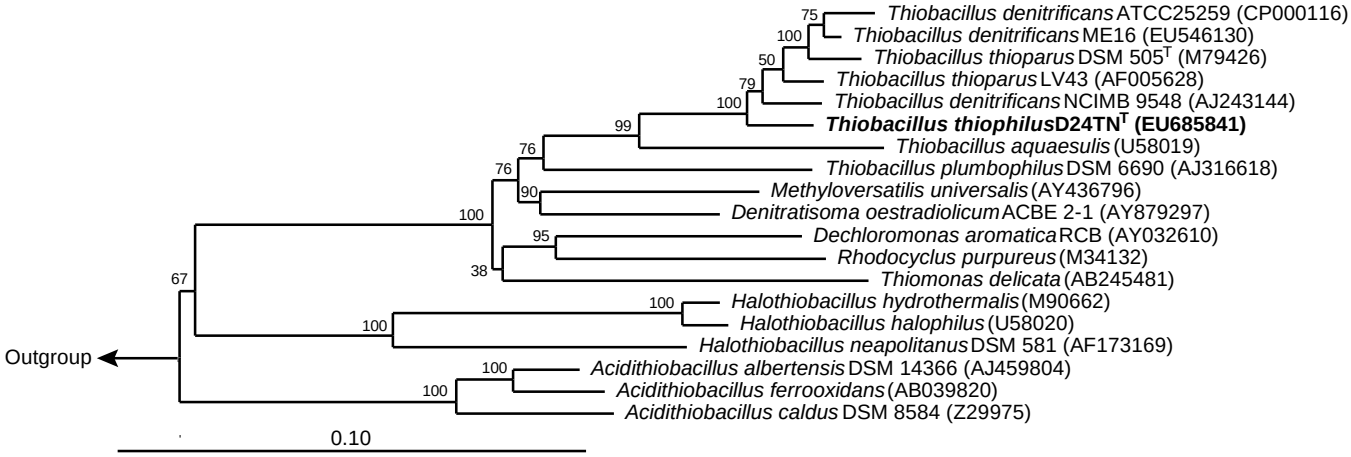

Figure 2.6: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain *Thiobacillus thiophilus* D24TN^T sp. nov. and some other related taxa. Bootstrap values (percentages of 1000 resamplings) are given at branching points. The bar indicates 10 % estimated sequence divergence.

2.3.5 CO_2 fixation potential and activity

The strain proved positive for *cbbL* type green-like (EU746410) and *cbbM* (EU746411) genes (Fig. 2.7 and Fig. 2.8). Sequence similarity to *cbbL*G sequences of *Thiobacillus thioparus* (DQ390449) and *Thiobacillus denitrificans* (L42940) was 91.9% and 88.1% respectively, sequence similarity to *cbbM* sequences of *Thiobacillus thioparus* (EU746412) and *Thiobacillus denitrificans* (NC007404; L37437) was 88.4% with each. It tested negative for *cbbL* type red-like. To investigate whether D24TN^T is actually using the Calvin cycle for CO_2 fixation, RT-PCR was used to detect transcription of the *cbb* genes. Both, aerobically as well as anaerobically grown cells showed to transcribe the *cbbM* and green-like *cbbL* gene.

Additionally, enzyme activity tests were performed, measuring RubisCO activity in cell extracts. RubisCO activity could be measured in aerobically (11.2 nmol min^{-1} (mg cell protein)$^{-1}$) as well as in anaerobically (2 nmol min^{-1} (mg cell protein)$^{-1}$) grown cells of D24TN^T.

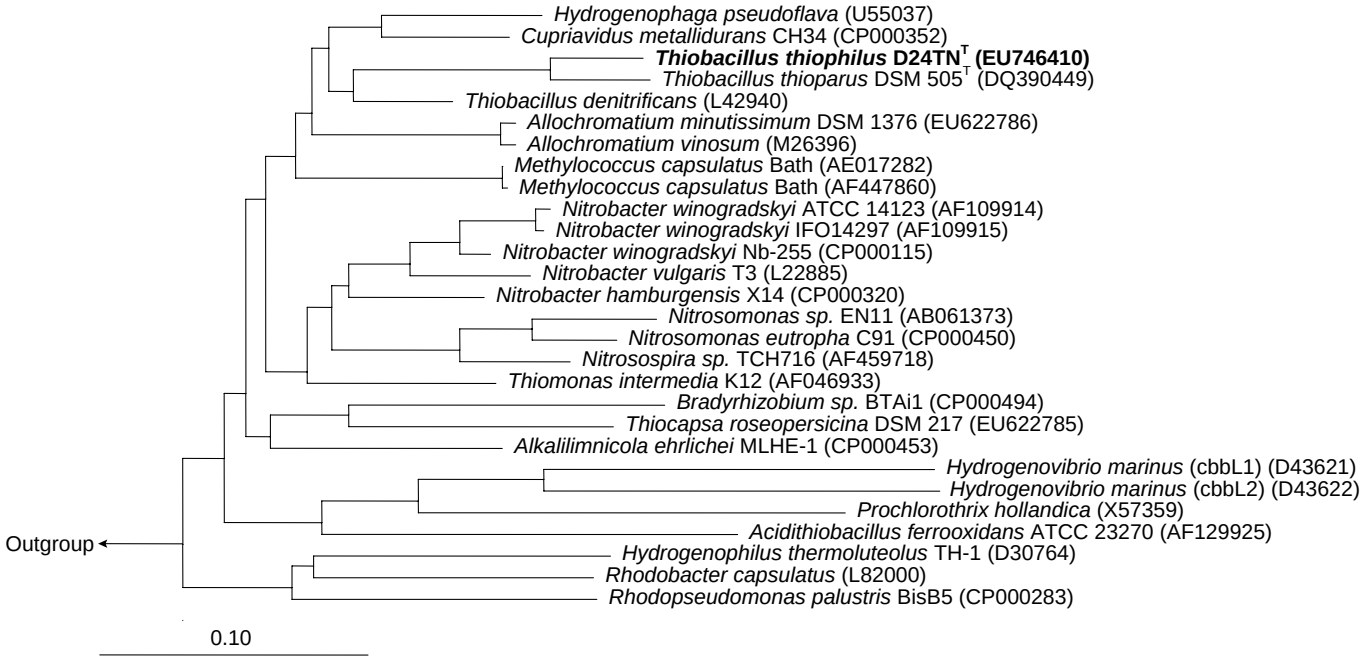

Figure 2.7: Phylogenetic tree of green-like *cbbL* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the red-like *cbbL* sequence of *Cupriavidus necator* (U20584) was used. The *cbbL* sequence of the isolate *Thiobacillus thiophilus* sp. nov. D24TN^T is shown in bold. Scale bar represents 0.10 changes per nucleotide position.

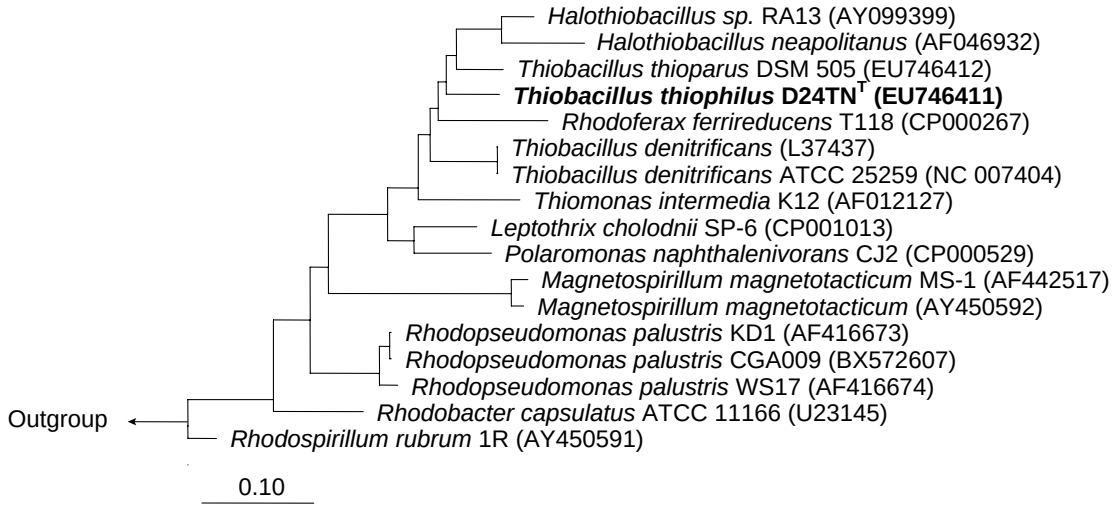

Figure 2.8: Phylogenetic tree of *cbbM* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the green-like *cbbL* sequence of *Nitrobacter vulgaris* T3 (22885) was used. The *cbbM* sequence of the isolate *Thiobacillus thiophilus* sp. nov. D24TN^T is shown in bold. Scale bar represents 0.10 changes per nucleotide position.

2.3.6 Description of *Thiobacillus thiophilus* sp. nov.

Thiobacillus thiophilus (thi.o'phi.lus. Gr. n. thion sulfur; Gr. adj. philos, loving; N.L. masc. adj. thiophilus sulfur-loving).

The type strain, D24TN^T (= DSM 19892^T = JCM 15047^T), was isolated from sediment deriving from a tar oil contaminated aquifer. D24TN^T is an obligate chemolithoautotrophic strain, oxidizing thiosulfate. Cells are gram-negative, aerobically and anaerobically growing small rods (1.8-2.5 μm in length and 0.5-0.8 μm in diameter). Oxidase and catalase positive. No aerobic growth on agar plates, anaerobic grown colonies are circular, smooth, shiny, convex and yellow in colour with a lighter-coloured fringe after 14 days incubation. No spore formation and no formation of aggregates. D24TN^T grows as a facultative anaerobic chemolithoautotroph on thiosulfate using nitrate as final electron acceptor. It furthermore grows as an aerobic chemolithoautotroph on thiosulfate. It shows no heterotrophic growth. Growth temperature lies between -2°C and 30°C, with an optimal temperature range between 24°C and 30°C. PH range is from 6.3 to 8.7, optimum lies between 7.5 and 8.3. Growth was observed under saline conditions to an upper NaCl concentration of 2% (w/v). The G+C content is 61.5 mol% as determined by HPLC. Based on 16S rRNA gene sequence analysis, D24TN^T belongs to the class β-Proteobacteria. It shows 97.6% 16S rRNA gene sequence similarity to its closest relative, *Thiobacillus denitrificans*, but the DNA–DNA hybridization value of 24.8% proves that the two species are clearly distinct from each other.

2.4 References

Alfreider, A., Vogt, C., Hoffmann, D., and Babel, W. (2003) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecol* **45**: 317-328.

Anneser, B., Einsiedl, F., Meckenstock, R.U., Richters, L., Wisotzky, F., and Griebler, C. (2008) High-resolution monitoring of biogeochemical gradients in a tar oil-contaminated aquifer. *Appl Geochem* **23**: 1715-1730.

Badger, M.R., and Bek, E.J. (2008) Multiple RubisCO forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J Exp Bot* **59**: 1525-1541.

Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M. *et al.* (2006) The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. *J Bacteriol* **188**: 1473-1488.

Cashion, P., Holder-Franklin, M.A., McCully, J., and Franklin, M. (1977) A rapid method for the base ratio determination of bacterial DNA *Anal Biochem* **81**: 461-466.

De Ley, J., Cattoir, H., and Reynaerts, A. (1970) The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**: 133-142.

Gabor, E., de Vries, E., and Janssen, D. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiol Ecol* **4**: 153-163.

Hügler, M., Huber, H., Stetter, K.O., and Fuchs, G. (2003) Autotrophic CO_2 fixation pathways in archaea (Crenarchaeota). *Arch Microbiol* **179**: 160-173.

Huß, V.A.R., Festl, H., and Schleifer, K.H. (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**: 184–192.

Kelly, D.P., and Wood, A.P. (2000) Confirmation of *Thiobacillus denitrificans* as a species of the genus *Thiobacillus*, in the beta-subclass of the *Proteobacteria*, with strain NCIMB 9548 as the type strain. *Int J Syst Evol Micr* **50**: 547-550.

Kinkle, B.K., and Kane, T.C. (2000) Chemolithoautotrophic micro-organisms and their potential role in subsurface environments. In *Ecosystems of the World*. Wilkens, H., Culven, D.C., and Humphreys, W.F. (eds). Amsterdam: Elsevier, pp. 309-318.

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005) Impact of different *in vitro* electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ Microbiol* **71**: 6664-6672.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Lueders, T., Manefield, M., and Friedrich, M.W. (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73-78.

Madsen, E., and Ghiorse, W.C. (1993) Groundwater microbiology: subsurface ecosystem processes. In *Aquatic Microbiology: an ecological approach*. Ford, T.E. (ed). Boston: Blackwell Scientific Publications, pp. 167-213.

Mesbah, M., Premachandran, U., and Whitman, W.B. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**: 159–167.

Olsen, G.J., Matsuda, H., Hagstrom, R., and Overbeek, R. (1994) fastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**: 41-48.

Robertson, L.A., and Kuenen, J.G. (2006) The Genus *Thiobacillus*. In *The Prokaryotes*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., and Stackebrandt, E. (eds). Berlin: Springer, pp. 812-827.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406-425.

Schmitt, M.E., Brown, T., and Trumpower, B. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisia*. *Nucleic Acids Res* **18**: 3091-3092.

Selesi, D., Schmid, M., and Hartmann, A. (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71**: 175-184.

Vlasceanu, L., Popa, R., and Kinkle, B.K. (1997) Characterization of *Thiobacillus thioparus* LV43 and its distribution in a chemoautotrophically based groundwater ecosystem. *Appl Environ Microbiol* **63**: 3123-3127.

Wayne, L., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I. *et al.* (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Sys Bacteriol* **37**: 463-464.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697-703.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New York: Springer, pp. 3352-3378.

3. Chemolithoautotrophy in an organically polluted aquifer – Potential for CO_2 fixation and *in situ* bacterial autotrophic activity

3.1 Introduction

All ecosystems are ultimately based on inputs of carbon and energy provided by autotrophic organisms which can be found in almost all environments (Kinkle and Kane, 2000). Thus, biological CO_2 fixation is one of the world's most important biochemical processes with the photosynthesis of green plants, algae and cyanobacteria (phototrophs) being responsible for the major part of global net CO_2 fixation. However, other microorganisms (chemolithoautotrophs) also play an important role in autotrophic CO_2 fixation. Autotrophic microorganisms are found within most bacterial groups and there are even numerous representatives within the *Archaea* (Hügler, 2003). To date, four CO_2 fixation pathways are established, *i.e.* the Calvin-Benson-Bassham cycle (Calvin cycle) (Bassham and Calvin, 1957), the reductive tricarboxylic acid cycle (reductive TCA cycle) (Evans *et al.*, 1966), the reductive acetyl-CoA pathway (Wood *et al.*, 1986) and the 3-hydroxypropionate cycle (Holo, 1989), with the Calvin cycle being the most intensively studied and probably the most abundant one. The only enzyme responsible for the actual CO_2 fixation in this cycle is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). Four different forms of RubisCO are known so far, all varying in structure, catalytic properties and O_2 sensitivity (Tabita, 1988; Tabita *et al.*, 2008). However, the existence of further CO_2 fixation pathways is very likely. Only recently evidence was obtained for a fifth and a sixth pathway in *Archaea* (Berg *et al.*, 2007; Huber *et al.*, 2008).

Since it was shown that the terrestrial subsurface is densely colonized by microorganisms (Ghiorse and Wilson, 1988), the energetic basis of its biomass and activity often has been questioned. Still, the prevailing opinion is that heterotrophic microorganisms dominate subsurface ecosystems. That for, many groundwater investigations concentrated on heterotrophs, especially in connection with natural attenuation and biodegradation (Kinkle and Kane, 2000). However, when looking at the amounts of organic carbon available to microorganisms, heterotrophic activity in pristine aquifers and especially in the deep subsurface is thought to be carbon limited, hinting at chemolithoautotrophic CO_2 fixation as an alternative strategy. Extensive research has been conducted on microbial communities in deep, mostly fractured basalt biospheres, both ground water and marine, in the quest for surface-independent ecosystems based on hydrogen driven lithoautotrophic microbial communities (Stevens and McKinley, 1995; Stevens, 1997; Anderson *et al.*, 1998; Pedersen,

2000; Chapelle *et al.*, 2002; Nealson *et al.*, 2005). However, the CO_2 fixation potential as well as actual activities hardly have been shown. The other extreme constitute organically polluted aquifers with a high load of complex mixtures of toxic and persistent organic compounds. Faced with the organic overload, bacteria degrading contaminants must get rid of electrons, while others not able to degrade the pollutants are in the same situation then the ones in pristine systems (Kinkle and Kane, 2000; Alfreider *et al.*, 2003). In summary, the existence and the role of chemolithoautotrophic CO_2 fixation in the terrestrial subsurface is hardly known. The subsurface complies with all requirements for autotrophic processes. Electron donors such as molecular hydrogen, reduced nitrogen (NO_2^- and NH_4^+), sulfur (*e.g.* $S_2O_3^{2-}$ and H_2S), metals (*e.g.* Fe^{2+} and Mn^{2+}) or carbon compounds (*e.g.* CO and CH_4) and electron acceptors such as oxygen, nitrate or sulfate are available in varying combinations always together with plenty of inorganic carbon (Shively *et al.*, 1998; Labrenz *et al.*, 2005). Thus, the oxidation of inorganic compounds may easily replace solar radiation as energy source. By now, not only the occurrence of numerous chemotrophic processes in the subsurface has been proven (*e.g.* nitrification, sulphide oxidation), but also the importance of chemolithoautotrophic bacteria in subsurface biogeochemical cycles has been indicated (Kinkle and Kane, 2000; Chapelle *et al.*, 2002; Alfreider *et al.*, 2003; Griebler and Lueders, 2008).

The aim of the study was to prove the CO_2 fixation potential within the microbial communities in an anoxic tar oil contaminated aquifer by means of functional gene analysis (*cbbL, cbbM* and *acl* genes) and to link this potential with *in situ* autotrophic activities as evaluated by different isotope and fatty acid approaches. Furthermore a new chemolithoautotrophic bacterial strain was isolated and characterized and subsequently looked for its role and distribution in the aquifer.

3.2 Material and Methods

3.2.1 Bacterial strains

Bacterial strains used as positive or negative controls are listed in Table 3.1. They were cultured as recommended by the German Collection of Microorganisms and Cell Cultures GmbH type culture collection (Braunschweig, Germany).

Table 3.1: Reference bacteria

Species	Strain[a]	*acl* [b]	*cbbM*[b]	*cbbL*[b]
Thiobacillus denitrificans	DSM 12475	-	+	+ (green-like)
Thiobacillus thioparus	DSM 505	-	+	+ (green-like)
Thiomicrospira denitrificans	DSM 1251	+	-	-
Cupriavidus necator	DSM 13513	-	-	+ (red-like)
Xanthobacter autotrophicus	DSM 432	-	-	+ (red-like)

[a]DSM, Deutsche Sammlung von Mikroorganismen

[b]+ PCR product of expected size; - no amplification

3.2.2 Site description, sediment and water sample collection

The investigated aquifer is situated at a former gasworks site in the river Rhine valley in Düsseldorf-Flingern, Germany. Large amounts of tar oil phase released into the subsurface during operation and break-down of the plant caused a severe organic contamination of the aquifer. Today's concentrations of monoaromatic hydrocarbons account for about 20-100 mg L^{-1} in the contaminant plume, while the concentration of individual polycyclic aromatic hydrocarbons, *i.e.* naphthalene, account for up to 10 mg L^{-1} (Eckert *et al.*, 2005; Anneser *et al.*, 2008). Sediments from the quaternary homogenous sandy aquifer were collected during a well drilling campaign in June 2005, and water samples were taken in July 2007 from a multi-level well located in the direct neighbourhood of the drilling spot. To protect the sediment samples from contact with oxygen, liners were removed from the borehole under argon atmosphere and further processed in a box continuously flushed with argon gas. Until further processing sediment samples for enrichment of bacteria were saturated with anoxic water from the same aquifer and stored in sterile glass bottles at 4°C in darkness. For extraction of genomic DNA, sediment samples were placed in sterile plastic tubes, shock frozen in dry ice and stored at -20°C till further processing. Water samples were filled in sterile 50 mL plastic tubes and stored at 4°C in darkness until further usage. The individual depths and sample codes of sediment and water samples can be retrieved from Figure 3.1.

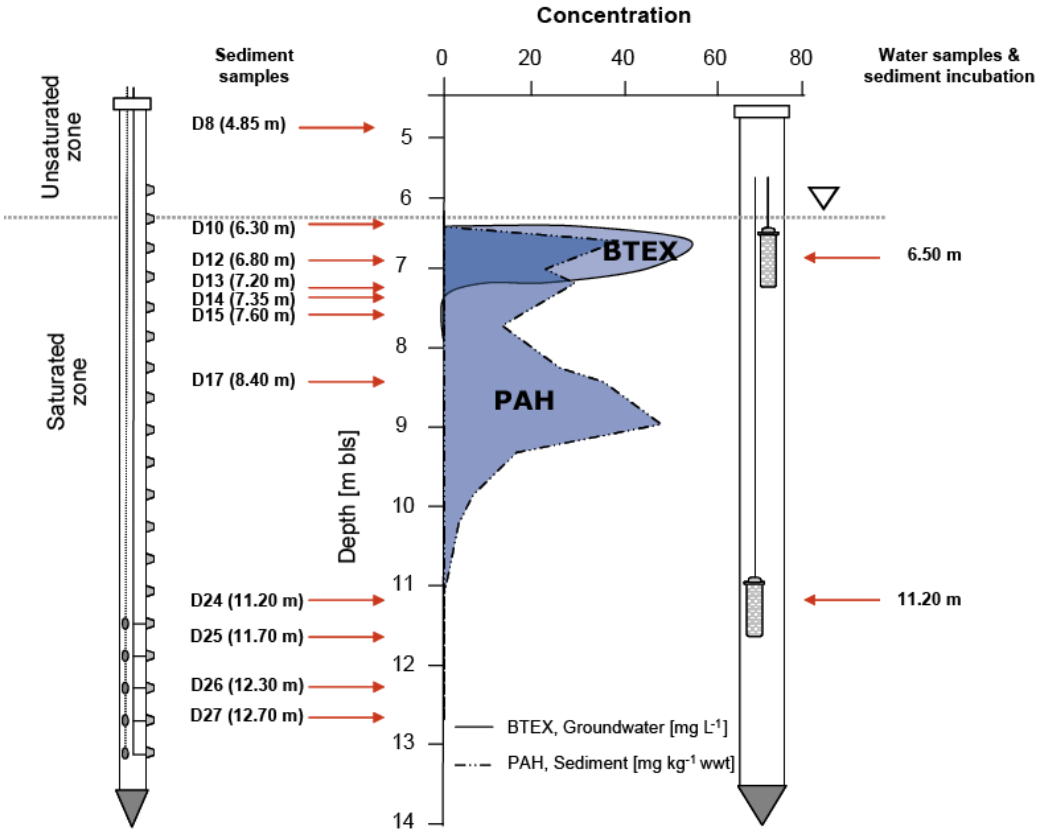

Figure 3.1: Schematic sketch of the spatial distribution of main groups of contaminants (*i.e.* BTEX and PAHs) within the tar oil contaminated aquifer. Arrows indicate the depths where sediment was subsampled for enrichment cultures as well as diverse molecular analysis. On the right hand the positions of sediment exposure for colonization and subsequent PLFA and FISH-MAR analysis are depicted.

3.2.3 Extraction of chromosomal DNA

Extraction of genomic DNA from sediment samples was performed using a modified protocol from Lueders *et al*. (2004) and Gabor *et al*. (2003). Genomic DNA from pure cultures was extracted and purified applying the FastDNA spin kit for soil (MP Biomedicals) according to the manufacturer's instructions. The integrity and yield of extracted nucleic acids was checked by standard agarose gel electrophoresis and ethidium bromide staining, as well as by UV quantification (NanoDrop ND-1000 Spectrophotometer).

3.2.4 Marker genes for CO_2 fixation - Amplification of *cbbL*, *cbbM* and *acl* genes

The primer sets for form I RubisCO *cbbL*G1-F/*cbbL*G1-G, *cbbL*R1-F/*cbbL*R1-R (Selesi *et al.*, 2005) and form II RubisCO *cbbM*-f/*cbbM*-r (Alfreider *et al.*, 2003) were used to amplify the RubisCO genes, applying following cycling parameters: an initial denaturation for 4 min at 94°C followed by 32 cycles of 1 min at 94°C, 1 min at 62°C (*cbbL*G) or 57°C (*cbbM*;

*cbbL*R) and 1 min at 70°C, concluding with a 10 min extension at 70°C. For amplification of the ATP citrate lyase (*acl*) genes we used different primer sets in a 32-cycle PCR at an annealing temperature of 54°C. Here, primers applied were F2/R5 (Hügler *et al.*, 2005) for the *acl* α-subunit (*aclA*) and 892F/1204R (Campbell *et al.*, 2003) and 275F/1204R (Campbell *et al.*, 2003; Takai *et al.*, 2005) for the *acl* β-subunit (*aclB*) with PCR conditions according to Campbell *et al.* (2003). For a survey of all used primers see Table 3.2. All amplification products were analyzed on 2% agarose gels (Biozym) by horizontal gel electrophoresis to ensure correct size (*cbbLG* 1.1 kb, *cbbLR* 0.8 kb, *cbbM* 0.5 kb, *aclA* 1.0 kb, *acl*B 0.4 kb and 1.0 kb) and visualized by UV excitation after staining with ethidium bromide.

Table 3.2: Primers used for amplification of *cbbM*, *cbbL*, *aclA* and *aclB* genes

Primer	Primer sequence (5'-3')	Reference
cbbM-f	GGC AAC ATC ATC AAG CCC AAG	(Alfreider *et al.*, 2003)
cbbM-r	TCT TGC CGT AGC CCA TGG TGC	(Alfreider *et al.*, 2003)
cbbLR1F	AAG GAY GAC GAG AAC ATC	(Selesi *et al.*, 2005)
cbbLR1R	TCG GTC GGS GTG TAG TTG AA	(Selesi *et al.*, 2005)
cbbLG1F	GGC AAC GTG TTC GGS TTC AA	(Selesi *et al.*, 2005)
cbbLG1R	TTG ATC TCT TTC CAC GTT TCC	(Selesi *et al.*, 2005)
aclA F2	TGC ATA GCA ATH GGN GGN GA	(Hügler *et al.*, 2003)
aclA R5	CCG ATA GAN CCR TCN ACR TT	(Hügler *et al.*, 2003)
aclB-F (892)	TGG ACM ATG GTD GCY GGK GGT	(Campbell *et al.*, 2003)
aclB-F (275)	TAG AGG ATG CRG CTA AWT GGA TTG ATG A	(Takai *et al.*, 2005)
aclB-R (1204)	ATA GTT KGG SCC ACC TCT TC	(Campbell *et al.*, 2003)

3.2.5 Clone libraries - Cloning and screening of environmental RubisCO coding genes

Amplicons of the expected sizes (1100 bp for green-like *cbbL*, 820 bp for red-like *cbbL* and 505 bp for *cbbM* genes) from sediment samples were purified with the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were ligated into the vector pGEM®-T (Promega) and transformed into competent *Escherichia coli* cells of strain JM 109 (Promega). Colonies were picked and the plasmid DNA was purified heating the clones for 10 min at 99°C in H_2O_{dest}.

To get a first overview over the diversity of the constructed clone libraries, restriction fragment length polymorphism (RFLP) was used for screening of the clones with correctly sized inserts. PCR products of clones harbouring *cbbM* or green-like *cbbL* inserts were

hydrolyzed with 2 U of the restriction endonucleases *Rsa*I and *Msp*I (Fermentas), and for PCR products of clones with red-like *cbbL* inserts, the restriction enzyme *Bsa*I (Fermentas) was used. Restriction fragments were analyzed electrophoretically in 2% (w/v) agarose gels.

3.2.6 Sequencing and phylogenetic analysis

Plasmids containing *cbbL* or *cbbM* inserts from sediment DNA were used directly for sequencing. Sequencing was performed as previously described by Selesi *et al.* (2005). The newly gained *cbbL* and *cbbM* nucleotide sequences were imported into the *cbbL* database implemented in the ARB software package (Ludwig *et al.*, 2004). Closest relatives to *cbbL* and *cbbM* nucleotide sequences were obtained using NCBI's sequence similarity search tool BLASTN.

3.2.7 ^{13}C-$CaCO_3$ field experiment – Phospholipid fatty acid analysis

Small cylindric cages consisting of stainless steel grids (pore size 0.2 mm) were filled with sterilized sand (grain size 0.20 - 2.24 mm). One gram of ^{13}C labelled $CaCO_3$ (99 atom.%; Isotec^{TM}) or 1 g non-labelled $CaCO_3$ (Sigma) (as control), respectively, were filled in a dialysis tubing embedded in the middle of the cage (flat width 10 mm, wall thickness 28 µm, MWCO 8,000 – 10,000). The cages were first pre-soaked in anoxic H_2O_{dest} water and then placed, as two pairs containing one labelled and a control, into a fully screened groundwater monitoring well at a depth of 6.5 m (= 0.2 m below groundwater level) and 11.2 m below soil horizon. Incubation lasted for two months. Afterwards the sediment was withdrawn and stored in sterile plastic tubes at -20 °C until further processing. The determination of the saturated (SATFA), monounsaturated (MUFA) and polyunsaturated (PUFA) phospholipid fatty acids was performed as described by Zelles *et al.* (1995). PLFA of labelled and non-labelled samples were extracted in three replicates. An aliquot of the phospholipid fraction, equivalent to 10 g dry matter was used for fatty acid analysis. The fatty acids were measured as fatty acid methyl esters (FAME) using a gas chromatograph (equipped with a BPX-70 column; SGE GmbH, 60 m x 0.25 mm x 0.25 µm, coated with 70 % of cyanopropyl polysilphenylene-siloxane) linked with a mass spectrometer (GC/MS, Agilent Technologies). The isotopic composition of the FAME was detected in a DeltaPlusAdvantage Isotope Ratio Mass Spectrometer (IRMS, Thermo Electron Cooperation) after combustion (GC Combustion III, Thermo Electron Cooperation). Identification of fatty acids was carried out by comparing the obtained mass spectra with established fatty acid libraries (Solvit) using MSD Chemstation (Version D.02.00.237). Standard nomenclature was used for PLFA (Frostegard

et al., 1993). ^{13}C results are expressed in $\delta^{13}C$ compared to the international carbon standard Vienna – Pee Dee Belemnite (V-PDB) as described in Werner and Brand (2001). n19:0 was used as an internal standard to calculate fatty acid contents as well as to correct the $\delta^{13}C$-values of the individual fatty acids.

3.2.8 Bacterial cell counts

Total counts of bacteria in water and sediment samples were conducted via epifluorescence microscopy and flow cytometry, respectively. Samples were fixed in paraformaldehyde (4%) for at least 2 h at 4°C. Cell counts of water samples were performed as described by Anneser *et al.* (2008). Attached bacteria were released from sediment in a swing mill (Retsch, MM 200) shaking the samples for 3 min at a frequency of 20 $L*s^{-1}$. 1.5 mL of the supernatant were subsequently pipetted on top of a cold 5 mL Nykodenz™ (Nykomed) solution (1.3 g mL^{-1} final density, pH 8) and centrifuged for density gradient separation (Centrikon T-2190) for 60 min at 4°C and 11.000 rpm to separate bacterial cells from debris and remaining sediment particles. The second and the third millilitre from the top were collected for subsequent flow cytometric analysis. Samples were stained with 10 µL of SYBR green I (30 $\mu L*mL^{-1}$) for 10 min at 4°C in the dark. Fluorescently labelled beads (TruCount beads, BD Biosciences) were used as internal standard. Cell numbers were determined in triplicates in a LSR II flow cytometer (Becton Dickinson) and corrected for cell release efficiency and loss during density gradient centrifugation.

3.2.9 FISH-MAR

Incubation with radiolabelled bicarbonate

^{14}C-labelled sodium bicarbonate (specific activity 1.813 GBq/mmol = 49 mCi/mmol, PerkinElmer) was used as substrate. Water and sediment samples from two different depths, *i.e.* 6.5 m and 11.2 m below soil horizon, were analysed. Sediment samples derived from the ^{13}C-PLFA experiment, while water samples were freshly taken from the same well after the exposed sediments have been removed. 1.4-1.6 g sediment (wet weight) were transferred to 2 mL tubes filled with 500 µL aquifer water from the same depth. Water samples (14 mL) were transferred into sterile 15 mL plastic tubes. 40 mCi [^{14}C] sodium bicarbonate solution was added and samples carefully mixed (final [^{14}C] sodium bicarbonate concentration: $7.4*10^8$ Bq/µL in sediment and $1.04*10^8$ Bq/µL in water samples). The samples were incubated for 14 days at 16°C (*in situ* temperature) in the dark. All experiments were performed in duplicates.

To account for possible adsorption phenomena, formaldehyde fixed sediment and water samples amended with radioactive substrate served as controls.

After the incubation, samples were fixed with 4% paraformaldehyde over night at 4°C. Since sediment particles are highly autofluorescent and therefore interfere with FISH analysis, it was necessary to dislodge the bacterial cells from sediment particles. Therefore, the samples were vortexed 45 seconds at full speed. After settlement of particles, the supernatant was collected. Subsequently, water and sediment samples were washed two times with phosphate buffered saline (PBS) (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) in order to remove the excess soluble radioactive substrate and fixative. All washing steps consisted of: centrifugation of the samples at 14.000 rpm for 3 min, removal of the supernatant, addition of further washing buffer and resuspension of the pellet. Finally, pellets were resuspended in a 1x PBS/Ethanol$_{absolute}$-solution and stored at -20°C.

Oligonucleotide probes and probe design

All rRNA-targeting oligonucleotide probes used for FISH in this study are listed in Table 3.4. All probes were purchased from MWG Biotech AG.

To design probes specific for isolate *Thiobacillus thiophilus* D24TN^T (= DSM 19892^T = JCM 15047^T) and its closest relatives, all 16S sequences of the next relatives which were available from the National Center for Biotechnology Information sequence database (NCBI) were used to establish a 16S database of D24TN relatives by the use of the ARB software package (Ludwig *et al.*, 2004). For probe design the function "probe match" implemented in the ARB software package was used. Two probes, *i.e.* D24TN_443, a species specific probe for the isolate *Thiobacillus sp.* D24TN and D24TN_825, a probe specific for the genus *Thiobacillus* (Table 3.3) were designed.

Fluorescent *in situ* hybridization and microautoradiographic procedure

To prevent loss of sample material, all slides and coverslips were precoated with Poly-L-Lysine (Sigma Aldrich) according to the manufacturer's instructions. *In situ* hybridization was performed as described previously (Manz *et al.*, 1992). After *in situ* hybridization an microautoradiographic procedure with autoradiographic emulsion LM-1 (Amersham International) was performed as described by Andreasen and Nielsen (1997). Exposure time for the samples was 14 days. A model LSM 510 scanning confocal microscope (Zeiss) was used to record optical sections. The formation of silver grains in the autoradiographic film covering a sample was observed in the transmission mode.

Table 3.3: FISH probes applied targeting rRNA

Probe	Target organisms target group	Binding position[1]	Target sequence 5'-3'	% FA[2]	Reference
EUB338 I	*Bacteria* without *Planctomyce-tales,* without V*errucomicrobiales*	16S 338 - 355	GCT GCC TCC CGT AGG AGT	Var.	(Amann *et al.*, 1990)
HGC69a	Bacteria with high G + C content	23S 1901-1918	TAT AGT TAC CAC CGC CGT	20	(Roller *et al.*, 1994)
Alf968	α- Proteobacteria, without *Rickettsiales*	16S 19 - 35	GGT AAG GTT CTG CGC GTT	20	(Neef *et al.*, 1998)
Alf1B	α-Proteobacteria	16S 19 - 35	CCT TCG YTC TGA GCC AG	20	(Manz *et al.*, 1992)
Bet42a	β- Proteobacteria	23S 1027-1043	GCC TTC CCA CTT CGT TT	35	(Manz *et al.*, 1992)
Gam42a	γ- Proteobacteria	23S 1027-1043	GCC TTC CCA CAT CGT TT	35	(Manz *et al.*, 1992)
D24TN_443	*Thiobacillus thiophilus* D24TN^T and species closely related to *Thiobacillus*	16S 443 - 458	GCG TAC CGT TTC GTT CCG	Var.	this study
D24TN_825	*Thiobacillus sp.* and species closely related to *Thiobacillus*	16S 825 - 843	CAC TCC CCC AAC AAC CAG	Var.	this study

[1] Position in rRNA of *E. coli*

[2] % FA: used formamide concentration (%, v/v) in hybridization buffer

3.2.10 Enrichment cultures - Isolation and cultivation of new bacterial strains

For isolation of chemolithoautotrophic aquifer bacteria enrichment cultures were started under various autotrophic conditions. Growth medium was a tenfold diluted anoxic Widdel freshwater medium (Widdel and Bak, 1992) supplemented with different combinations of electron donors (S^0, $S_2O_3^{2-}$, NH_4^+, S^{2-}, Fe^{2+}, H_2) and electron acceptors (NO_3^-, O_2, S^0, CO_2, SO_4^{2-}) (table 3.4).

The redox indicator Resazurin (1 mg L^{-1}; Sigma-Aldrich) was added to guarantee anoxic conditions throughout the incubations. Initially, the medium (50 mL in 120 mL serum bottles) was inoculated with 10 g of fresh sediment. The headspace was replaced by N_2/CO_2 (80:20 v/v) and bottles were sealed with butyl stoppers (Ochs). Electron acceptors and electron donors deriving from sterile aqueous stock solutions were amended via syringes injected through the butyl stoppers. The first series of enrichment cultures were incubated at 16°C (*in situ* temperature) in the dark for 3-4 months and before transferred to fresh medium when visibly turbid. For purification of growing cells two dilution series were performed under the same culturing conditions. After purification, the cultures were transferred every 2-3 weeks and cell growth was monitored by measuring optical density at 580 nm (OD_{580}; Varian) and microscopic observations (Zeiss).

Table 3.4: Experimental conditions for enrichment cultures. Each given electron donor/acceptor combination was applied on sediment from 6.30 m, 7.60 m and 11.20 m below soil horizon

Microbial process	Electron donor	Electron acceptor	Carbon source	Temperature
Sulfur oxidation	S^0	O_2	CO_2	16°C
Thiosulfate oxidation	$S_2O_3^{2-}$	O_2	CO_2	16°C
Nitrification	NH_4^+	O_2	CO_2	16°C
Anaerobic sulfide oxidation	S^{2-}	NO_3^-	CO_2	16°C
Anaerobic iron oxidation	Fe^{2+}	NO_3^-	CO_2	16°C
Denitrification	H_2	NO_3^-	CO_2	16°C
Sulfur reduction	H_2	S^0	CO_2	16°C
Sulfate reduction	H_2	SO_4^{2-}	CO_2	16°C
Methanogenesis	H_2	CO_2	CO_2	16°C
Acetogenesis	H_2	CO_2	CO_2	16°C
Anaerobic thiosulfate oxidation	$S_2O_3^{2-}$	NO_3^-	CO_2	16°C

3.2.11 Isolate D24TN^T - Marker genes for CO_2 fixation and RNA isolation / RT-PCR

For amplification of the RubisCO and the ATP citrate lyase genes the same primer sets and the same cycling conditions were used as described above. PCR products with the correct size amplified from D24TN^T DNA extracts were used for direct sequencing. The gained *cbbL* and *cbbM* nucleotide sequences were brought in to the *cbbL* database implemented in the ARB software package (Ludwig *et al.*, 2004).

For total RNA isolation 50 mL densely aerobically and anaerobically grown cultures were centrifuged (4,000 rpm; 15 min). Preparation was performed as described by Schmitt *et al.* (1990). To obtain pure RNA without DNA, 25 µL DNA/RNA extract were digested with *DNase*I (20 U; Fermentas). AccessQuick™ RT-PCR System (Promega) was used to monitor the transcription of RubisCO form I and II genes using the primers as described in the method section "marker genes for CO_2 fixation". Reactions were carried out according to the manufacturer's instructions. RT-PCR parameters were 30 min at 45°C and 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57 and 62°C (depending on the specific primer pair), and 1 min at 68 °C, followed by 1 cycle of 5 min at 68 °C. Amplification products were separated by electrophoresis on 2% agarose gels in 1x TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA), stained with ethidium bromide, and visualized under UV light. For each RT-PCR, a negative control PCR without AMV reverse transcriptase was performed to rule out DNA contamination.

3.2.12 Isolate D24TN^T - Enzyme assays

Photometric enzyme assays were carried out in 0.5 mL glass cuvettes, other assays in 1 mL glass vials. Anaerobic enzyme assays were performed with N_2 gas as headspace in rubber-stoppered glass-cuvettes or stoppered vials. All assays and extraction buffers were evacuated and gassed with nitrogen gas to remove oxygen. Compounds of the assay were added with gas-tight syringes. All assays were performed at 25°C, which lies in the range of the optimal growth temperature of D24TN^T. All assay mixtures had a total volume of 500 µL.

Cell extracts were prepared using a mixer-mill (type MM2, Retsch). Per 0.2 g of wet cells 800 µL buffer (100 mM Tris/HCl, pH 7.8, 3 mM 1,4-dithioerythritol and 1 mg DNaseI per 10 mL of buffer) were added.

After addition of 0.8 g beads (diameter 0.1-0.25 mm) the solution was treated for 8 min at 30 Hz in the mixer-mill, followed by a centrifugation step (10 min, 14.000 rpm, 4°C). The supernatant was used for enzyme tests. For the anoxic preparation of cell extracts anoxic buffer and stoppered glass-vials were used. Glass beads were added in an anaerobic chamber. Protein concentration in cell extracts was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Photometric Assays

Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm (ε_{365nm} (NADH = 3.4 x 10^3 M^{-1} cm^{-1}).

Isocitrate dehydrogenase

The isocitrate-dependent oxidation of $NADP^+$ was monitored. The assay mixture contained 100 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM $NADP^+$, 5 mM β-mercaptoethanol and 10 mM D/L-isocitrate. The addition of isocitrate started the reaction.

Malate dehydrogenase

The oxaloacetate-dependent oxidation of NADH was monitored. The assay mixture contained 100 mM Tris/HCl, pH 7.8, 0.3 mM NADH and 1 mM oxaloacetate. The addition of oxaloacetate started the reaction.

ATP citrate lyase

ATP citrate lyase activity was determined by coupling the reaction to endogenous L-malate dehydrogenase activity, which oxidizes NADH. The citrate-, CoA- and MgATP-dependent

oxidation of NADH was monitored. The assay mixture contained 100 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 3 mM ATP, 0.5 mM CoA, 0.4 mM NADH and 3 mM D-citrate. The reaction was started by the addition of citrate. To ensure that L-malate dehydrogenase activity in this coupled assay was not limiting, this enzyme activity was measured under the same conditions by adding 1 mM oxaloacetate.

Radioactive assay

Carboxylation reactions with $^{14}CO_2$ were followed by measuring the substrate-dependent incorporation of ^{14}C from ^{14}C-bicarbonate into acid-stable products. The samples were incubated for 1, 2 and 5 min. The addition of 20 µL of 6 M HCl stopped the reaction. Shaking the samples in open scintillation vials for 5 h removed volatile $^{14}CO_2$. The amount of ^{14}C in liquid samples was determined by scintillation counting (5 min per sample) using 5 mL scintillation cocktail. As blank and control served two experiments in which substrate and extract, respectively, were omitted. The molar amount of product formed was calculated from the amount of radioactivity fixed into acid-stable labelled products taking into account the final specific radioactivity of added ^{14}C-bicarbonate.

Ribulose 1,5-bisphosphate carboxylase/ oxygenase

The assay was performed under aerobic and strictly anaerobic (with N_2 gas in the headspace) conditions. The assay mixture contained 100 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithioerythritol, 10 mM $NaHCO_3$, 2 kBq [^{14}C] Na_2CO_3 (Sigma) and 1 mM ribulose 1,5-bisphosphate. The reaction was started by addition of ribulose 1,5-bisphosphate.

3.3 Results

3.3.1 Marker genes for CO_2 Fixation

Amplification of cbbL, cbbM and acl genes from aquifer sediment samples

DNA was extracted from sediments freshly collected from various depths (Fig. 3.1) in a tar oil-contaminated aquifer. To evaluate the potential for bacterial CO_2 fixation, the presence of marker genes from the Calvin cycle, *i.e.* the RubisCO genes (*cbbL* and *cbbM*), and the reductive TCA cycle, *i.e.* ATP citrate lyase genes (*acl*), has been investigated using PCR amplification of the *cbbL*, *cbbM* and *acl* genes. Form I RubisCO green-like *cbbL* genes were detected in 9 out of 11 depths examined (no presence detected in sampling sites D8 and D17). Red-like *cbbL* genes were only found in DNA extracts from the BTEX-contaminated area, (sampling sites D10 and D12). Form II RubisCO *cbbM* genes were not detectable in D8, D17 and D26, but present in all other samples. The ATP citrate lyase *(acl*) genes could not be detected in any of the investigated sediment samples.

Green-like cbbL clone libraries and sequences

Clone libraries were constructed of right-sized (about 1034 bp) PCR products amplified from sediment samples from selected depths (D10, D12, D15, D24 and D27). To gain insight into the diversity of the 134 retrieved clones, RFLP analysis using the restriction enzymes *Rsa*I and *Msp*I was performed. The sequences revealed 15 different RFLP patterns in total with only one pattern occurring in all green-like *cbbL* clone libraries. D10 showed highest RFLP pattern diversity with 6 different patterns. Lowest was found in D12 with only one pattern. Of each RFLP pattern at least one clone was sequenced, 2-3 clones of *cbbL* sequences with RFLP patterns of high abundance. Concerning the green-like *cbbL* clone library D24 all 13 clones were sequenced, independent of their RFLP pattern. A total of 53 green-like *cbbL* clones from the different samples were sequenced. Clones showing the same RFLP pattern proved to be identical concerning their *cbbL* sequences. The sequences were named with a "D" for Düsseldorf, followed by the depth designation, with an added "gl" for sequences of the green-like *cbbL* library and the clone number. The sequence similarities of all sequenced clones ranged between 79.5% and 100%. The phylogenetic tree for green-like *cbbL* nucleotide sequences (Fig. 3.2) shows that most of the sediment clone sequences build a large cluster (cluster I), with the highest degree of relatedness to the sequence of an uncultivated aquifer clone (AY099392; 89.5-99.0%).

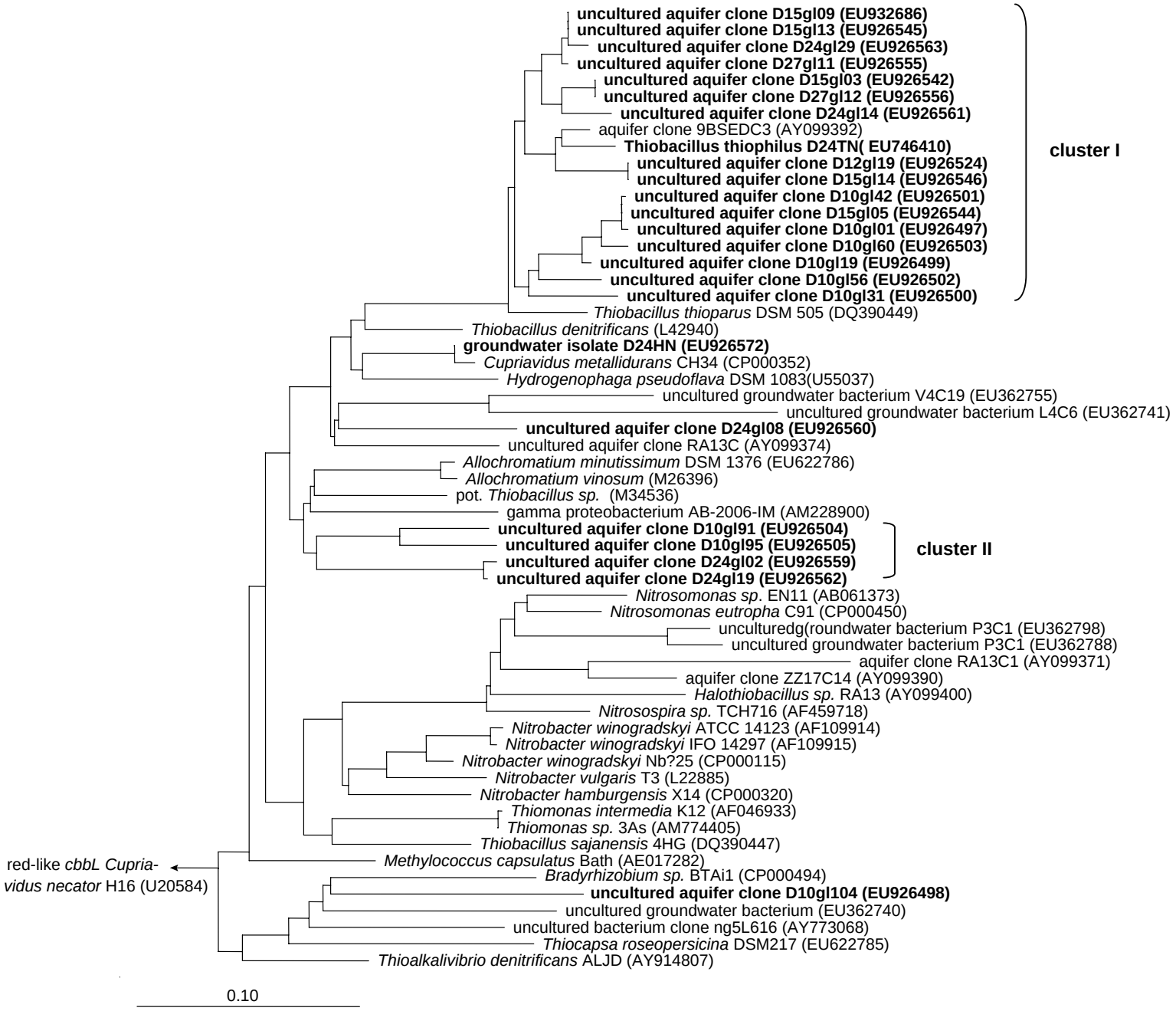

Fig. 3.2: Phylogenetic tree of green-like *cbbL* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the red-like *cbbL* sequence of *Cupriavidus necator* H16 (U20584) was used. All environmental clone sequences are shown in bold; the *cbbL* sequences of the isolates D24HN and *Thiobacillus thiophilus* D24TN^T are shown in bold and are shaded in grey. Scale bar represents 0.10 changes per nucleotide position.

A second, smaller cluster (cluster II) comprises only four sequences, which are most closely related to the sequence of *Thiobacillus sp.* (M34536, 85.3-86.3%). Only two sequences appear somewhere else in the tree, one having a *cbbL* sequence closely related to a sequence of an uncultured groundwater bacterium (AY09937; 87.0%), the other one closely related to the *cbbL* sequence of *Thioalkalivibrio denitrificans* ALJD (AY914807; 80.4%).

Red-like cbbL clone libraries and sequences

PCR products about 820 bp long indicated the presence of red-like *cbbL* genes in DNA extracts of sediment samples of D10 and D12. The two red-like *cbbL* clone libraries (D10rl and D12rl) contained a total of 163 clones having right sized insert. For RFLP analysis the enzyme *Bsa*I (Fermentas) was used, resulting in 19 different RFLP patterns. Only four of them could be found in both depths. Of each RFLP pattern at least one clone was sequenced, 2-3 clones of *cbbL* sequences with RFLP patterns of high abundance, in total 52 clones of the two clone libraries. The sequence similarities ranged from being identical to 74.5% sequence similarity. The sequences formed two clusters; seven sequences appeared somewhere else in the phylogenetic tree (Fig. 3.3). Sequences of cluster I were most closely related to the red-like *cbbL* sequence of the nitrogen fixing α-Proteobacterium *Rhizobium radiobacter* (AY572468; 82.1-88.4%). Sequences of cluster II exhibited the closest relation to the red-like *cbbL* sequence of an uncultivated forest soil proteobacterium (AY422906; 80.3-86.2%). Depth-specific clustering could not be observed (Fig. 3.3).

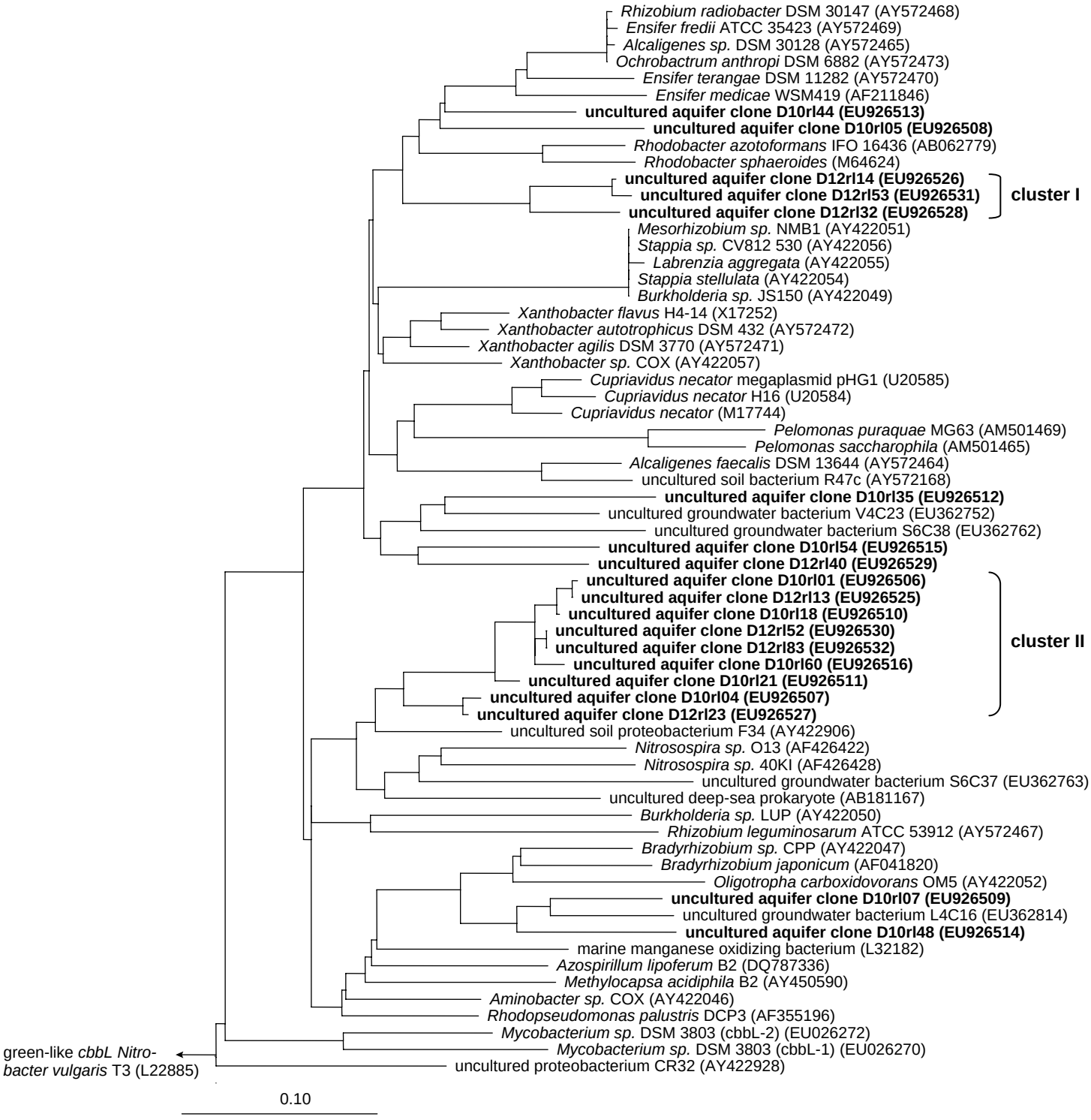

Fig. 3.3: Phylogenetic tree of red-like *cbbL* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the green-like *cbbL* sequence of *Nitrobacter vulgaris* T3 (L22885) was used. All environmental clone sequences are shown in bold. Scale bar represents 0.10 changes per nucleotide position.

CbbM clone libraries and sequences

PCR products of correct size (about 505 bp) obtained using the *cbbM* specific primers were selected to build clone libraries from D10, D12, D15, D24 and D27 samples (D10M, D12M, D15M, D24M and D27M). For RFLP analysis 244 clones with the right-sized inserts were digested with the restriction enzymes *Rsa*I and *Msp*I. 32 different RFLP patterns were found in all libraries, only two patterns could be found in every clone library. The highest RFLP pattern diversity could be found in D15 (15 patterns), the lowest in D27 (8 patterns). Of each RFLP pattern at least one clone was sequenced, but 2-3 clones of RFLP patterns with high abundance. As in the green- and red-like *cbbL* clones libraries, sequence analysis proved that sequences showing the same RFLP pattern were identical. All 35 clones of the *cbbM* library of D24 were sequenced. In total, 109 *cbbM* clones from the different samples were analysed. The sequence similarities ranged between 51%-100%. A phylogenetic tree for *cbbM* nucleotide sequences (Fig. 3.4) shows that the sediment clone sequences are distributed all over the tree without clustering and without any depth related distribution.

Fig. 3.4: Phylogenetic tree of *cbbM* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the green-like *cbbL* sequence of *Nitrobacter vulgaris* T3 (L22885) was used. All environmental clone sequences are shown in bold; the *cbbM* sequence of the isolates D15HSO, D24HN and *Thiobacillus thiophilus* D24TN^T are shown in bold and shaded in grey. Scale bar represents 0.10 changes per nucleotide position.

3.3.2 ^{13}C –PLFA

From the two sediment cages containing ^{13}C-labelled bicarbonate dedicated for microbial colonization, which were incubated at 6.5 m (highly contaminated zone) and 11 m (zone with minor contamination) in a fully screened monitoring well, the tube exposed at 11 m could unfortunately not be retrieved from the aquifer. Thus PLFA analysis for the detection of *in situ* CO_2 fixation could only be performed on sediment incubated close to the groundwater table (Fig. 3.1). Even though total bacterial counts in groundwater and attached to the sediment accounted for $1.2*10^6$ cells cm^{-3} and about $2.4*10^7$ cells cm^{-3}, respectively, the amount of detectable PLFA in the colonized sediment was rather low with about 5 $nmol*g^{-1}$ dry weight. Seven saturated fatty acids and three monounsaturated fatty acids could be found (Table 3.6), whereas no polyunsaturated fatty acids could be detected. Distinct ^{13}C-labelling was only found in branched 16:0 and monounsaturated 16:1ω7, both 16 chained fatty acids which are indicative for gram-positive bacteria as well as for sulfate-reducing gram-negative bacteria (O'Leary and Wilkinson, 1988; Zelles, 1999a). These fatty acids were found enriched by approx. 2 ‰δ^{13}C compared to non-spiked control samples.

Table 3.6: PLFA analysis

PLFA[1]	Microbial groups	^{13}C label	Reference
SATFA[2]			
br13:0	bacterial origin, gram +, -	-	Zelles (1999b; 1999a)
i15:0	bacterial origin, gram -	-	Frostegard & Baath (1996)
a15:0	bacterial origin, gram +	-	Frostegard & Baath (1996)
br16:0	bacterial origin, gram +, sulphate-reducing gram -	+	Zelles (1999b; 1999a); O'Leary & Wilkinson (1988)
cy17:0	bacterial origin, gram -	-	Zelles (1999b); Frostegard & Baath (1996)
br18:0	bacterial origin, gram +, sulphate-reducing gram -	-	Zelles (1999b; 1999a)
cy19:0	bacterial origin, gram -	-	Zelles (1999b); Frostegard & Baath (1996)
MUFA[3]			
16:1ω7	bacterial origin, gram -	+	Kaur *et al.* (2005); Zelles (1999a)
18:1ω7 & 18:1ω9 (mixpeak)	bacterial origin, gram -	(-)	Lu *et al.* (2007)

[1] phospholipid fatty acids

[2] saturated fatty acids

[3] monounsaturated fatty acids

3.3.3 FISH-MAR

Analysis of water samples from 6.5 m and 11 m depth, corresponding to the depths of sediment exposure, exhibited good fluorescence signals with group specific probes selected. Both depths showed about 40% of the EUBI labelled cells to be Gam42a labelled, a hint for γ-Proteobacteria possibly being important in this aquifer. However, total cell counts were found considerably low with $1.2 * 10^6$ and $4.4 * 10^5$ cells mL^{-1} in 6.5m and 11m, respectively. In sediment samples, low cell numbers along with the fluorescence of non-bacterial particles residual after detachment strongly interfered with fluorescent signals of the probes, anticipating its reliable analysis. Incubation of samples with ^{14}C bicarbonate and microautoradiographic analysis did not show the appearance of silver grains doubtless associated with individual cells hinting at $^{14}CO_2$ fixation. Preceding tests showed that the two probes designed for the detection of strain D24TN cells, worked well resulting in bright fluorescence if hybridisation buffers with up to 60% formamide were used. However, probe D24TN_443 was not selectively targeting strain D24TN but also tagged closely related species (*Thiobacillus denitrificans*), independent from the used formamide concentration, as tests using *Thiobacillus denitrificans*, *Pseudomonas putida* and $D24TN^T$ showed. Unfortunately, our FISH analysis revealed only a few cells weakly fluorescing in water samples from 6.5 m, but no signal at all for the 11m samples.

3.3.4 Chemolithoautotrophic Isolates

Bacteria were enriched from different depths (D10, D15, and D24) under various chemolithoautotrophic conditions (Table 3.3). During enrichment and isolation it became evident that some cultures grew much better in medium with increased ionic strength. Apart from this, the growth of most enrichment cultures stopped after several transfers or during dilution series for purification with few exceptions. From D15 one culture could be enriched and sustained under acetogenic/methanogenic conditions. Additionally, a sulphate-reducing strain (D15HSO) was isolated and purified. The latter, after direct sequencing of the 16S rRNA sequence, showed a close relation to *Thiobacillus sp.* strain Q (99.6%; AJ289884). It also proved positive for RubisCO form II (*cbbM*) with 87.9% relation to the *cbbM* sequence of an uncultured groundwater bacterium (EU362821). Furthermore, we were able to isolate and purify two more strains from D24. Strain D24HN was obtained under denitrifying conditions, a facultative chemolithoautotroph exhibiting faster growth under heterotrophic than under autotrophic conditions. Direct sequencing revealed a close relatedness to the 16S of an uncultured *β*-Proteobacterium (98.9%; EF562548) and *Ferribacterium limneticum* strain

cda-1 (98.9%; Y17060). It proved positive for RubisCO form I green-like (*cbbL*G) and RubisCO form II (*cbbM*). Strain D24TN^T is a thiosulfate-oxidizer which belongs to the genus *Thiobacillus*, being closely related to *T. denitrificans* (97.6%) and *T. thioparus* (97.5%) (for further details see Chapter 2).

3.3.5 Marker genes and enzymes of isolate D24TN^T – CO_2 fixation potential and activity

Strain D24TN^T, isolated under thiosulfate oxidizing conditions, was further analyzed. It proved to be a facultative anaerobic, obligate chemolithoautotrophic organism. Oxygen and nitrate were used as electron acceptor; from all tested electron donors only thiosulfate and tetrathionate could be utilized. When the culture was transferred in medium containing organic carbon but no thiosulfate, the cells could use organic carbon neither as carbon nor as electron source. The strain was described as a new species with the scientific name *Thiobacillus thiophilus* D24TN^T (DSM19892 = JCM 15047) (for a detailed description of D24TN^{T}see Chapter 2). When testing D24TN^T for CO_2 fixation potential via the Calvin cycle and the reductive TCA cycle, it proved positive for the functional marker genes RubisCO form I (green-like *cbbL*; 91.9% sequence similarity to the green-like *cbbL* sequence of *Thiobacillus thioparus* DSM 505; DQ390449) and RubisCO form II (*cbbM*; 88.4% sequence similarity to the *cbbM* sequence of *Halothiobacillus sp.* RA13; AY099399); the ATP citrate lyase genes could not be detected.

To find out if the isolate actually uses the Calvin cycle for CO_2 fixation RT-PCR was applied to detect transcription of the *cbb* genes. Both, aerobically as well as anaerobically grown cells showed to transcribe the *cbbM* and green-like *cbbL* genes. Furthermore, cell extracts of D24TN^T grown anaerobically and aerobically were tested for the occurrence of autotrophic CO_2 fixation via the Calvin cycle by enzyme activity measurements of RubisCO, the carboxylating enzyme of this cycle. Additionally, cell extracts were also tested for the activity of ATP citrate lyase, the citrate cleaving enzyme of the reductive TCA cycle. Malate dehydrogenase and isocitrate dehydrogenase activity was also tested. These enzymes are not only important enzymes in the reductive TCA cycle but also occur in the oxidative citric acid cycle or in a horseshoe-type citric acid cycle for biosynthetic purposes. The activity of both enzymes could be measured in cell extracts of aerobically and anaerobically grown cells, with isocitrate dehydrogenase clearly $NADP^+$ dependent (specific activity: 102 and 50 nmol min^{-1} (mg cell protein)$^{-1}$, respectively). The specific activity of NADH dependent malate dehydrogenase was 17.3 and 22.0 nmol min^{-1} (mg cell protein)$^{-1}$ for aerobically and anaerobically grown cells. ATP citrate lyase activity could not be detected, neither with nor

without addition of exogenous malate dehydrogenase. RubisCO activity was detected in cell extracts from both aerobically and anaerobically grown cells. The specific activity of RubisCO was 11.2 nmol min^{-1} (mg cell protein)$^{-1}$ in the aerobically cultured cells and 2.0 nmol min^{-1} (mg cell protein)$^{-1}$ in anaerobically grown cells of D24TN^T (Table 3.5).

Table 3.5: Specific activities [nmol min^{-1} (mg cell protein)$^{-1}$] of key enzymes of autotrophic CO_2 fixation pathways in D24TN^T

Enzyme activity	D24TN^T aerobically grown	D24TN^T anaerobically grown
Assay temperature (°C)	25	25
Calvin cycle		
Ribulose 1,5-bisphosphate carboxylase	11.2	2.0
Reductive TCA cycle		
ATP citrate lyase	-	-
Reductive/oxidative TCA cycle		
Isocitrate dehydrogenase		
NAD^+	-	-
$NADP^+$	102.2	50.0
Malate dehydrogenase		
NADH	17.3	22.0
NADPH	-	-

-, no activity detected; nd, not determined

3.4 Discussion

Aquifers are regarded as heterotrophic systems dependent on organic input from the surface. In terms of CO_2 this means the saturated subsurface is regarded as a source rather than a sink. Heterotrophic microbial communities in oligotrophic groundwater ecosystems often seem to be limited by organic carbon. At several spots investigated the DOC concentration in water is found constant with depth from several meters below the groundwater table to several hundreds of meters further down. Hence one may speculate on a significant role of chemolithoautotrophy to support growth of indigenous communities and maintenance of microbial biomass. Besides the very oligotrophic systems, aquifers impacted by high loads of organics such as petroleum hydrocarbons are another extreme. In such cases, organic carbon is present in surplus but rather persistent to biodegradation. These environments, however, also contain the reduced ingredients (*e.g.* H_2, NH_4^+, NO_2^-, $S_2O_3^{2-}$, H_2S, S^0, Fe^{2+}, Mn^{2+}, CO, CH_4, CH_3OH) for a number of chemotrophic processes to gain energy for growth of microorganisms on CO_2 as carbon source (Alfreider *et al.*, 2003). Thus autotrophic CO_2 fixation may substantially contribute to the overall carbon flow in subsurface environments.

Apart from that, it was shown that to a certain extent CO_2 is required for heterotrophic growth, it is estimated that CO_2 fixation by anaplerotic reactions during heterotrophic growth accounts for up to 10% of cell carbon (Krebs, 1941; Perez and Matin, 1982; Sonntag *et al.*, 1995). *Thiobacillus sp.*, for example, was shown to incorporate more than 10% of the cell carbon from CO_2 during mixotrophic as well as heterotrophic growth (Perez and Matin, 1982). Moreover, during carboxylation reactions, recently shown to be performed also by aerobic *Rhodococcus* and *Xanthobacter* species (Ensign *et al.*, 1998; Clark and Ensign, 1999), CO_2 substantially contribute to biomass formation. In soils, CO_2 fixation was shown to be of relevance accounting for 2-5% of the net respiration (Miltner *et al.*, 2005), mainly attributed to heterotrophic microbes, as was indicated by a linear correlation between respiration and CO_2 fixation. In organically polluted aquifers CO_2 fixation thus may be suggested one additional way for mixotrophic and heterotrophic bacteria to get rid of electrons. So far, information on the role of autotrophic, mixotrophic and heterotrophic CO_2 fixation in subsurface ecosystems is scarce (Alfreider *et al.*, 2003; Miltner *et al.*, 2005; Selesi *et al.*, 2005).

3.4.1 CO_2 fixation potential - Calvin cycle and reductive TCA cycle

The occurrence and high diversity of the RubisCO genes was already demonstrated in different systems: in agricultural soils (Selesi *et al.*, 2005; Tolli and King, 2005; Selesi *et al.*, 2007), in pine forest soil (Tolli and King, 2005), in a hypersaline anoxic basin (van der Wielen, 2006), in deep-sea habitats (Elsaied and Naganuma, 2001) and in intertidal marine and littoral lake sediments (Nigro and King, 2007), to list only a few. To our knowledge only one study exists so far for aquifers which tackled the occurrence a CO_2 fixation potential indicated by the presence of RubisCO genes (Alfreider *et al.*, 2003). In those contaminated, mostly anoxic aquifers a high diversity of *cbbL* sequences was found, some of them closely related to *cbbL* sequences of *Halothiobacillus*, *Thiobacillus*, *Acidithiobacillus*, *Hydrogenophaga* and *Nitrosomonas*, but also numerous only slightly related to already known RubisCO sequences, making the assessment of the physiology and ecological role of the potential autotrophs more complicated (Alfreider *et al.*, 2003). Retrieved *cbbM* clone sequences showed less diversity and were mostly closely related to sequences of the genera *Halothiobacillus* and *Thiobacillus*. Our study in a tar oil contaminated aquifer revealed the large subunit of RubisCO form I and form II being detectable close to the contaminant plume core as well as in areas less contaminated. The diversity of the obtained green-like *cbbL* sequences was quite low with most sequences closely related to the *cbbL* sequences of *Thiobacillus thioparus* (DQ390449) and *Thiobacillus sp.* (M34536). The retrieved red-like *cbbL* clone sequences showed a similar low diversity, with most clone sequences being similar to *cbbL*R sequences of the genus *Nitrosospira* (AF426428) and *Rhizobium radiobacter* (AY572468).

Interestingly, the red-like *cbbL* genes could only be detected in sediment samples deriving from the plume core (D10 and D12). This might be a hint that organisms harbouring those genes are less sensitive to the contaminants present in the aquifer or are even able to use those organic carbon compounds as carbon and/or energy sources. Some of the retrieved red-like *cbbL* sequences were relatively close related (82.3-88.7% sequence similarity) to the *cbbL*R sequences of *Ochrobactrum anthropi* (AY572473), *Burkholderia sp.* (AY422049; AY422050) (Fig. 3.3), the 16S rRNA gene sequences of bacteria belonging to those genera have already been detected in petroleum oil in stockpiles (Yoshida *et al.*, 2005).

In contrast, *cbbM* sequences were quite diverse without forming cluster but being dispersed over the phylogenetic tree (Fig. 3.2), showing relatedness to *cbbM* sequences of various bacterial genera, *e.g. Halothiobacillus*, *Thiobacillus*, *Polaromonas* and *Leptothrix*. The large subunit gene of RubisCO is suited to phylogenetic analysis (Watson and Tabita, 1997), but

since there is a discrepancy between phylogenies based on *cbb* genes and based on the 16S rRNA gene, probably due to horizontal gene transfer of *cbbL*, sequences showing a very close relatedness or even identical sequences not necessarily have to derive from the same genera or organisms (Delwiche and Palmer, 1996; Alfreider *et al.*, 2003; Selesi *et al.*, 2005). Consequently, the low diversity of the retrieved green- and red-like *cbbL* sequences does not automatically mean a low diversity of the bacterial community in total. We may also have to consider the contribution of PCR biases by unequal accessibilities of target sequences, unequal efficiencies during amplification of related sequences or interferences due to contaminants to the low diversity observed (Wawrik *et al.*, 2002). Since only *cbb* sequences available in the databases could be used for the construction of *cbbL* and *cbbM* primers, distantly related *cbbL* and *cbbM* sequences might not be detected by those primers.

The abundance and diversity of the detected *cbb* genes hint at a significant potential for CO_2 fixation via the Calvin cycle within the aquifers microbial communities. However DNA-diversity data only is not proving the actual carbon fixation activity. Even though CO_2 fixation is this cycle's main function, it is also used as an important electron sink during photoheterotrophic growth of phototrophs (Wang *et al.*, 1993; Kusian and Bowien, 1997), making it possible, that under certain conditions some of the chemotrophs harbouring *cbb* genes might similarly use the cycle for electron-dissipating (Kusian and Bowien, 1997). Moreover, some or even all of the microorganisms might just still harbour the *cbb* genes as evolutionary relicts, without taking use of it.

The access to functional genes involved in CO_2 fixation via the reductive TCA cycle allow to test an alternative pathway, so far only found in anaerobic and microaerobic microorganisms, *i.e.* members of the *Chlorobiaceae* (Evans *et al.*, 1966), ε-Proteobacteria (Hügler *et al.*, 2005), Aquificales (Beh *et al.*, 1993; Hügler *et al.*, 2007) or the δ-Proteobacterium *Desulfobacter hydrogenophilus* (Schauder *et al.*, 1987). The *acl* genes, encoding ATP citrate lyase, one of the key enzymes of this cycle, have already been detected in microorganisms inhabiting deep-sea hydrothermal vent environments (Campbell and Cary, 2004; Voordeckers *et al.*, 2008). Since the investigated aquifer was anoxic, we tried to detect microorganisms with the potential to use this cycle. However, with the primer sets applied in this study, detection of the functional genes was not successful in any of the investigated samples. Besides the possibility that there were no organisms present harbouring those genes, the result might also be caused by the restricted specificity of the primer sets.

3.4.2 CO_2 fixation activity in situ

To further prove or disprove the occurrence and importance of autotrophic CO_2 fixation in this aquifer, measurements of actual autotrophic activity in water and sediment samples are needed. This is not an easy task, as in the subsurface one is confronted with relative low cell numbers, low metabolic activities, and the difficult accessibility of the system (Griebler and Lueders, 2008). This point was subsequently addressed by enrichment of several bacterial strains, the exposure of ^{13}C-bicarbonate and phospholipid fatty acid (PLFA) analysis and fluorescence *in situ* hybridization combined with microautoradiography (FISH-MAR).

^{13}C-labelled $CaCO_3$ was exposed together with sterile sediment to prove CO_2-fixation activity *in situ*. After two months of incubation in groundwater, phospholipid fatty acids were extracted from the microbial biomass of the colonized sediments. The PLFA approach is well suitable for estimating the amount of active microbial biomass and analyzing the composition of microbial communities in environmental samples (Ben-David *et al.*, 2004). This approach is based on its great structural diversity, coupled with high biological specificity (Zelles, 1999b). Lipid profiles can thus provide insight into the microbial community structure based on the relative abundance of certain PLFAs which considerably differ among specific groups. ^{13}C-labelled bacterial PLFAs thus not only confirm the presence of an active microbial community, but enables to distinguish the active members of the microbial community (Evershed *et al.*, 2006). Even though cell counts suggested a successful colonization of the exposed sediments, PFLA concentration was low. In total seven saturated fatty acids and three monounsaturated fatty acids could be detected in our samples. Both detected 16-C fatty acids showed an incorporation of ^{13}C-carbon derived from ^{13}C-$CaCO_3$. Those PLFAs are indicative for gram-positive bacteria as well as for sulfate-reducing gram-negative bacteria. Chemolithoautotrophs are distributed in both of these groups (Aggag and Schlegel, 1973; Moussard *et al.*, 2004; Zavarzina *et al.*, 2007). Interestingly, sulfate reduction is not only most prominent in the plume core (Anneser *et al.*, 2008), additionally, 16S rRNA gene sequences of sulfate-reducers have also been detected in the aquifer (Winderl *et al.*, 2007). CO_2 fixation in some gram-positive autotrophic bacteria is performed via the reductive Acetyl-CoA pathway (Thauer, 2007), therefore the detection of ^{13}C-labelled bacterial PLFAs indicative for gram-positive bacteria might be a hint for the occurrence of the reductive Acetyl-CoA pathway in this aquifer. The incorporation of ^{13}C-label into the PFLAs is a direct hint for bacterial CO_2-uptake activity in the aquifer.

While FISH, based on small subunit rRNA sequence analysis, allows the phylogenetic identification and *in situ* detection of individual microbial cells (Amann *et al.*, 1995), the

microautoradiography potentially reveals an idea of growth and function of microorganisms directly in nature (Brock and Brock, 1966). Attempts to detect actual autotrophic activity applying fluorescence *in situ* hybridization combined with microautoradiography failed (FISH-MAR; Lee *et al.* (1999)). The good results from the FISH analysis indicate that bacteria in this aquifer were considerably active, but a detection of radiolabelled cells was not possible. There are several reasons possible to be responsible, including insufficient concentration of ^{14}C-labelled bicarbonate, unfavourable conditions during incubation of samples in the lab and an overall low CO_2 fixation activity.

3.4.3 Chemolithoautotrophic isolates and isolate *Thiobacillus thiophilus* D24TN^T

New chemolithoautotrophic organisms were successfully enriched from the freshly collected sediment samples, with three pure cultures isolated so far. Although the enrichments were obtained using media with low ionic strength (to mimic groundwater), some of the cultures regained activity after having stopped growing (*i.e.* D15HCO) or subsequently grew faster than before (D24TN, D15HSO) when transferred to medium with higher salt concentrations. Two of the three isolates belong to the genus *Thiobacillus*. Moreover, most of the retrieved *cbbL*G clone sequences also showed a high relatedness to the *cbbL*G sequences of *Thiobacilli*. This is in accordance with the results of Winderl *et al.* (2008) who found *Thiobacillus* related clones to be prominent, *i.e.* 6 % clone frequency in bacterial 16S rRNA gene clone libraries, in two depths of the investigated aquifer (6.3 m bls = capillary fringe and 11.20 m = less contaminated zone), including the depth from which the isolates were obtained. Two species of *Thiobacillus, T. denitrificans* (Baker *et al.*, 1998; Beller *et al.*, 2006) and *T. thiophilus* D24TN^T (Chapter 2), were shown to be able to fix CO_2 via the Calvin cycle under anoxic conditions, using nitrate as electron acceptor and sulfide and/or thiosulfate as electron donor. Transcription of the *cbbM* and green-like *cbbL* genes, as well as the synthesis of RubisCO via enzyme tests could be proved for the later strain (Chapter 2). In the aquifer investigated, nitrate was repeatedly detected at low concentration, while sulphide was prominent (Anneser *et al.*, 2008) and indication for the presence of thiosulfate was gained (F. Einsiedl, pers. comm.). In summary, these lines of evidence may be quoted a hint that *Thiobacillus* is abundant in certain areas of the aquifer and may there contribute to CO_2 fixation. In a pioneering study, Alfreider *et al.* (2003) detected and isolated *Thiobacilli* related bacteria in a BTEX (Benzene, Toluene, Ethylbenzene and Xylene isomers) contaminated aquifer. Furthermore, numerous *cbb* sequences related to *cbb* sequences of *Thiobacilli* could be found (Alfreider *et al.*, 2003).

Besides the potential for autotrophic CO_2 fixation first evidence for the importance of this process *in situ* could be collected. Lines of evidence include, (i) the isolation of a new strain, *i.e. T. thiophilus* D24TN^T, carrying *cbbM* and green-like *cbbL* genes which both were transcribed, (ii) presence of *Thiobacilli* in the aquifer, (iii) incorporation of ^{13}C-label from carbonate into PLFAs of bacteria indigenous to the aquifer.

3.5 References

Aggag, M., and Schlegel, H.G. (1973) Studies on a gram-positive hydrogen bacterium, *Nocardia opaca* strain 1b. *Arch Microbiol* **88**: 299-318.

Alfreider, A., Vogt, C., Hoffmann, D., and Babel, W. (2003) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecol* **45**: 317-328.

Amann, R., Schleifer, K.H., and Ludwig, W. (1995) Phylogenetic Identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.

Amann, R.I., Binder, B.J., Olson, S.W., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919-1925.

Anderson, R.T., Chapelle, F.H., and Lovley, D.R. (1998) Evidence against hydrogen-based microbial ecosystems in basalt aquifers. *Science* **281**: 976-977.

Andreasen, K., and Nielsen, P.H. (1997) Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. *Appl Environ Microbiol* **63**: 3662-3668.

Anneser, B., Einsiedl, F., Meckenstock, R.U., Richters, L., Wisotzky, F., and Griebler, C. (2008) High-resolution monitoring of biogeochemical gradients in a tar oil-contaminated aquifer. *Appl Geochem* **23**: 1715-1730.

Baker, S.H., Jin, S., Aldrich, H.C., Howard, G.T., and Shively, J.M. (1998) Insertion mutation of the form I cbbL gene encoding ribulose bisphosphate carboxylase/oxygenase (RuBisCO) in *Thiobacillus neapolitanus*: Results in expression of form II RuBisCO, loss of carboxysomes, and an increased CO_2 requirement for growth. *J Bacteriol* **180**: 4133-4139.

Bassham, J.A., and Calvin, M. (1957) *The path of carbon in photosynthesis* Englewood Cliffs: Prentice-Hall Inc.

Beh, M., Strauss, G., Huber, R., Stetter, K.O., and Fuchs, G. (1993) Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaebacterium *Thermoproteus neutrophilus*. *Arch Microbiol* **163**: 306 - 311.

Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M. *et al.* (2006) The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. *J Bacteriol* **188**: 1473-1488.

Ben-David, E.A., Holden, P.J., Stone, D.J.M., Harch, B.D., and Foster, L.J. (2004) The use of phospholipid fatty acid analysis to measure impact of acid rock drainage on microbial communities in sediments. *Microb Ecol* **48**: 300-315.

Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in *Archaea*. *Science* **318**: 1782 - 1786.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Brock, T., and Brock, M.L. (1966) Autoradiography as a tool in microbial ecology. *Nature* **209**: 734-736.

Campbell, B.J., and Cary, S.C. (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. *Appl Environ Microbiol* **70**: 6282-6289.

Campbell, B.J., Stein, J.L., and Cary, S.C. (2003) Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. *Appl Environ Microbiol* **69**: 5070-5078.

Chapelle, F.H., O'Neill, K., Bradley, P.M., Methe, B.A., Ciufo, S.A., Knobel, L.L., and Lovley, D.R. (2002) A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* **415**: 312-315.

Clark, D.D., and Ensign, S.A. (1999) Evidence for an inducible nucleotide-dependent acetone carboxylase in *Rhodococcus rhodochrous* B276. *J Bacteriol* **181**: 2752-2758.

Delwiche, C.F., and Palmer, J.D. (1996) Rampant horizontal transfer and duplication of RubisCO genes in eubacteria and plastids. *Mol Biol Evol* **13**: 873-882.

Eckert, P., Richters, L., Wisotzky, F., Schulte, U., Eisenberg, V., Griebler, C. *et al.* (2005) Monitoring und Bewertung von stimulierten Selbstreinigungsprozessen (NA) an einem BTEX- und PAK-belasteten Porengrundwasserleiter. In *Statusseminar 2005 - KORA: Kontrollierter natürlicher Rückhalt und Abbau von Schadstoffen bei der Sanierung kontaminierter Grundwässer und Böden, Frankfurt*. Frankfurt/Main: BMBF pp. 83-97.

Elsaied, H., and Naganuma, T. (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**: 1751-1765.

Ensign, S.A., Small, F.J., Allen, J.R., and Sluis, M.K. (1998) New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. *Arch Microbiol* **169**: 179-187.

Evans, M.C., Buchanan, B.B., and Arnon, D.I. (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. *P Natl Acad Sci USA* **55**: 928-934.

Evershed, R.P., Crossman, Z.M., Bull, I.D., Mottram, H., Dungait, J.A.J., Maxfield, P.J., and Brennand, E.L. (2006) C-13-Labelling of lipids to investigate microbial communities in the environment. *Curr Opin Biotech* **17**: 72-82.

Frostegard, A., and Baath, E. (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* **22**: 59-65.

Frostegard, A., Tunlid, A., and Baath, E. (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Appl Environ Microbiol* **59**: 3605-3617.

Gabor, E., de Vries, E., and Janssen, D. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiol Ecol* **4**: 153-163.

Griebler, C., and Lueders, T. (2008) Microbial biodiversity in groundwater ecosystems. *Freshwater Biol* **early online doi:10.1111/j.1365-2427.2008.02013.x**.

Holo, H. (1989) *Chloroflexus aurantiacus* secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO_2 and acetate. *Arch Microbiol* **151**: 252-256.

Huber, H., Gallenberger, M., Jahn, U., Eylert, E., Berg, I.A., Kockelkorn, D. *et al.* (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum *Ignicoccus hospitalis*. *Proc Natl Acad Sci USA* **105**: 7851-7856.

Hügler, M. (2003) Autotrophe CO_2-Fixierung in thermophilen Mikroorganismen. Freiburg: Albert-Ludwigs-Universität Freiburg i. Br., pp. 127

Hügler, M., Krieger, R.S., Jahn, M., and Fuchs, G. (2003) Characterization of acetyl-CoA/propionyl-CoA carboxylase in *Metallosphaera sedula* - Carboxylating enzyme in the 3-hydroxypropionate cycle for autotrophic carbon fixation. *Eur J Biochem* **270**: 736-744.

Hügler, M., Wirsen, C.O., Fuchs, G., Taylor, C.D., and Sievert, S.M. (2005) Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria. *J Bacteriol* **187**: 3020-3027.

Hügler, M., Huber, H., Molyneaux, S.J., Vetriani, C., and Sievert, S.M. (2007) Autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle in different lineages within the

phylum *Aquificae*: evidence for two ways of citrate cleavage. *Environ Microbiol* **9**: 81-92.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., and Kaushik, R. (2005) Phospholipid fatty acid - A bioindicator of environment monitoring and assessment in soil ecosystem. *Curr Sci* **89**: 1103-1112.

Kinkle, B.K., and Kane, T.C. (2000) Chemolithoautotrophic micro-organisms and their potential role in subsurface environments. In *Ecosystems of the World*. Wilkens, H., Culven, D.C., and Humphreys, W.F. (eds). Amsterdam: Elsevier, pp. 309-318.

Krebs, H.A. (1941) Carbon dioxide assimilation in heterotrophic organisms. *Nature* **147**: 560-563.

Kusian, B., and Bowien, B. (1997) Organization and regulation of cbb CO_2 assimilation genes in autotrophic bacteria. *FEMS Microbiol Rev* **21**: 135-155.

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005) Impact of different *in vitro* electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ Microbiol* **71**: 6664-6672.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.H., and Wagner, M. (1999) Combination of fluorescent *in situ* hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. *Appl Environ Microbiol* **65**: 1289-1297.

Lu, Y.H., Abraham, W.R., and Conrad, R. (2007) Spatial variation of active microbiota in the rice rhizosphere revealed by in situ stable isotope probing of phospholipid fatty acids. *Environ Microbiol* **9**: 474-481.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Lueders, T., Manefield, M., and Friedrich, M.W. (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73-78.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593-600.

Miltner, A., Kopinke, F.D., Kindler, R., Selesi, D.E., Hartmann, A., and Kastner, M. (2005) Non-phototrophic CO_2 fixation by soil microorganisms. *Plant Soil* **269**: 193-203.

Moussard, H., L'Haridon, S., Tindall, B.J., Banta, A., Schumann, P., Stackebrandt, E. *et al.* (2004) *Thermodesulfatator indicus* gen. nov., sp nov., a novel thermophilic chemolithoautotrophic sulfate-reducing bacterium isolated from the Central Indian Ridge. *Int J Syst Evol Microbiol* **54**: 227-233.

Nealson, K.H., Inagaki, F., and Takai, K. (2005) Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): do they exist and why should we care? *Trends Microbiol* **13**: 405-410.

Neef, A., Amann, R., Schlesner, H., and Schleifer, K.H. (1998) Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* **144**: 3257-3266.

Nigro, L.M., and King, G.M. (2007) Disparate distributions of chemolithotrophs containing form IA or IC large subunit genes for ribulose-1,5-bisphosphate carboxylase/oxygenase in intertidal marine and littoral lake sediments. *FEMS Microbiol Ecol* **60**: 113-125.

O'Leary, W.M., and Wilkinson, S.G. (1988) Gram-positive bacteria. In *Microbial Lipids*. Ratledge, C., and Wilkinson, S.G. (eds). London: Academic Press, pp. 117-201.

Pedersen, K. (2000) Exploration of deep intraterrestrial microbial life: current perspectives. *FEMS Microbiol Lett* **185**: 9-16.

Perez, R.C., and Matin, A. (1982) Carbon dioxide assimilation by *Thiobacillus novellus* under nutrient-limited mixotrophic conditions. *J Bacteriol* **150**: 46-51.

Roller, C., Wagner, M., Amann, R., Ludwig, W., and Schleifer, K.H. (1994) In situ probing of gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotids. *Microbiology* **140**: 2849-2858.

Schauder, R., Widdel, F., and Fuchs, G. (1987) Carbon assimilation pathways in sulfate-reducing bacteria II. Enzymes of a reductive citric acid cycle in the autotrophic Desulfobacter hydrogenophilus. *Arch Microbiol* **148**: 218-225.

Schmitt, M.E., Brown, T., and Trumpower, B. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisia*. *Nucleic Acids Res* **18**: 3091-3092.

Selesi, D., Schmid, M., and Hartmann, A. (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71**: 175-184.

Selesi, D., Pattis, I., Schmid, M., Kandeler, E., and Hartmann, A. (2007) Quantification of bacterial RubisCO genes in soils by *cbbL* targeted real-time PCR. *J Microbiol Meth* **69**: 497-503.

Shively, J.M., van Keulen, G., and Meijer, W.G. (1998) Something from almost nothing: Carbon dioxide fixation in chemoautotrophs. *Annu Rev Microbiol* **52**: 191-230.

Sonntag, K., Schwinde, J., de Graaf, A.A., Marx, A., Eikmanns, B.J., Wiechert, W., and Sahm, H. (1995) ^{13}C NMR studies of the fluxes in the central metabolism of Corynebacterium glutamicum during growth and overproduction of amino acids in batch cultures. *Appl Microbiol Biot* **44**.

Stevens, T. (1997) Lithoautotrophy in the subsurface. *FEMS Microbiol Rev* **20**: 327-337.

Stevens, T.O., and McKinley, J.P. (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270**: 450-454.

Tabita, F.R. (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol Rev* **52**: 155–189.

Tabita, F.R., Satagopan, S., Hanson, T.E., Kreel, N.E., and Scott, S.S. (2008) Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *J Exp Bot* **59**: 1515-1524.

Takai, K., Campbell, B.J., Cary, S.C., Suzuki, M., Oida, H., Nunoura, T. *et al.* (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of Epsilonproteobacteria. *Appl Environ Microbiol* **71**: 7310-7320.

Thauer, R.K. (2007) A Fifth Pathway of Carbon Fixation. *Science* **318**: 1732-1733.

Tolli, J., and King, G.M. (2005) Diversity and structure of bacterial chemolithotrophic communities in pine forest and agroecosystem soils. *Appl Environ Microbiol* **71**: 8411-8418.

van der Wielen, P. (2006) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes in the $MgCl_2$-dominated deep hypersaline anoxic basin discovery. *FEMS Microbiol Lett* **259**: 326-331.

Voordeckers, J.W., Do, M.H., Hügler, M., Ko, V., Sievert, S.M., and Vetriani, C. (2008) Culture dependent and independent analyses of 16S rRNA and ATP citrate lyase genes: a comparison of microbial communities from different black smoker chimneys on the Mid-Atlantic Ridge. *Extremophiles* **12**: 627-640.

Wang, X., Falcone, D.L., and Tabita, F.R. (1993) Reductive pentose phosphate-independent CO_2 fixation in Rhodobacter sphaeroides and evidence that ribulose bisphosphate carboxylase/oxygenase activity serves to maintain the redox balance of the cell. *J Bacteriol* **175**: 3372-3379.

Watson, G.M.F., and Tabita, F.R. (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* **146**: 13-22.

Wawrik, B., Paul, J.H., and Tabita, F.R. (2002) Real-time PCR quantification of *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase) mRNA in diatoms and pelagophytes. *Appl Environ Microbiol* **68**: 3771-3779.

Werner, R.A., and Brand, W.A. (2001) Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun Mass Sp* **15**: 501-519.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New York: Springer, pp. 3352-3378.

Winderl, C., Schaefer, S., and Lueders, T. (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. *Environ Microbiol* **9**: 1035-1046.

Winderl, C., Anneser, B., Griebler, C., Meckenstock, R.U., and Lueders, T. (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. *Appl Environ Microbiol* **74**: 792-801.

Wood, H.G., Ragsdale, S.W., and Pezacka, E. (1986) CO_2 fixation into acetyl-CoA: a new pathway of autotrophic growth. *Trends Biochem Sci* **11**: 14-18.

Yoshida, N., Yagi, K., Sato, D., Watanabe, N., Kuroishi, T., Nishimoto, K. *et al.* (2005) Bacterial communities in petroleum oil in stockpiles. *J Biosci Bioeng* **99**: 143-149.

Zavarzina, D.G., Sokolova, T.G., Tourova, T.P., Chernyh, N.A., Kostrikina, N.A., and Bonch-Osmolovskaya, E.A. (2007) *Thermincola ferriacetica* sp. nov., a new anaerobic, thermophilic, facultatively chemolithoautotrophic bacterium capable of dissimilatory Fe(III) reduction. *Extremophiles* **11**: 1-7.

Zelles, L. (1999a) Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol Fertil Soils* **29**: 111-129.

Zelles, L. (1999b) Identification of single cultured micro-organisms based on their whole-community fatty acid profiles, using an extended extraction procedure. *Chemosphere* **39**: 665-682.

Zelles, L., Bai, Q.Y., Rackwitz, R., Chadwick, D., and Beese, F. (1995) Determination of phospholipid- and lipopolysaccharide-derived fatty acids as an estimate of microbial biomass and community structures in soils. *Biol Fert Soils* **19**: 115-123.

4. Detection of autotrophic potential and enrichment of new chemolithoautotrophic microorganisms at the Test Field Scheyern

4.1 Introduction

At first the subsurface was believed to be a mainly abiotic system, where living organisms are not present. It seemed unlikely that microorganisms were able to inhabit such seemingly barren environment. For a long period, microorganisms found in subsurface samples were thought to be surface contaminants deriving from drilling and sampling processes or being transported along with water flow. However, many studies proved most subsurface ecosystems being full of life, a fact well accepted nowadays (Beloin *et al.*, 1988; Fliermans and Balkwill, 1989; Chapelle and Lovley, 1990; Phelps *et al.*, 1994; Fredrickson and Onstott, 1996). Investigations further showed that microorganisms are not just inhabiting those environments but are actively shaping environmental conditions such as mineralogy and groundwater composition by conducting and mediating a wide range of redox reactions (Phelps *et al.*, 1994; Lovley and Chapelle, 1995; Pedersen, 1997; Ehrlich, 1998) and therefore play the most important role in biogeochemical cycles of those habitats (Labrenz *et al.*, 2005). Aquatic and near-surface terrestrial environments have already been well investigated for quite some time. In contrast to this, our understanding of the ecology of deeper subsurface microorganisms is a relatively recent development (Jones *et al.*, 1989; Chandler *et al.*, 1998) and there still exists comparatively little knowledge about subsurface organisms in general and the ecosystems they live in (Alfreider *et al.*, 1997). However, due to intensifying usage of groundwater and an increasing number of sites significantly contaminated, it became more important over the years to gain new and more detailed information about groundwater ecosystems, processes and the organisms living within (Ghiorse, 1997). Thus, during the last years several studies dealt with investigations of microbial communities in pristine and contaminated subsurface environments (Chandler *et al.*, 1998; Fredrickson *et al.*, 2004; Nazina *et al.*, 2004; North *et al.*, 2004; Tiago *et al.*, 2004; Fields *et al.*, 2005; Nedelkova, 2005).

Subsurface systems are usually oligotrophic environments. Normally, there is no direct plant and animal input from the surface (Brockman and Murray, 1997) and most dissolved organic carbon (DOC) and organic materials are in general already reduced significantly within the soil and unsaturated zone before reaching the primary aquifer. Additionally the total absence of light leaves all subsurface systems entirely dependent on chemical energy sources (Goldscheider *et al.*, 2006). On one hand, those conditions may pose major limitations for

heterotrophic microorganisms. On the other hand, groundwater ecosystems are often offering all necessary components for chemolithoautotrophic bacterial communities, *i.e.* electron donors like molecular hydrogen, reduced nitrogen (NO_2^- and NH_4^+), sulfur (*e.g.* $S_2O_3^{2-}$ and H_2S), metals (*e.g.* Fe^{2+} and Mn^{2+}) or carbon compounds (*e.g.* CO and CH_4) and electron acceptors like oxygen, nitrate or sulfate are available as well as plenty of inorganic carbon due to biological and geological processes (Shively *et al.*, 1998; Labrenz *et al.*, 2005).

In recent years an increasing interest in autotrophic microorganisms in the subsurface was developed (Alfreider *et al.*, 2003) and the occurrence of a number of autotrophic processes (iron reduction, sulphide oxidation) in the subsurface was proven (Pedersen, 1997; Stevens, 1997). Even the existence of surface independent biospheres based on chemolithoautotrophy has been considered (Pedersen, 1997; Stevens, 1997; Takai *et al.*, 2004).

The basis for chemolithoautotrophy is energy and inorganic carbon. The energy is gained in redox reactions as already mentioned above. For the fixation of inorganic carbon it takes special pathways. So far there are five CO_2 fixation pathways known: The Calvin-Benson-Bassham cycle (Calvin cycle) (Bassham and Calvin, 1957), the reductive tricarboxylic acid cycle (Evans *et al.*, 1966), the reductive Acetyl CoA pathway (Wood *et al.*, 1986), the 3-hydroxypropionate cycle (Holo, 1989) and the 3-hydroxypropionate/4-hydroxybutyrate pathway (Berg *et al.*, 2007). And only recently evidence was obtained for a new, sixth pathway (Huber *et al.*, 2008). The Calvin cycle seems to be the most abundant and important cycle (Fuchs *et al.*, 1987; Selesi, 2003), which means at the same time that most information is available for this cycle. In this cycle, ribulose-1,5 bisphosphate carboxylase/oxygenase (RubisCO) is the only enzyme responsible for the CO_2 fixation. All genes exclusively encoding Calvin cycle enzymes are named *cbb* genes (Tabita *et al.*, 1992), *cbbL* standing for the gene encoding the large subunit of form I RubisCO and *cbbM* for the gene encoding the large subunit of form II RubisCO (Alfreider *et al.*, 2003). There are four natural forms of RubisCO known which differ in structure, catalytic property and O_2 sensitivity (Tabita, 1988; Selesi *et al.*, 2005). Form I RubisCO is the most abundant one. It is composed of eight large and eight small subunits (L_8S_8), occurring in photo- and chemoautotrophic organisms. It is further divided in two major forms: green-like and red-like *cbbL* (Watson and Tabita, 1997). The green-like group is containing sequences deriving from cyanobacteria, plants, green algae and representatives of the α-, β- and γ-Proteobacteria, the red-like group contains sequences deriving from non-green algae and representatives of the α- and β-Proteobacteria (Shively *et al.*, 1998). Form II consists only of large subunits (L_x) (Watson and Tabita, 1997), its amino acid sequence showing 25-30% identity to the large subunit of form I (Kellog and Juliano,

1997) and can also be found in photo- and chemoautotrophs. One important biochemical feature of the form II enzyme is its poor affinity to CO_2, implying that the form II enzyme operates exclusively at high CO_2 and low O_2 concentrations (Alfreider *et al.*, 2003). The RubisCO large subunit gene is a highly conserved gene, with extended sequence data base available. Therefore, it seems to be ideally suited for the phylogenetic analysis of autotrophic bacteria in the environment without prior cultivation (Watson and Tabita, 1997; Alfreider *et al.*, 2003). Only recently the widespread potential of CO_2 fixation in microbial communities via the presence of RubisCO large subunit genes was shown in investigations in an organically contaminated aquifer and in agricultural soil (Watson and Tabita, 1997; Alfreider *et al.*, 2003; Selesi *et al.*, 2005).

The aim of this study was to investigate the diversity of the *cbb* form I and form II genes in different depths of an aquifer situated in an area influenced by agriculture to obtain first information about the CO_2 fixation potential based on RubisCO. Additionally new chemolithoautotrophic bacteria were enriched and isolated from freshly drilled sediments and subsequently tested for the occurrence of the RubisCO genes.

4.2 Material and Methods

4.2.1 Sampling site and sampling procedure

Sediment samples derive from the aquifer below the agriculturally managed "Testfield Scheyern" (TFS), situated in Southern Germany, near Munich (Fig. 4.1).

Drilling of sediments took place down to a depth of 30 m below soil horizon in fall 2006, approximately 5 m upgradient of an already exiting multi-level well, ML II (Fig. 4.1).

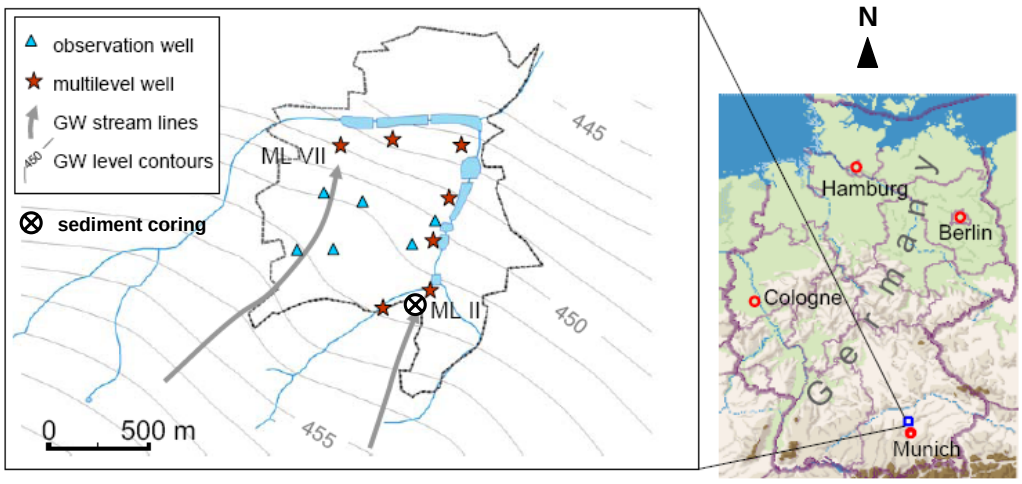

Figure 4.1: Map of the Test Field Scheyern located in southern Germany (taken from Schwientek, 2008)

To minimize oxidation of the partly reduced sediments the drill core PVC liners (1 m length, 100 mm diameter) were cut longitudinal within a box continuously flushed with argon gas. To avoid sample contamination the outer sediment of the core was removed. Samples dedicated for enrichment cultures were transferred directly into sterile glass bottles. Sediments were overlaid with oxygen free distilled water and stored at 4°C in darkness. Samples for DNA extraction and clone libraries were collected in sterile plastic tubes and kept at -20°C until further processing. For detection of CO_2 fixation potential sediment samples from 18 depths below soil surface were analysed in total (Table 4.1).

For the start up of enrichment cultures, sediments from the following depths below soil surface were used: 5.0 m, 12.3 m, 17.6 m, 22.6 m and 29.6 m, respectively (Table 4.1; Fig. 4.2). The depths were selected on the basis of the concentrations of selected anions and DOC concentrations at ML II (Fig. 4.2). Nitrate concentrations showed the most pronounced variability. A NO_3^- rich section with concentrations of up to 20.8 mg L^{-1} was found between 16 and 26 m depth, above and below this section NO_3^- concentrations were partly below the detection limit of 0.5 mg L^{-1}.

Table 4.1: Sediment samples taken for detection of CO_2 fixation potential

Name	Depth*	Material
Sy3	3.0	sand (moderately silty)
Sy5	5.0	sand (moderately silty)
Sy5.9	5.9	sand (moderately silty)
Sy7	7.0	sand (moderately silty)
Sy9	9.7	sand (moderately silty)
Sy10	10.6	sand (moderately silty)
Sy12	12.3	sand (moderately silty)
Sy13	13.6	clay (silty, sandy)
Sy15	15.6	clay (silty, sandy)
Sy17	17.6	sand (fine to medium)
Sy19	19.6	sand (fine to medium)
Sy20	20.6	sand (fine to medium)
Sy21	21.6	sand (fine to medium)
Sy24	24.6	clay (silty, sandy)
Sy25	25.6	clay (silty, sandy)
Sy27	27.6	sand (fine to medium)
Sy28	28.6	sand (fine to medium)
Sy29	29.6	sand (fine to medium)

* m below soil surface

Sulfate concentrations in groundwater varied between 34.4 and 19.2 mg L^{-1}, chloride concentrations were between 10.6 and 2.3 mg L^{-1}. Dissolved organic carbon (DOC) concentrations were very low (≤ 1 mg L^{-1}), only between 4 and 8 m depth the concentrations reached up to 3.9 mg L^{-1} (Fig. 4.2) (Schwientek, 2008).

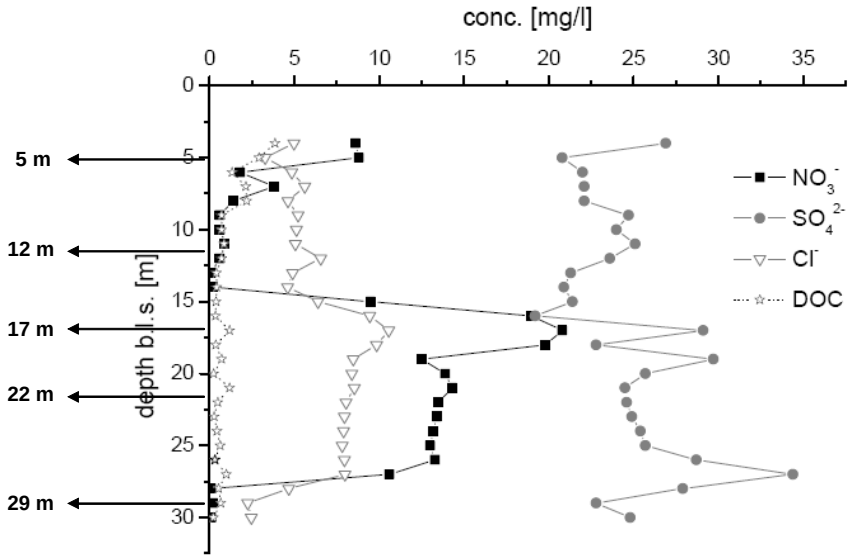

Figure 4.2: Vertical concentration profiles of selected anions and DOC concentrations at ML II, about 5 m downgradient from the sediment drilling point. Arrows indicate the depths where sediment was sampled for enrichment cultures (modified from Schwientek, 2008).

4.2.2 Bacterial strains

All bacterial strains used as positive or negative control in all conducted experiments are listed in Table 4.2. They were cultured as recommended by the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany).

Table 4.2: Bacterial strains

Species	Strain[a]	cbbM[b]	cbbL[b]
Thiobacillus denitrificans	DSM 12475	+	+ (green-like)
Cupriavidus necator	DSM 13513	-	+ (red-like)
Xanthobacter autotrophicus	DSM 432	-	+ (red-like)

[a]DSM, Deutsche Sammlung von Mikroorganismen
[b]+ PCR product of expected size; - no amplification

4.2.3 DNA extraction, PCR, cloning

Extraction of chromosomal DNA

Extraction of genomic DNA from sediment samples (7-8 g sediment per sample) was performed using a modified protocol from Lueders *et al*. (2004) and Gabor *et al*. (2003). Genomic DNA from pure cultures was extracted and purified applying the FastDNA spin kit for soil (MP Biomedicals) according to the manufacturer's instructions. The integrity and yield of extracted nucleic acids was checked by standard agarose gel electrophoresis and ethidium bromide staining, and by UV quantification (NanoDrop ND-1000 Spectrophotometer).

Marker genes for CO_2 Fixation - Amplification of cbbL and cbbM genes

The primer sets for form I RubisCO *cbbL*G1-F/*cbbL*G1-G, *cbbL*R1-F/*cbbL*R1-R (Selesi *et al.*, 2005) and the primer set for form II RubisCO *cbbM*-f/*cbbM*-r (Alfreider *et al.*, 2003) were used to amplify the RubisCO genes, cycling parameters were applied as described in Chapter 3. All amplification products were analyzed electrophoretically on 2% agarose gels (Biozym) by horizontal gel electrophoresis to ensure correct size (*cbbLG* 1.1 kb, *cbbLR* 0.8 kb, *cbbM* 0.5 kb) and were visualized by UV excitation after staining with ethidium bromide.

Clone libraries - Cloning of environmental clones

PCR products of the expected sizes (1100 bp for green-like *cbbL*, 820 bp for red-like *cbbL* and 505 bp for *cbbM* genes) from sediment samples were purified with the MinElute PCR Purification Kit (Quiagen) according to the manufacturer's instructions. Purified PCR

products were ligated in the vector pGEM®-T (Promega) and transformed into competent *Escherichia coli* cells Strain JM 109 (Promega). Colonies were picked and the plasmid DNA was purified heating the clones for 10 min at 99°C in H_2O_{dest}.

Screening of environmental clones

Restriction fragment length polymorphism (RFLP) was used for screening of the clones with correctly sized inserts. PCR products of clones harbouring *cbbM* or green-like *cbbL* inserts were hydrolyzed with 2 U of the restriction endonucleases RsaI and MspI (Fermentas), and for PCR products of clones with red-like *cbbL* inserts, the restriction enzyme BsaI (Fermentas) was used. Restriction fragments were analyzed electrophoretically in 2% (w/v) agarose gels.

4.2.4 Sequencing and phylogenetic analysis

Plasmids containing *cbbL* or *cbbM* inserts from sediment DNA were used directly for sequencing. Vector specific primers (M13 reverse and T7 promotor) were applied to sequence both strands. The plasmids were sequenced in an ABI Prism 377 automated sequencer (Applied Biosystems) by use of a Big Dye Terminator sequencing kit (Applied Biosystems).

The newly gained *cbbL* and *cbbM* nucleotide sequences were analysed using the ARB software package (Ludwig *et al.*, 2004). Closest relatives to *cbbL* and *cbbM* nucleotide sequences were obtained using NCBI's sequence similarity search tool BLASTN. Phylogenetic analyses based on nucleotide sequences were performed by applying maximum likelihood, neighbour joining and maximum parsimony methods by use of the respective tools in the ARB software. Alignments were checked visually. 16S rRNA gene sequence phylogenetic analyses were performed as described above.

4.2.5 Enrichment cultures - Isolation and cultivation of new bacterial strains

For the enrichment and isolation of chemolithoautotrophic bacteria from various depths of the aquifer, enrichment cultures were started under different autotrophic conditions. 10 g of wet sediment was filled into 120 mL serum bottles containing about 50 mL diluted Widdel freshwater medium (containing 2.52 g L^{-1} sodium-hydrocarbonate, 100 mg L^{-1} NaCl, 50 mg L^{-1} potassium chloride, 40 mg L^{-1} magnesium-dichloride hexahydrate, 25 mg L^{-1} ammonium chloride, 20 mg L^{-1} potassium-dihydrophosphate, 15 mg L^{-1} calcium-dichloride dihydrate, trace elements, and vitamins) (dilution 1:10; pH 7.3, anoxic; Table 4.3) (Widdel and Bak, 1992). The gasphase was replaced by N_2/CO_2 (80:20 v/v) and bottles were sealed with butyl stoppers (Ochs). The redox indicator Resazurin (1 mg L^{-1}; Sigma-Aldrich) was added to

confirm anoxic conditions. Different combinations of electron donors (S^{2-}, H_2) and electron acceptors (NO_3^-, Ferrihydrate, SO_4^{2-}) were set up (Table 4.4). The individual solutions deriving from sterile aqueous stocks were directly injected with a syringe through the butyl stopper. Primary enrichment cultures were incubated at 16°C in the dark for 3-4 months and transferred to fresh medium when visibly turbid. For purification of the most abundant cell types in enrichment cultures two dilution series (dilutions up to 10^{-11}) were performed under the same culturing conditions. After purification the cultures were transferred every 2-3 weeks. Cell growth was monitored measuring optical density at 580 nm (OD_{580}; Varian) and regular microscopic observations.

Table 4.3: Composition of the Widdel freshwater medium (Widdel and Bak, 1992)

Components	**Concentrations (mg L^{-1})**	
	dilution 1:10	dilution 1:2
NaCl	100	500
$MgCl_2$ * 6 H_2O	40	200
KH_2PO_4	20	100
NH_4Cl	25	125
KCl	50	250
$CaCl_2$ * $2H_2O$	15	75

Table 4.4: Conditions setup for the enrichment of chemolithoautotrophic bacteria from the Scheyern aquifer

Microbial process	**Electron donor**		**Electron acceptor**		**Carbon source**	**Temperature**
Anaerobic sulfide oxidation	S^{2-}	5 mM	NO_3^-	10 mM	CO_2	16°C
Anaerobic iron reduction	H_2	~ 0.5 bar	FH*	40 mM	CO_2	16°C
Denitrification	H_2	~ 0.5 bar	NO_3^-	10 mM	CO_2	16°C
Sulfate reduction	H_2	~ 0.5 bar	SO_4^{2-}	10 mM	CO_2	16°C

* FH = Ferrihydrate

4.3 Results

4.3.1 Amplification of *cbbL* and *cbbM* genes from aquifer sediment samples

CbbM genes were detectable in 10 out of 18 investigated depths, mostly showing a strong PCR signal (Table 4.5).

Table 4.5: Detection of the *cbbL* and *cbbM* genes

Depth*	*cbbM*	*cbbL*G	*cbbL*R
3.0	(+)	-	-
5.0	+	-	(+)
5.9	+	(+)	(+)
7.0	+	(+)	+
9.7	+	-	-
10.6	-	-	(+)
12.3	-	-	-
13.6	(+)	-	-
15.6	-	-	-
17.6	-	-	-
19.6	(+)	-	-
20.6	+	-	-
21.6	-	-	-
24.6	+	-	+
25.6	-	-	-
27.6	-	-	-
28.6	-	-	-
29.6	+	-	-

* m below soil surface; + PCR product; (+) very weak PCR product, not confirmed by cloning and sequencing; - no PCR product

Green-like *cbbL* genes on the other hand could only be detected at 5.9 and 7.0 m, and here the PCR products revealed a weak signal. Red-like *cbbL* genes were detectable at 5.0, 5.9, 7.0, 10.6 and 24.6 m, whereas a strong PCR product could only be retrieved with DNA extracts from samples of 7.0 and 24.6 m below soil horizon (bsh). Changes in the PCR conditions for optimization did not prove successful.

4.3.2 *CbbM* clone libraries and sequences

The *cbbM* clone libraries constructed from three different depths *i.e.* Sy5, Sy7 and Sy24 revealed 88 out of 136 clones had inserted a right sized fragment (64.7%). In total 47 *cbbM* clones from the different samples were analysed. The sequences were named with “Sy” for Scheyern, followed by the depth designation, with an added “M” for sequences of the *cbbM*

library and the clone number; sequence similarities of all sequenced clones ranged between 82.6-100%.

In clone library Sy5M only 10 clones had inserted correctly sized fragments. In this case all clones were sequenced. Eight of the sequences built a cluster with similarities between 99.4-100%, being closely related to *Thiobacillus thiophilus* D24TN (sequence relatedness 99.4-99.8%) (Fig. 4.3). Two of the sequences exhibited a different relatedness. Sy5M02 showed a sequence similarity of 86.5% to the *cbbM* sequence of *Thiobacillus thioparus* DSM 505 and Sy5M10 had an only 88.9% sequence relatedness to the *cbbM* sequence of *Thiobacillus thiophilu*s D24TN. The second *cbbM* clone library (Sy7M) consisted of 45 clones, with 20 clones having right sized inserts. All clones were sequenced. The sequences showed a relatedness of 98.8-100%, all belonging to the same cluster as the clone sequences of Sy24M and Sy5M (Fig. 4.3).

Clone library Sy24M comprised of 74 clones, 60 clones proved to have inserted a right sized fragment. For RFLP analysis the inserts were restricted with the restriction enzymes *Rsa*I (Fermentas) and *Msp*I (Fermentas). Six different RFLP patterns were found. Of each RFLP pattern at least one clone was sequenced, 2-3 clones of *cbbL* sequences with RFLP patterns of high abundance In total, 19 clones were sequenced and the sequences showed a very high sequence similarity (99.4-100%). All sequences belonged to the same cluster as the sequences of the library Sy5M (Fig. 4.3).

4.3.3 Red-like *cbbL* clone libraries and sequences

All 28 clones of the clone library Sy7rl having right sized (about 820 bp) inserts were sequenced. Sequence similarities ranged between 74.6-100%. The sequences formed two distinct clusters, one sequence was located somewhere else in the phylogenetic tree, with 81.2% sequence relatedness to *Xanthobacter sp.* COX (AY422057) (Fig. 4.4). Sequences of cluster I showed sequence similarity between 83.5-85.0% to the red-like *cbbL* sequence of *Nitrosospira sp.* (AF426419), while sequences of cluster II appeared to be quite isolated, containing only *cbbL*R sequences of the investigated aquifer sediment samples. The sequences of cluster II were most closely related (81.1-84.7%) to the red-like *cbbL* sequence of *Rhodopseudomonas palustris* (AF355196) (Fig. 4.3).

In case of the green-like *cbbL* clones, the obtained PCR products were in comparison with the primer dimers too weak to use them for the building of clone libraries.

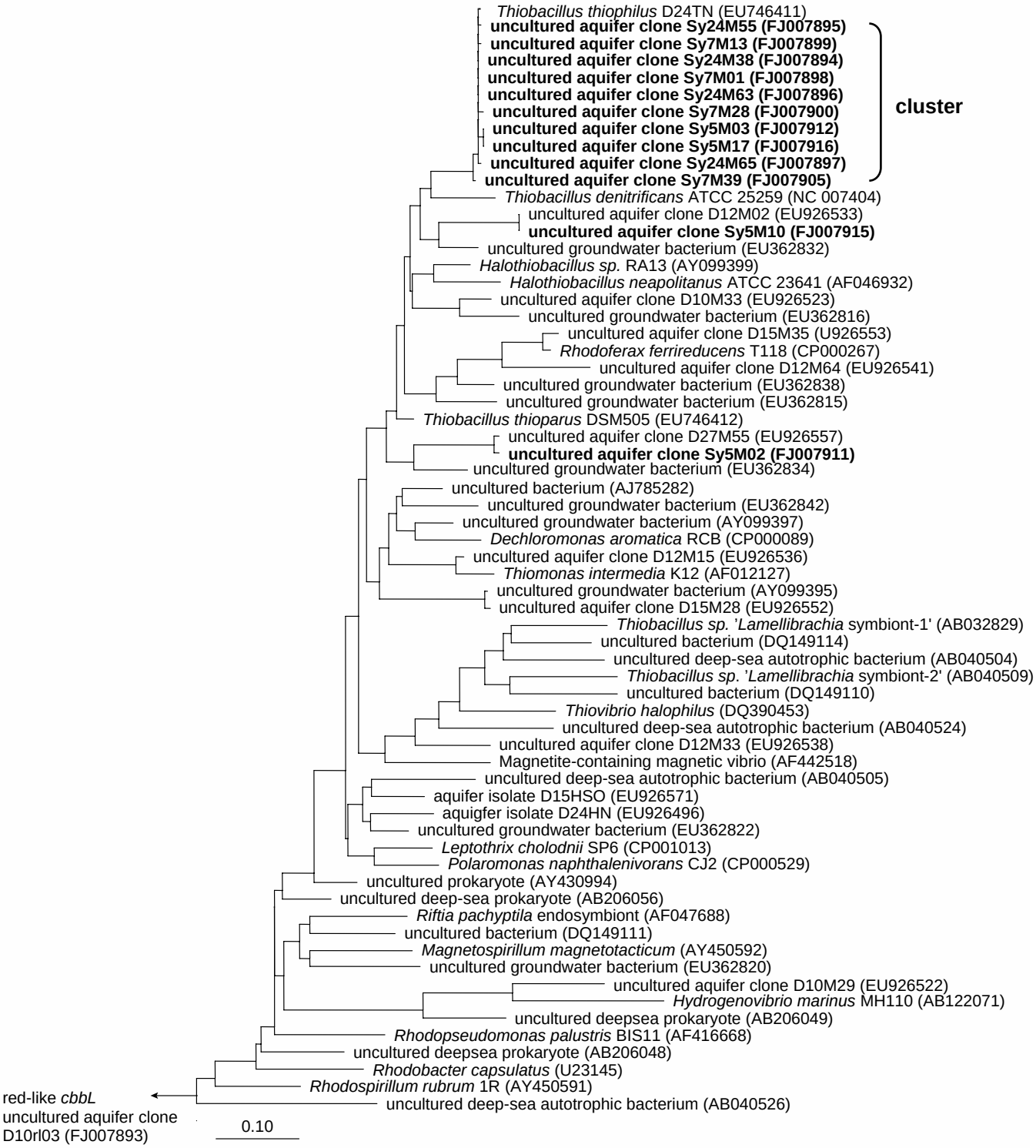

Figure 4.3: Phylogenetic tree of *cbbM* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the red-like *cbbL* sequence of an uncultured aquifer clone (FJ007893) was used. All environmental clone sequences are shown in bold. Scale bar represents 0.10 changes per nucleotide position.

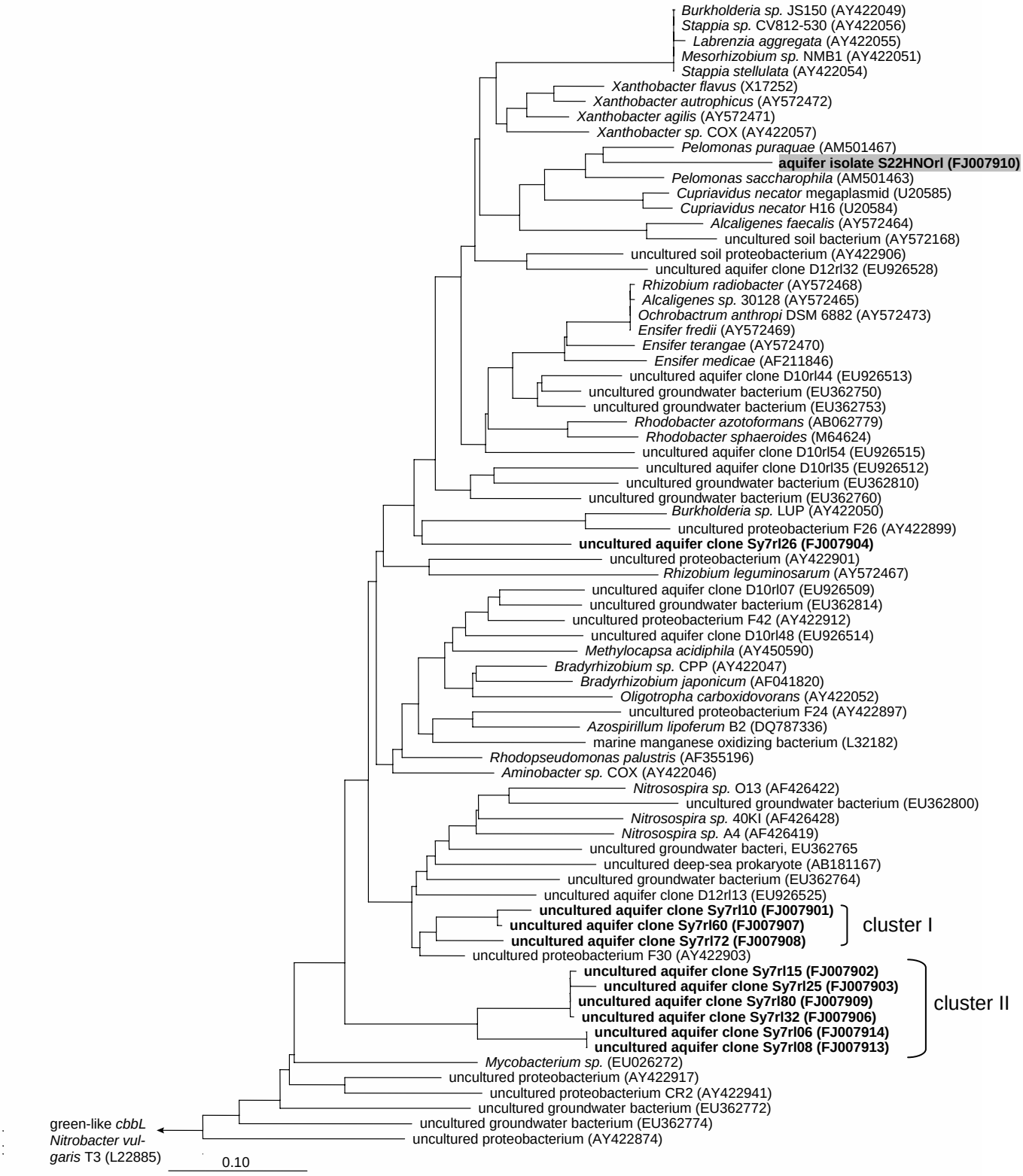

Figure 4.4: Phylogenetic tree of red-like *cbbL* genes. A consensus tree was constructed by neighbour-joining, maximum parsimony and maximum likelihood methods. As outgroup for tree calculations the green-like *cbbL* sequence of *Nitrobacter vulgaris* (L22885) was used. All environmental clone sequences are shown in bold; the sequence of isolate Sy22HNO is shown in bold and shaded in gray. Scale bar represents 0.10 changes per nucleotide position.

4.3.4 Enrichment cultures

The first enrichment culture obtained originated from 22.6 m depth and successfully grew under denitrifying conditions with hydrogen as electron donor (Table 4.4). It became visibly turbid already after 10 days. After three dilution series with dilutions up to 10^{-11} the most abundant cell type was isolated (Sy22HNO). Cells were small rods, 1.7-2.0 µm in length and 1.0–1.3 µm in diameter. The isolate Sy22HNO proved to be a facultative anaerobic and facultative chemolithoautotrophic organism. 16S rRNA sequence of the strain was most closely related to the genus *Acidovorax* with 99.2% sequence similarity to *Acidovorax defluvii* (Fig. 4.5). The isolate also proved positive for the red-like *cbbL* gene with 85.3% sequence similarity to the sequence of *Pelomonas puraquae* (AM501467) (Fig. 4.4).

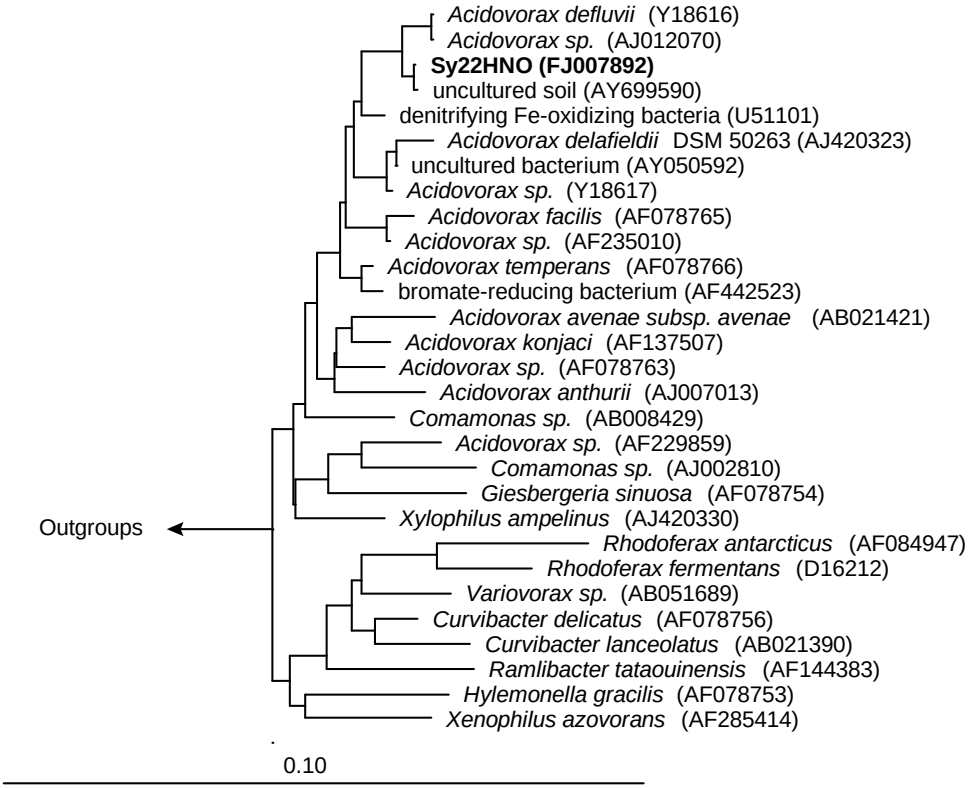

Figure 4.5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain Sy22HNO and some other related taxa. The bar indicates 10% estimated sequence divergence.

Eight other enrichment cultures showed visible growth after four months of incubation. Enrichments were obtained under all tested chemolithoautotrophic conditions with one exception. Under anaerobic sulfide oxidizing conditions no growth in any of the incubations (Table 4.5) occurred.

Table 4.5: Results enrichment cultures

Depth*	Anaerobic sulfide oxidation	Anaerobic iron reduction	Denitrification	Sulfate reduction
5	-	+	-	-
12	-	-	+	+
17	-	+	+	-
22	-	-	+	+
29	-	-	+	+

* m below soil surface; + growth; - no growth

Although the experience from the past showed that bacteria from the subsurface are more successfully enriched in low ionic strength medium, it became evident during the incubations that several cultures initially growing well slowed down or even stopped growing with increased number of transfers. An increase in the mineral concentration (Widdel freshwater medium dilution 1:2 instead of 1:10; same electron donor and electron acceptor concentrations; Table 4.3) could re-induce growth in some of these enrichments.

4.4 Discussion

Although subsurface systems for a long time have been considered sterile and later exclusively heterotrophic, there is growing evidence for the presence and importance of individual chemolithoautotrophic processes in aquifers. The potential of CO_2 fixation in natural microbial communities was investigated at an agriculturally influenced site – the Test Field Scheyern. Several bacterial cultures under different chemolithoautotrophic conditions were enriched and evidence for autotrophic metabolism was obtained through specific PCR-amplification of the marker genes and sequence analysis.

4.4.1 Clone libraries and phylogenetic analysis

With respect to cultivable relatives, the *cbbM* sequences of all retrieved clones were most closely related to the *cbbM* sequences of two *Thiobacillus* species: *Thiobacillus thioparus* DSM 505 (EU746412) and *Thiobacillus thiophilus* D24TN (EU746411) (sequence similarity 86.5-99.8%). Closest related environmental sequences belonged to clones of a tar oil contaminated aquifer with sequence relatedness between 88.9-100%. To our knowledge this is the first study looking for CO_2 fixation potential via the Calvin cycle not in an organically polluted but in an aquifer influenced by agriculture and increased nitrate concentrations. Studies in organically contaminated groundwater ecosystems already retrieved many *Thiobacillus* related sequences (Alfreider *et al.*, 2003; Chapter 3). Those results might be a hint that representatives of the genus *Thiobacillus* are important in those systems, especially since not only sequences closely related to *Thiobacillus* were detected, but also *Thiobacillus* species were isolated out of those and other aquifers (Vlasceanu *et al.*, 1997; Alfreider *et al.*, 2003; Chapter 3). In all three studies the same primer combination was used. The primers have been constructed using *cbbM* sequences of *Thiobacillus denitrificans*, *Thiobacillus intermedius*, *Halothiobacillus intermedius* and several closely related *cbbM* gene sequences more (Alfreider *et al.*, 2003). This may lead to the conclusion that those primers might be selective for *Thiobacillus* sequences and fail to detect more distant related *cbbM* sequences. All retrieved red-like *cbbL* sequences build two clusters with one exception; sequence Sy07rl26 was located somewhere else in the phylogenetic tree (Figure 4.3). The environmental red-like *cbbL* sequence (AY422903) most closely related to all Sy7rl clone sequences originated from a 300 year old forest soil volcanic deposit. Looking at cultured organisms, the Scheyern clone sequences showed the highest relatedness to the sequences of *Xanthobacter sp.* COX (AY422057), *Rhodopseudomonas palustris* (AF355196) and *Bradyrhizobium sp.* (AY422047). Interestingly, in another study conducted in the same area dealing with the molecular diversity of the *cbbL* genes in soil, also red-like *cbbL* sequences

related to the *cbbL* sequences of *Xanthobacter* and *Bradyrhizobium* species have been found (Pattis, 2002). Species of those genera have been isolated from waste-water, soil, sludge and plant roots. *Xanthobacter* and *Bradyrhizobium* species have also been detected in contaminated groundwater (Connon *et al.*, 2005). However, due to the incongruity of the *cbbL* phylogeny with the phylogeny based on 16S rRNA genes (Delwiche and Palmer, 1996), those sequences may belong to those organisms and their relatives, but it is also possible, that they belong to microorganisms of completely different genera (Selesi *et al.*, 2005). Interestingly, none of the detected red-like *cbbL* sequences showed any close phylogenetic relatedness to the sequence of the isolate Sy22HNO or its relatives. A possible explanation could be that Sy22HNO and its relatives are, compared to other microorganisms carrying the *cbb* genes, only present in low numbers and therefore not represented in the clone library. Alternatively, their *cbb* sequences are not at all or only to a little extent amplified during PCR, possibly due to biases of the PCR-based techniques as already illustrated earlier (Chapter 3).

4.4.2 Enrichment cultures

Nine bacterial cultures could successfully be enriched. With an exception in the uppermost sediment depth, denitrifying cultures were obtained from the Scheyern aquifer. The successful enrichment of cultures under denitrifying conditions is in agreement with results of Schwientek (2008) who found denitrification to be an important redox process in this aquifer, which he proved by chemical and isotopic data (Fig. 4.2 and Fig. 4.6).

The local depletion of nitrate in the depths between 9 m to 14 m, around 19 m and below 27 m was characterized by elevated $\delta^{15}N$ values of NO_3^-, indicating denitrification in the groundwater at those depths (Schwientek, 2008). However, also sediments from zones with less isotopic evidence and plenty of nitrate harboured denitrifiers which could be enriched. According to phylogenetic analysis of the 16S rRNA gene the closest relative of the isolate Sy22HNO was *Acidovorax defluvii*, a denitrifyer isolated from wastewater treatment plant sludge; several other species of this genus have been detected already in contaminated groundwater ecosystems and wastewater (Connon *et al.*, 2005; Fan *et al.*, 2008; Heylen *et al.*, 2008). Interestingly, sulfate reducers could be obtained from the same depths as the denitrifiers with an exception with 17 m sediment (Table 4.5), although here isotopic sign for sulfate reduction was obtained (Fig. 4.6). Eleven out of 20 enrichment cultures did not show any growth or stopped growing after several inoculations. Even though special enrichment media adjusted to environmental conditions (Widdel and Bak, 1992; Bartscht *et al.*, 1999) have been used. It is well known that most viable bacteria are still not cultivable (Boivin-Jahns *et al.*, 1996).

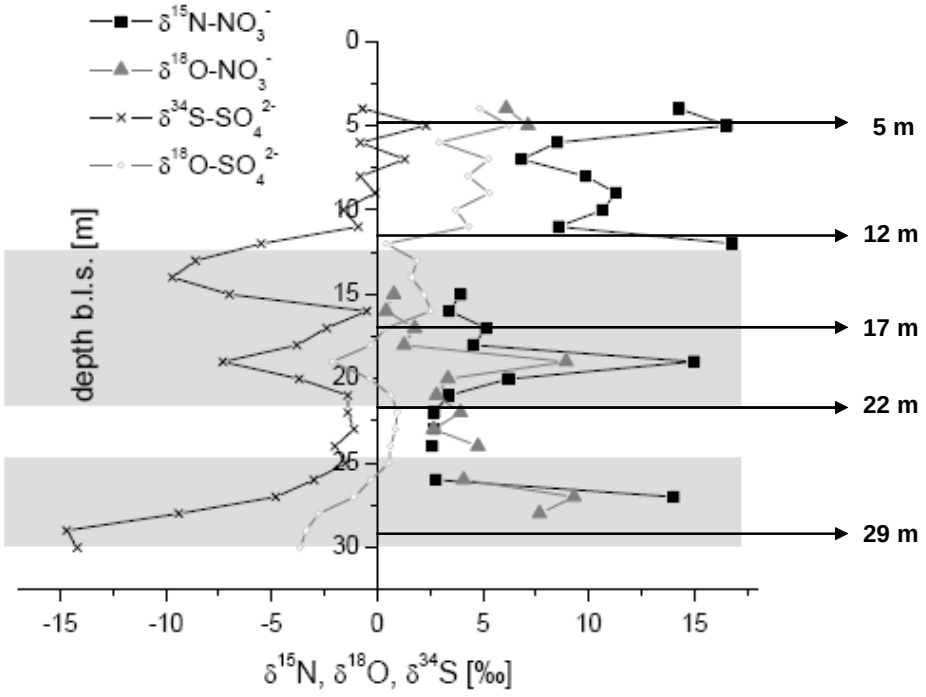

Figure 4.6: Vertical profiles of $\delta^{15}N$ and $\delta^{18}O$ of nitrate and $\delta^{34}S$ and $\delta^{18}O$ of sulfate in groundwater at ML II. Arrows indicate the depths where sediment was sampled for enrichment cultures The shaded bars represent lenses of silt and clay (modified from Schwientek, 2008)

Estimations reveal that so far less than 10% of the microorganisms in pristine groundwater can be cultivated under laboratory conditions (Goldscheider *et al.*, 2006). Some of the cultures which stopped growth could be reanimated by increasing the mediums ionic strength (Widdel freshwater medium dilution 1:2 instead of 1:10; Table 4.3). While the low salt concentration at the beginning is complemented by the presence of the sediment inoculum, this is lost during repeated transfers. Another possible explanation for cultures stopping growth could be syntrophic interactions in groundwater communities, leaving the single bacterial strains unable to survive due to the lack of syntrophic partners.

4.4.3 Chemolithotrophy and CO_2 fixation in the Scheyern aquifer

Dissolved organic carbon (DOC) is a typical electron donor and carbon source for heterotrophic microorganisms. In contrast, chemolithoautotrophic microorganisms depend on sufficient amounts of inorganic carbon and electron donors in form of reduced inorganic compounds. Most uncontaminated groundwater ecosystems are oligotrophic, most organic carbon compounds have already been oxidized in the unsaturated zone or along the groundwater flow path. The mean DOC concentration in such oligotrophic ecosystems lies between 0.2 and 2.0 mg L^{-1} (Griebler and Mösslacher, 2003). The investigated aquifer at TFS is also oligotrophic with DOC concentrations generally below 1 mg L^{-1}. Only between 4 and 8

m depth DOC-concentrations are reaching up to 3.9 mg L^{-1} (Fig. 4.2). The aquifer is rich in carbonate and cation concentrations are fairly constant ranging around 150 mg L^{-1} for calcium, 50 mg L^{-1} for magnesium, 7.5 mg L^{-1} for sodium, and 1.5 mg L^{-1} for potassium. Electron acceptors such as oxygen, nitrate or sulfate attain to the system from the surface either naturally or by anthropogenic application. Organic and inorganic electron donors are available in the aquifer in form of TOC (total organic carbon), pyrite and ferrous iron, respectively, relatively enriched within the lenses of clay and silt (Schwientek, 2008). Altogether all requirements for autotrophic processes are given and according to chemical and isotopic analysis performed by Schwientek (2008), denitrification occurs at three separate sections (between 9 m and 14 m, around 19 m and between 27 m and 30 m) in the aquifer with pyrite as electron donor, producing sulfate. Those findings fit quite well to the results of this study concerning the detection of CO_2 fixation potential via the Calvin cycle. *CbbM* genes have not only been detected in the unsaturated zone but also at 9.7 m, 13.6 m, 19.6 m, 20.6 m, 24.6 m and 29.6 m depth, almost exactly the depths in which the occurrence of denitrification was proven (Schwientek, 2008). Most obtained *cbbM* sequences were closely related to the *cbbM* sequences found in representatives of the genus *Thiobacillus*. Some known representatives of this genus are obligate chemolithoautotrophic denitrifiers using various inorganic compounds as electron donors *e.g.* hydrogen sulfide, thiosulfate or pyrite. The detection of red-like *cbbL* sequences in at least some sediment samples is a hint that besides chemolithoautotrophic denitrification other autotrophic processes might go on in the aquifer. The successful enrichment cultures under various chemolithoautotrophic conditions are a further indication for the presence of autotrophs in this aquifer. Interestingly, all enrichment cultures under denitrifying conditions were successful, with the enrichment culture deriving from sediment from 5.0 m being the only exception. Enrichments under sulfate-reducing conditions were successful with sediment deriving from 12.0 m, 22.0 m and 29.0 m depth. In those depths sulfate is produced due to the denitrification using pyrite as electron donor.

In summary, the data presented by Schwientek (2008) in combination with the detection of CO_2 fixation potential via the Calvin cycle and the successful chemolithoautotrophic enrichment cultures in this study are a strong hint that autotrophic processes might be abundant and important in this oligotrophic aquifer. An important future task will be the proof of chemolithoautotrophic activities *in situ*.

4.5 References

Alfreider, A., Krossbacher, M., and Psenner, R. (1997) Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Res* **31**: 832-840.

Alfreider, A., Vogt, C., Hoffmann, D., and Babel, W. (2003) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecol* **45**: 317-328.

Bartscht, K., Cypionka, H., and Overmann, J. (1999) Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. *FEMS Microbiol Ecol* **28**: 249-259.

Bassham, J.A., and Calvin, M. (1957) *The path of carbon in photosynthesis* Englewood Cliffs: Prentice-Hall Inc.

Beloin, R.M., Sinclair, J.L., and Ghiorse, W.C. (1988) Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. *Microb Ecol* **16**: 85-97.

Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in *Archaea*. *Science* **318**: 1782 - 1786.

Boivin-Jahns, V., Ruimy, R., Bianchi, A., Daumas, S., and Christen, R. (1996) Bacterial diversity in a deep-subsurface clay environment. *Appl Environ Microbiol* **62**: 3405 - 3412.

Brockman, F.J., and Murray, C.J. (1997) Subsurface microbiological heterogeneity: current knowledge, descriptive approaches and applications. *FEMS Microbiol Rev* **20**: 231-247.

Chandler, D.P., Wagnon, C.A., and Bolton, H. (1998) Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. *Appl Environ Microbiol* **64**: 669-677.

Chapelle, F.H., and Lovley, D.R. (1990) Rates of microbial metabolism in deep coastal plain aquifers. *Appl Environ Microbiol* **56**: 1865-1874.

Connon, S.A., Tovanabootr, A., Dolan, M., Vergin, K., Giovannoni, S.J., and Semprini, L. (2005) Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater. *Environ Microbiol* **7**: 165-178.

Delwiche, C.F., and Palmer, J.D. (1996) Rampant horizontal transfer and duplication of RubisCO genes in eubacteria and plastids. *Mol Biol Evol* **13**: 873-882.

Ehrlich, H.L. (1998) Geomicrobiology: its significance for geology. *Earth Sci Rev* **45**: 45-60.

Evans, M.C., Buchanan, B.B., and Arnon, D.I. (1966) A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. *P Natl Acad Sci USA* **55**: 928-934.

Fan, H., Su, C., Wang, Y., Yao, J., Zhao, K., Wang, Y. *et al.* (2008) Sedimentary arsenite-oxidizing and arsenate-reducing bacteria associated with high arsenic groundwater from Shanyin, Northwestern China. *J Appl Microbiol* **105**: 529 - 539.

Fields, M.W., Yan, T.F., Rhee, S.K., Carroll, S.L., Jardine, P.M., Watson, D.B. *et al.* (2005) Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid-uranium waste. *FEMS Microbiol Ecol* **53**: 417-428.

Fliermans, C.B., and Balkwill, D. (1989) Microbial life in deep terrestrial subsurfaces. *BioScience* **39**: 370-377.

Fredrickson, J.K., and Onstott, T.C. (1996) Microbes deep inside the earth. *Sci Am* **275**: 68-73.

Fredrickson, J.K., Zachara, J.M., Balkwill, D.L., Kennedy, D., Li, S.M.W., Kostandarithes, H.M. *et al.* (2004) Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford Site, Washington State. *Appl Environ Microbiol* **70**: 4230-4241.

Fuchs, G., Lange, S., Rude, E., Schaefer, S., Schauder, R., Scholtz, R., and Stupperich, E. (1987) Autotrophic CO_2 fixation in chemotrophic anaerobic bacteria. In *Microbial growth on C1 compounds*. Van Versefeld, H.W., and Duine, J.A. (eds). Dordrecht, pp. 39-43.

Gabor, E., de Vries, E., and Janssen, D. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiol Ecol* **4**: 153-163.

Ghiorse, W.C. (1997) Subterranean life. *Science* **275**: 789-790.

Goldscheider, N., Hunkeler, D., and Rossi, P. (2006) Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeol J* **14**: 926-941.

Griebler, C., and Mösslacher, F. (2003) *Grundwasser-Ökologie*. Wien: Facultas Universitätsverlag.

Heylen, K., Lebbe, L., and De Vos, P. (2008) *Acidovorax caeni* sp nov., a denitrifying species with genetically diverse isolates from activated sludge. *Int J Syst Evol Micr* **58**: 73-77.

Holo, H. (1989) *Chloroflexus aurantiacus* secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO_2 and acetate. *Arch Microbiol* **151**: 252-256.

Huber, H., Gallenberger, M., Jahn, U., Eylert, E., Berg, I.A., Kockelkorn, D. *et al.* (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum *Ignicoccus hospitalis*. *Proc Natl Acad Sci USA* **105**: 7851-7856.

Jones, R., Beeman, R., and Suflita, J. (1989) Anaerobic metabolic processes in the deep terrestrial subsurface. *Geomicrobiol J* **7**: 117-130.

Kellog, E.A., and Juliano, N.D. (1997) The structure and function of RuBisCO and their implications for systematic studies. *Am J Bot* **84**: 413-428.

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005) Impact of different *in vitro* electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ Microbiol* **71**: 6664-6672.

Lovley, D.R., and Chapelle, F.H. (1995) Deep subsurface microbial processes. *J Geophys* **33**: 365-381.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Lueders, T., Manefield, M., and Friedrich, M.W. (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73-78.

Nazina, T.N., Kosareva, I.M., Petrunyaka, V.V., Savushkina, M.K., Kudriavtsev, E.G., Lebedev, V.A. *et al.* (2004) Microbiology of formation waters from the deep repository of liquid radioactive wastes Severnyi. *FEMS Microbiol Ecol* **49**: 97-107.

Nedelkova, M. (2005) Microbial diversity in ground water at the deep-well monitoring site S15 of the radioactive waste depository Tomsk-7, Siberia, Russia. Freiberg: Technische Universität Bergakademie Freiberg, pp. 144

North, N.N., Dollhopf, S.L., Petrie, L., Istok, J.D., Balkwill, D.L., and Kostka, J.E. (2004) Change in bacterial community structure during *in situ* biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Appl Environ Microbiol* **70**: 4911-4920.

Pattis, I. (2002) Einfluß einer H_2-Behandlung auf die Bodenmikroflora und die molekulare Diversität der *cbbL*-(RubisCO) Gene. Diplomarbeit. München: Ludwig-Maximilians-Universität, pp. 109

Pedersen, K. (1997) Microbial life in deep granitic rock. *FEMS Microbiol Rev* **20**: 399-414.

Phelps, T.J., Murphy, E.M., Pfiffner, S., and White, D.C. (1994) Comparison between geochemical and biological estimates of subsurface microbial activity. *Microb Ecol* **28**: 335-349.

Schwientek, M. (2008) Biogeochemical characterization of the groundwater system at the Test Field Scheyern using environmental isotopes. PhD-Thesis. Freiburg: Albert-Ludwigs-Universität, pp. 150

Selesi, D. (2003) Diversität und Abundanz des Ribulose-1,5-bisphosphat Carboxylase/Oxygenase (RubisCO)-Gens *cbbL* autotropher Bakterien in Agrarböden. PhD-Thesis. München: Ludwig-Maximilians-Universität, pp. 140

Selesi, D., Schmid, M., and Hartmann, A. (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71**: 175-184.

Shively, J.M., van Keulen, G., and Meijer, W.G. (1998) Something from almost nothing: Carbon dioxide fixation in chemoautotrophs. *Annu Rev Microbiol* **52**: 191-230.

Stevens, T. (1997) Lithoautotrophy in the subsurface. *FEMS Microbiol Rev* **20**: 327-337.

Tabita, F.R. (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol Rev* **52**: 155–189.

Tabita, F.R., Gibson, J.L., Bowien, B., Dijkhuizen, L., and Meijer, W.G. (1992) Uniform designation for genes of the Calvin-Benson-Bassham reductive pentose phosphate pathway of bacteria. *FEMS Microbiol Lett* **78**: 107-110.

Takai, K., Gamo, T., Tsunogai, U., Nakayama, N., Hirayama, H., Nealson, K.H., and Horikoshi, K. (2004) Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. *Extremophiles* **8**: 269-282.

Tiago, I., Chung, A.P., and Verissimo, A. (2004) Bacterial diversity in a nonsaline alkaline environment: heterotrophic aerobic populations. *Appl Environ Microbiol* **70**: 7378-7387.

Vlasceanu, L., Popa, R., and Kinkle, B.K. (1997) Characterization of *Thiobacillus thioparus* LV43 and its distribution in a chemoautotrophically based groundwater ecosystem. *Appl Environ Microbiol* **63**: 3123-3127.

Watson, G.M.F., and Tabita, F.R. (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A molecule for phylogenetic and enzymological investigation. *FEMS Microbiol Lett* **146**: 13-22.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New York: Springer, pp. 3352-3378.

Wood, H.G., Ragsdale, S.W., and Pezacka, E. (1986) CO_2 fixation into acetyl-CoA: a new pathway of autotrophic growth. *Trends Biochem Sci* **11**: 14-18.

5. General conclusions and outlook

Chemolithoautotrophic bacteria were first described more than 100 years ago by Winogradsky (1890). However, for quite some time they were regarded as unimportant and thus neglected. Nowadays it is well known that autotrophic processes are important in ecosystem ecology and elemental cycles (Labrenz *et al.*, 2005), with the Calvin cycle being the most abundant one of all six known CO_2 fixation pathways so far (Tabita, 1999; Badger and Bek, 2008).

One objective of this thesis was to elucidate if CO_2 fixation potential via the Calvin cycle can be detected in different groundwater ecosystems either organically depleted (Testfield Scheyern, agricultural area) or organically contaminated (Tar oil field Düsseldorf-Flingern, former gasworks site), and whether site- or depth-specific differences can be identified regarding the diversity of this autotrophic potential. Therefore primers specific for the *cbb* genes - genes encoding RubisCO, the key enzyme of the Calvin cycle (Alfreider *et al.*, 2003; Selesi *et al.*, 2005) - were applied. Today only a few studies exist investigating autotrophy in different ecosystem, some of them dealing with the presence of the *cbb* genes (Pedersen, 1997; Stevens, 1997; Alfreider *et al.*, 2003; Selesi *et al.*, 2005; Tolli and King, 2005). To our knowledge, so far only one study exists that deals with the detection of the RubisCO genes in groundwater (Alfreider *et al.*, 2003). In the contaminated aquifer at Testfield Düsseldorf-Flingern the *cbb* genes were found in areas close to the contaminant plume centre as well as in areas with low contaminant concentrations. The diversity of the obtained green-like and red-like *cbbL* sequences was low, whereas the diversity of the *cbbM* sequences was clearly higher. However, the predominant majority of all retrieved *cbb* sequences was closely related to the *cbbL* and *cbbM* sequences belonging to the genus *Thiobacillus*. Additionally, the occurrence of CO_2 fixation potential via the reductive Tricarboxylic Acid cycle (reductive TCA cycle) was investigated at this site. Therefore primers specific for the *acl* genes (genes encoding the ATP citrate lyase, one of the key enzymes of the reductive TCA cycle) were applied (Campbell and Cary, 2004; Hügler *et al.*, 2005; Takai *et al.*, 2005). The detection of those genes was not possible in any of the investigated samples. Even though it might be possible that there are no organisms using the reductive TCA cycle present in the investigated aquifer, it seems more likely that the used primers are too selective, not detecting all different *acl* genes.

At the organically poor site, the Testfield Scheyern, most *cbbM* sequences detected were also closely related to the *cbb* sequences of *Thiobacillus ssp.*, hinting that this genus might be of importance in groundwater ecosystems. This hypothesis is further supported by the study of

Alfreider *et al.* (2003), who detected also *Thiobacillus*-related *cbb* sequences in a contaminated aquifer. Green-like *cbbL* genes could hardly be detected at all. The red-like *cbbL* sequences were most closely related to a sequence originating forest soil volcanic deposit and to sequences belonging to genera of which some species already have been detected in waste-water, soil, sludge, groundwater and plant roots (Connon *et al.*, 2005).

C*bb* sequence clustering related to sediment depth could not be observed in the contaminated, or in the pristine aquifer. Interestingly, even though the *cbbM* sequences of the two investigated aquifers were quite similar, only few identical sequences could be found in both, indicating diverse and differing autotrophic communities.

Of course detection of CO_2 fixation potential is not a direct hint to autotrophic activity *in situ*. In groundwater ecosystems the main problems concerning activity measurements are the relatively low cell numbers and slow metabolic rates. However, to prove that actual autotrophic activity is taking place in those systems a selected set of methods was applied to certain subsets of the samples. Fluorescence *in situ* hybridization combined with microautoradiography (FISH-MAR) was applied on water and sediment samples deriving from the contaminated aquifer. Even though good fluorescent signals were retrieved, indicating that the groundwater bacteria were active, radiolabelled cells were not detected. Besides the possible reason that no CO_2 fixing bacteria were present in the samples, other possible reasons could be the insufficient concentration of ^{14}C-labelled bicarbonate applied, unfavourable conditions during incubation of samples in the lab or an overall low CO_2 fixation activity. Another approach tested to detect CO_2 fixation was the analysis of phospholipids fatty acids (PLFAs) after the incubation of sediment together with ^{13}C-labelled $CaCO_3$ in the contaminated aquifer. The PLFA approach is generally well suited for estimating the amount of active microbial biomass and analyzing the composition of microbial communities in environmental samples (Ben-David *et al.*, 2004). In the samples seven saturated fatty acids and three monounsaturated fatty acids could be detected. Those PLFAs are indicative for gram-positive bacteria as well as for sulfate-reducing gram-negative bacteria. Chemolithoautotrophs are distributed in both of these groups (Aggag and Schlegel, 1973; Moussard *et al.*, 2004; Zavarzina *et al.*, 2007). ^{13}C-carbon incorporation from ^{13}C-$CaCO_3$ was measured in both detected 16-C fatty acids, which is a possible hint for bacterial CO_2-uptake in the aquifer. Of course, it has to be kept in mind that also heterotrophic microorganisms are performing CO_2 fixation to some extent which may not easily be distinguished from autotrophic CO_2 fixation.

Besides the cultivation-independent approach by molecular methods the enrichment of microbes is a useful tool to get an idea about the presence of autotrophic organisms.

Enrichment attempts with sediment material from both investigated sites started under obligate chemolithoautotrophic conditions were partly successful. Some of the cultures obtained showed good and fast growth, in some cases cultures started growing again after being transferred to a medium with higher salt concentrations, others never showed any growth or stopped growing after some time. This might be due to insufficient media, but it has to be considered, that only little information about the living of environmental communities is available so far. Interestingly, two pure cultures isolated from the contaminated aquifer proved to belong to the genus *Thiobacillus*, a result further supporting the theory that *Thiobacilli* might be important in groundwater ecosystems. This hypothesis is further supported by the fact that *Thiobacilli* are widely distributed microorganisms, also being represented in groundwater ecosystems. The best known representative of this genus is *T. denitrificans*, obligate chemolithoautotrophic and able to couple the oxidation of inorganic sulfur compounds and the anaerobic oxidation of Fe(II) to denitrification. For example from an environmental perspective, this means that this microorganism can be useful for bioremediation of the major groundwater contaminant nitrate. Nitrate contamination of groundwater is a pervasive and high-priority concern in rural and urban areas throughout the industrialised world (Beller *et al.*, 2006). One of the isolates was further characterized, it was described as new *Thiobacillus* species, *Thiobacillus thiophilus* D24TN^T sp. nov., an obligate chemolithoautotrophic strain, able to grow under oxic and anoxic conditions.

One aim of this thesis was to detect CO_2 fixation potential in pristine and contaminated aquifers. The presented data prove that the potential for CO_2 fixation via the Calvin cycle can be detected in contaminated as well as in pristine aquifers. Potential for CO_2 fixation via the reductive TCA cycle could not be found, but primer modifications or new primer design might make it possible to detect a higher diversity of *acl* sequences. Even though the Calvin cycle might be the most abundant CO_2 fixation pathway worldwide, the occurrence and distribution of the other pathways should not be neglected. Unfortunately, only limited information about these pathways and the organisms using them is available and so far no primer sets exist to detect the functional genes encoding their key enzymes.

The second aim was to connect CO_2 fixation potential with actual autotrophic activity *in situ*. To build this connection proved complicated, autotrophic activity measurements are difficult due to low cell abundance and activity. PLFA analysis gave a hint about possible CO_2 incorporation, via FISH-MAR the general activity of groundwater microorganisms could be shown to a certain extent, detection of CO_2 fixation was not possible. Successful enrichment cultures under various chemolithoautotrophic conditions proved the occurrence of autotrophic

microorganisms in the investigated aquifers, giving a first hint about the possible diversity of the autotrophic bacterial communities in the investigated aquifers.

To gain further insights into the role of autotrophic microorganisms in groundwater ecosystems, more groundwater ecosystems have to be analysed. For future studies not only the occurrence of the other CO_2 fixation pathways should be investigated, also new and more sensitive methods for *in situ* autotrophic activity measurements in those systems are needed to be developed.

References

Aggag, M., and Schlegel, H.G. (1973) Studies on a gram-positive hydrogen bacterium, *Nocardia opaca* strain 1b. *Arch Microbiol* **88**: 299-318.

Alfreider, A., Vogt, C., Hoffmann, D., and Babel, W. (2003) Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecol* **45**: 317-328.

Badger, M.R., and Bek, E.J. (2008) Multiple RubisCO forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J Exp Bot* **59**: 1525-1541.

Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M. *et al.* (2006) The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. *J Bacteriol* **188**: 1473-1488.

Ben-David, E.A., Holden, P.J., Stone, D.J.M., Harch, B.D., and Foster, L.J. (2004) The use of phospholipid fatty acid analysis to measure impact of acid rock drainage on microbial communities in sediments. *Microb Ecol* **48**: 300-315.

Campbell, B.J., and Cary, S.C. (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. *Appl Environ Microbiol* **70**: 6282-6289.

Connon, S.A., Tovanabootr, A., Dolan, M., Vergin, K., Giovannoni, S.J., and Semprini, L. (2005) Bacterial community composition determined by culture-independent and -dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater. *Environ Microbiol* **7**: 165-178.

Hügler, M., Wirsen, C.O., Fuchs, G., Taylor, C.D., and Sievert, S.M. (2005) Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria. *J Bacteriol* **187**: 3020-3027.

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005) Impact of different *in vitro* electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ Microbiol* **71**: 6664-6672.

Moussard, H., L'Haridon, S., Tindall, B.J., Banta, A., Schumann, P., Stackebrandt, E. *et al.* (2004) *Thermodesulfatator indicus* gen. nov., sp nov., a novel thermophilic chemolithoautotrophic sulfate-reducing bacterium isolated from the Central Indian Ridge. *Int J Syst Evol Microbiol* **54**: 227-233.

Pedersen, K. (1997) Microbial life in deep granitic rock. *FEMS Microbiol Rev* **20**: 399-414.

Selesi, D., Schmid, M., and Hartmann, A. (2005) Diversity of green-like and red-like ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes (*cbbL*) in differently managed agricultural soils. *Appl Environ Microbiol* **71**: 175-184.

Stevens, T. (1997) Lithoautotrophy in the subsurface. *FEMS Microbiol Rev* **20**: 327-337.

Tabita, F.R. (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective. *Photosynthesis Research* **60**: 1-28.

Takai, K., Campbell, B.J., Cary, S.C., Suzuki, M., Oida, H., Nunoura, T. *et al.* (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of Epsilonproteobacteria. *Appl Environ Microbiol* **71**: 7310-7320.

Tolli, J., and King, G.M. (2005) Diversity and structure of bacterial chemolithotrophic communities in pine forest and agroecosystem soils. *Appl Environ Microbiol* **71**: 8411-8418.

Winogradsky, S. (1890) Recherches sur les organismes de la nitrification. *Annales de l'Institute Pasteur* **4**: 213-223.

Zavarzina, D.G., Sokolova, T.G., Tourova, T.P., Chernyh, N.A., Kostrikina, N.A., and Bonch-Osmolovskaya, E.A. (2007) *Thermincola ferriacetica* sp. nov., a new anaerobic, thermophilic, facultatively chemolithoautotrophic bacterium capable of dissimilatory Fe(III) reduction. *Extremophiles* **11**: 1-7.

Appendix

Table A.1: Growth rates of D24TN^T under a. oxic and b. anoxic conditions as shown in **Fig. 2.2;** Changes in optical density measured at wave length 580 nm. Cells counts performed via microscopy

a.

Time [h]	OD_{580nm}	Cells ml^{-1}	log cells ml^{-1}
0	0.101	$1.1*10^7$	7.06
24	0.118	$4.0*10^7$	7.60
29	0.123	$5.6*10^7$	7.75
53	0.142	$1.0*10^8$	8.01
77	0.156	$1.3*10^8$	8.10
96	0.165	$1.5*10^8$	8.16
168	0.191	$1.9*10^8$	8.27
194	0.206	$2.3*10^8$	8.35

b.

Time [h]	OD_{580nm}	Cells ml^{-1}	log cells ml^{-1}
0	0.077	$2.2*10^6$	6.35
24	0.085	$3.2*10^6$	6.51
48	0.090	$1.1*10^7$	7.05
67	0.099	$2.9*10^7$	7.46
90	0.107	$3.9*10^7$	7.59
116	0.116	$5.0*10^7$	7.70
145	0.123	$6.5*10^7$	7.81
169	0.124	$7.2*10^7$	7.86
188	0.129	$7.4*10^7$	7.87
232	0.145	$1.0*10^8$	8.01
256	0.145	$1.0*10^8$	8.01
328	0.145	$1.0*10^8$	8.01

Table A.2: Growth curves of aerobically a. and anaerobically b. grown cells of strain D24TN^T showing consumption of the electron donor thiosulfate and the electron acceptor nitrate, as well as the reaction products sulfate and nitrite. Cells were grown in a 1:2 diluted WS medium as shown in **Fig. 2.3**

a.

Time [h]	$S_2O_3^{2-}$ [mM]	SO_4^{2-} [mM]	NO_3^- [mM]	NO_2^- [mM]	log cells ml^{-1}
0	8.06	0.72	3.52	0	6.31
53	5.70	3.44	3.28	0	7.64
192	2.61	11.55	1.33	0.89	8.08

b.

Time [h]	$S_2O_3^{2-}$ [mM]	SO_4^{2-} [mM]	NO_3^- [mM]	NO_2^- [mM]	log cells ml^{-1}
0	8.18	0.78	3.66	0	6.32
53	6.20	1.33	2.50	0.46	6.63
192	4.23	6.00	0.54	2.17	7.34

Table A.3: Growth of the thiosulfate oxidizing strain D24TN^T at various pH values. The electron acceptor was oxygen, and the electron donor was thiosulfate as shown in **Fig. 2.4** Changes in optical density, measured at different pH values at wave length 580 nm.

Time [days]	OD_{580nm} at different pH values								
	6.0	**6.3**	**6.5**	**7.0**	**7.5**	**8.0**	**8.3**	**8.72**	**8.94**
0	0.088	0.088	0.085	0.086	0.087	0.088	0.085	0.082	0.087
2	0.088	0.099	0.103	0.097	0.096	0.095	0.093	0.090	0.086
4	0.094	0.110	0.102	0.114	0.121	0.129	0.135	0.117	0.085
7	0.096	0.112	0.115	0.135	0.159	0.204	0.222	0.145	0.086
9	0.093	0.112	0.121	nd*	nd	0.236	nd	0.169	0.083
11	0.086	0.114	0.126	nd	nd	nd	nd	0.190	0.083
14	0.091	0.114	0.130	0.209	0.251	nd	0.295	0.217	0.082
16	0.092	0.116	0.135	0.220	0.272	0.290	0.306	0.244	0.086
18	0.087	0.114	0.137	0.241	0.282	0.302	0.309	0.258	0.086
21	0.086	0.114	0.136	0.254	0.295	0.311	0.310	0.262	0.085

*no data

Table A.4: Growth of the thiosulfate oxidizing strain D24TN^T at various temperatures. The electron acceptor was oxygen, and the electron donor was thiosulfate as shown in **Fig. 2.5** Changes in optical density, measured duplicate incubations at different temperatures at wave length 580 nm.

Temp. [°C]	OD_{580nm} at different points in time [days]								
	0	**2**	**4**	**5**	**7**	**9**	**10**	**13**	**14**
-2	0.102	0.106	0.108	nd*	0.114	0.116	nd	nd	0.120
	0.102	0.104	0.106	nd	0.112	0.118	nd	nd	0.150
mean	**0.102**	**0.105**	**0.107**	**nd**	**0.113**	**0.117**	**nd**	**nd**	**0.135**
0	0.090	0.092	nd	0.096	0.102	0.107	nd	nd	0.133
	0.088	0.094	nd	0.093	0.096	0.102	nd	nd	0.128
mean	**0.089**	**0.093**	**nd**	**0.095**	**0.099**	**0.105**	**nd**	**nd**	**0.131**
4	0.089	0.096	0.104	nd	0.112	0.117	nd	nd	0.134
	0.095	0.102	0.111	nd	0.116	0.128	nd	nd	0.147
mean	**0.092**	**0.099**	**0.108**	**nd**	**0.114**	**0.123**	**nd**	**nd**	**0.141**
16	0.092	0.107	0.130	nd	0.163	0.196	nd	nd	0.236
	0.090	0.105	0.129	nd	0.165	0.203	nd	nd	0.248
mean	**0.091**	**0.106**	**0.130**	**nd**	**0.164**	**0.200**	**nd**	**nd**	**0.242**
24	0.092	0.124	0.169	nd	0.238	0.260	nd	nd	0.306
	0.092	0.116	0.152	nd	0.218	0.256	nd	nd	0.311
mean	**0.092**	**0.120**	**0.161**	**nd**	**0.228**	**0.258**	**nd**	**nd**	**0.309**
30	0.097	0.154	0.213	nd	0.249	0.276	nd	nd	0.308
	0.097	0.136	0.205	nd	0.247	0.278	nd	nd	0.314
mean	**0.097**	**0.145**	**0.209**	**nd**	**0.248**	**0.277**	**nd**	**nd**	**0.311**
33	0.094	0.096	nd	0.097	0.097	nd	0.095	0.095	0.095
	0.096	0.098	nd	0.101	0.096	nd	0.096	0.096	0.096
mean	**0.095**	**0.097**	**nd**	**0.099**	**0.097**	**nd**	**0.096**	**0.096**	**0.096**
35	0.096	0.098	0.098	nd	0.096	nd	0.096	0.095	0.095
	0.095	0.096	0.099	nd	0.094	nd	0.094	0.094	0.094
mean	**0.096**	**0.097**	**0.099**	**nd**	**0.095**	**nd**	**0.095**	**0.095**	**0.095**
37	0.095	0.100	0.096	nd	0.093	0.090	nd	nd	0.088
	0.091	0.093	0.092	nd	0.092	0.092	nd	nd	0.091
mean	**0.093**	**0.097**	**0.094**	**nd**	**0.093**	**0.091**	**nd**	**nd**	**0.090**

*no data

Curriculum vitae

Claudia Sabine Kellermann

* 13.01.1978, Munich, Germany

1997	Abitur at the Käthe-Kollwitz-Gymnasium, Munich, Germany
1998-2004	Studies of Biology; Ludwig-Maximilians-University Munich (LMU) Majors: systematic botany, physiological botany, zoology, ecology
2003-2004	Master Thesis (Diplom) at the LMU, Institute of Botany and Mycology, supervised by Prof. Dr. R. Agerer Title: "Investigation of bacterial communities on ectomycorrhizal fungi of *Fagus sylvatica* and *Picea abies* applying *in situ* hybridization and sequence analysis" (in German).
2005-2008	PhD Thesis at the Institute of Groundwater Ecology, Helmholtz Center Munich, Germany (Director Prof. Dr. R. Meckenstock), supervisors Dr. C. Griebler and Prof. Dr. A. Hartmann

Posters and Presentations

Kellermann, C, Selesi, D, Hartmann, A & Griebler, C (2008) Autotrophic CO_2 fixation in a tar oil contaminated aquifer. VAAM-Meeting, March 2008, Frankfurt/ Main. Oral presentation

Kellermann, C, Selesi, D, Hartmann, A & Griebler, C (2007) Occurrence and importance of autotrophic prokaryotes in a tar oil contaminated aquifer. 10th Symposium on aquatic microbial ecology (SAME10), September 2007, Faro Portugal. Poster presentation

Kellermann, C, Selesi, D, Hartmann, A & Griebler, C (2007) Occurrence and importance of autotrophic prokaryotes in a tar oil contaminated aquifer. VAAM-Meeting, April 2007, Osnabrück. Poster presentation.

Kellermann, C, Selesi, D, Hartmann, A & Griebler, C (2006) Autotrophy in Groundwater Ecosystems. VAAM-Meeting, March 2006, Jena. Poster presentation.

Publications

Kellermann, C & Griebler, C (2008) *Thiobacillus thiophilus* D24TN^T sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments. International Journal of Systematic and Evolutionary Microbiology (IJSEM), 59: 583-588

Kellermann, C, Selesi, D, Hartmann, A, Lee, N, Hügler, M, Esperschütz, J, & Griebler, C (2008) Chemolithoautotrophy in an organically polluted aquifer – Potential for CO_2 fixation and in situ bacterial autotrophic activity, *in preparation*

Acknowledgments

This work was financed by the Helmholtz Association.

Sincere thanks belong to my supervisor Dr. Christian Griebler for providing me support, ideas and inspiration during this work.

Many thanks to Prof. Dr. Anton Hartmann for inspiring meetings and his continuous support.

I also want to thank Prof. Dr. Rainer Meckenstock for the opportunity to perform this work at the Institute of Groundwater Ecology.

Special thanks belong to Sabine Schäfer and Michael Stöckl for their great technical advice and help.

Furthermore, I would like to thank Dr. Michael Schmid for providing continuous access to the ARB server of the AMP and Dr. Reiner Schroll and his working group for the possibility to conduct some of my experiments in their labs.

Many thanks belong to Dr. Michael Hügler, who taught me the art of enzyme assay analyses and granted me access to his laboratory.

I would like to thank Prof. Dr. Natuschka Lee for the possibility to perform the activity measurements in her laboratory and her help evaluating the results.

Also thanks belong to Dr. Jürgen Esperschütz for conducting PLFA analysis.

I am grateful to all my colleagues from the institute for creating an enjoyable working environment.

I want to thank Kathrin, Bettina, Robert, Sabine, CJ and Nidal for the nice and often funny time, and also for their continuous support in the office and in the lab.

And I especially want to thank my family for their endless support and faith.