

Searching for Branched Glycerol Dialkyl Glycerol Tetraether Membrane Lipid Producing Bacteria in Soil

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This research was conducted under the auspices of the Graduate School
SENSE (Socio-Economic and Natural Sciences of the Environment)

Searching for branched glycerol dialkyl glycerol tetraether membrane lipid producing bacteria in soil

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Thesis

submitted in fulfillment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. dr. M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Wednesday 24 October 2012

at 4:00 p.m. in the Aula

Rozelin Aydın

Searching for branched glycerol dialkyl glycerol tetraether membrane lipid producing bacteria in soil

164 pages.

Thesis, Wageningen University, Wageningen, NL (2012)

With references, with summaries in English and Dutch

ISBN 978-94-6173-379-5

Hayata yeniden tutunan biricik annecigime...

SUMMARY

Bacteria present in soil and peat bog environments were previously found to produce branched glycerol dialkyl glycerol tetraether membrane lipids (GDGTs) that are used as a paleoenvironmental proxy to estimate historic soil temperature and pH. Based on the composition and abundance of branched GDGTs, two indices were previously defined, of which the degree of Cyclisation of Branched Tetraethers (CBT) is related to soil pH and the degree of Methylation of Branched Tetraethers (MBT) is related to both soil pH and mean annual air temperature (MAT). It was hypothesized that bacteria produce branched GDGTs as a response to changes in pH and temperature. Members of the phylum *Acidobacteria* were proposed to produce branched GDGTs based on their abundance in peat environments, rich in GDGTs. Recently, it was shown that two representatives from subdivision 1 and 3, *Edaphobacter aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c, are able to produce these membrane lipids. However, environmental distribution of branched GDGTs is diverse indicating that members of additional subdivisions of *Acidobacteria* or yet other phyla might also be able to produce branched GDGTs.

The *Acidobacteria* constitute a diverse and ubiquitous phylum and its members play an important role especially in terrestrial environments. Information about the *Acidobacteria* was collected, including their phylogeny and taxonomy, their role in nature, genomic traits and methods applied for their isolation.

Soil is a very dynamic and complex ecosystem and soil organisms are major components of the soil. They are crucial for both soil structure and soil processes. The biological activity in the soil mainly occurs in the topsoil, which contains soil organisms and plant roots. Soil microorganisms including bacteria, archaea and fungi are responsible for nutrient cycling and decomposition of organic residues. Microbial activities are strongly influenced by a range of abiotic and biotic factors and by the interaction between different microorganisms. To get insight into the influence of environmental parameters on the bacterial diversity in relation to production of branched GDGTs, soil samples containing variable amounts of these lipids were taken from different locations, namely from two different hot springs (Surprise Valley, California, USA) and along the watershed of the Têt river (France).

Bacterial community composition was characterized by using high-throughput cultivation independent techniques as well as selective cultivation. It was shown that both pH and temperature strongly affected microbial community composition. Data presented in this thesis furthermore suggest that besides *Acidobacteria*, members of additional phyla might also be responsible for the observed production of branched GDGTs. Notably, occurrence and relative abundance of members of the *Alpha*- and *Deltaproteobacteria* and *Bacteroidetes* were related to the abundance of branched GDGTs. Furthermore, enrichment studies at different pH, temperature and with different substrates suggested that methanol might be a good carbon source to enrich for producers of branched GDGTs. Unfortunately, attempts towards the isolation of branched GDGTs producing bacteria were unsuccessful. However, a novel methylotrophic *Azospirillum* species, *Azospirillum methanolicus*, was isolated. In addition to that, novel methylotrophic candidate genera including *Acidobacteria* subdivision 1 and *Pedobacter* were identified from initial enrichment studies.

In conclusion, data presented in this thesis showed that temperature and pH have a strong effect on the microbial community composition in all soil studied here. Although branched GDGTs producing bacteria were not isolated, the data described in this thesis may help to design new strategies to isolate and identify target bacteria in the future.

KEYWORDS: Branched GDGTs, proxy, pH, temperature, *Acidobacteria*, methylotrophy, high-throughput techniques

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Aim and Outline

Aim and outline of the thesis

Even though branched GDGT membrane lipids are used as a paleoenvironmental proxy, limited information is available about the candidate bacteria that produce these types of lipids. Therefore, one of the aims of the research described in this thesis was to identify, culture and isolate branched GDGT producing bacteria. To this end, the effect of temperature and pH on the soil bacterial community composition was studied by using both culture dependent and culture independent techniques.

Previous studies have indicated that members of the phylum of *Acidobacteria* are prime candidates as bacterial producers of branched GDGT membrane lipids.

Chapter 2 provides an overview of the current knowledge regarding the taxonomic diversity of the *Acidobacteria*, as well as their ecological and physiological role in different habitats, and gives a description and evaluation of the cultivation strategies used to isolate members of this mysterious phylum.

Chapter 3 describes how environmental parameters influence the bacterial community composition in soils along temperature gradients associated with hot springs, with a special interest of identification of bacterial groups potentially responsible for the observed production of branched GDGTs. We conducted barcoded pyrosequencing analysis of PCR-amplified 16S rRNA gene fragments to characterize the bacterial communities in soil samples taken from two hot springs (Surprise Valley, California), which were previously shown to contain different amounts of branched GDGTs. A strong correlation was observed between environmental variables, including distance from the spring and temperature, and bacterial community composition.

We further aimed to provide additional leads towards the identity of bacteria able to synthesize branched GDGTs using molecular biological approaches in **Chapter 4**. Soil samples were taken along a temperature gradient in the Têt river watershed (France), which were previously shown to contain largely different amounts of branched GDGTs. Molecular analysis was done by clone library analysis and DGGE fingerprinting of 16S rRNA gene fragments.

Chapter 5 provides information on the microbial community structure of soil samples enriched with methanol at different pH and temperature. Methylotrophs are important key players in the global carbon cycle. One of the samples studied in Chapter 4, Teso 2, was used to enrich methylotrophic microorganisms at different pH

and temperature with methanol as the sole source of carbon and energy. We conducted 16S rRNA gene fragment pyrosequencing analysis and measured membrane PFLA and branched GDGT membrane lipids to assess the effect of pH and temperature on the methylotrophic community composition. Several microbial groups, including *Pedobacter* and *Acidobacteria* subdivision 1, were enriched, suggesting that these contain thus far unknown methylotrophs. Branched GDGTs were detected in one of the enrichment cultures.

In **Chapter 6** the isolation and characterization of a novel *Azospirillum* strain is described. The strain is a Gram negative, spiral, non-spore forming aerobic bacterium that was isolated from the Teso 2 soil sample with methanol as sole energy and carbon substrate. Based on 16S rRNA gene sequence analysis the strain is most closely related to *Azospirillum amazonense* (95%), *A. irakense* (91%), and *A. brasilense* (88%). As these strains show marked differences, we propose that the isolated strain represents a novel species within the genus *Azospirillum* for which the name *Azospirillum methanolicus* sp. nov. is proposed.

Finally, **Chapter 7** provides a summary and general discussion of the results presented in this thesis.

CHAPTER 1

General Introduction

Soil is a very diverse, dynamic and heterogeneous ecosystem that is vital for life. Soil contains minerals, trace elements and organic matter. Also, it can vary in texture, leading to the definition of different soil types such as clay, silt and sand. Soil contains numerous ecological niches for living organisms. Even a small amount of soil may contain millions of organisms and provides a suitable environment for a broad range of organisms, including bacteria, archaea, fungi, protozoans, nematodes, earthworms and arthropods, which all play an important role in this complex ecosystem, namely mineralization of organic matter and biotransformation of inorganic compounds, and hence contribute to the control of both local and global cycles of carbon, nitrogen, sulfur and phosphorus (Fang et al., 2005; Rousket al., 2010). Microorganisms, including bacteria, archaea and fungi, represent not only important key players in soil ecosystem functioning, but are also the most abundant and diverse groups (Gans et al., 2005). The number of prokaryotic genome equivalents was estimated to range between 2,000 and 18,000 per gram of soil (Nacke et al., 2011). Among these, bacteria are probably the best-studied group of microorganisms in the soil ecosystem (Grayston et al., 2004; Nacke et al., 2011). Over the past decades, especially the advent of cultivation independent approaches has provided a wealth of information regarding bacterial community composition, the abundance of different taxa and their possible function in different habitats. However, there are still big gaps in our understanding of the processes leading to the observed diversity in different ecosystems and to which extent environmental factors contribute to shaping the microbial communities. Microbial activities are strongly regulated by a range of abiotic and biotic factors, and by the interaction between different microorganisms. As small changes in environmental parameters can affect habitat functioning, it is essential to gain knowledge regarding i) the type of interactions that occur between organisms and with their environment in different habitats, ii) how microorganisms cope with environmental changes, and iii) what are the environmental boundary conditions for microbial populations to persist in natural environments? Such knowledge can also contribute to improving our chances for isolation and cultivation of specific microorganisms in the laboratory. With the advent of biomolecular strategies for the detection and characterization of microorganisms in environmental samples, it has become obvious that traditional cultivation-based approaches only

allow to scrape the tip of the iceberg of microbial diversity, and that only around 1% of soil microorganisms can be cultivated under laboratory conditions using traditional approaches (Daniel, 2005). Nevertheless, the ability to cultivate and isolate specific microorganisms from their natural habitat remains essential for a thorough characterization of their physiological properties. To this end, integrated cultivation-dependent and molecular strategies are increasingly used in order to gain more insight in the distribution of microorganisms in a broad range of different environments. Molecular analysis of cellular biomarkers, including membrane phospholipid measurements, ribosomal RNA (rRNA) gene based analyses, such as denaturing gradient gel electrophoresis (DGGE) and barcoded pyrosequencing of PCR-amplified 16S rRNA gene fragments, environmental metagenomics, proteomics, and stable isotope probing of lipids, proteins and nucleic acids can be applied in microbial ecology (Muyzer et al., 1993; Felske et al., 1996; Ludwig et al., 1997; Smit et al. 1997; Fierer & Jackson, 2006; DeSantis et al., 2007; Jones et al., 2009).

In an investigation of the microbial community composition in a variety of soils including forest, arid woodland, alpine and grassland soils, differing in their physical-chemical characteristics, nine dominant bacterial phyla were detected using 16S rRNA targeted analysis, including *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Firmicutes* (Janssen, 2006). This author also showed that *Proteobacteria* and *Acidobacteria* were the most dominant phyla in bacterial communities inhabiting the soils studied. In addition, it was addressed that the assessment of soil microbial abundances using rRNA-based methods might cause overestimation of some groups. The ever increasing numbers of uncultured bacterial groups, found in rRNA- based studies, urge to isolate and identify novel bacteria in order to determine the functional and genetic diversity more deeply. Recently, a number of innovative approaches were implemented to isolate strains belonging to the phyla of *Acidobacteria* and *Verrucomicrobia* (Sangwan et al., 2005; Sait et al., 2006). It turned out that many representatives of these phyla can be cultured by using modified high-throughput techniques (Joseph et al., 2003; Nichols et al., 2010). Furthermore, many of the new isolates do not possess close cultured relatives. Often the novel isolates have a

very slow growth rate and in some cases, formation of colonies on solidified media can take months rather than days. Both high-throughput cultivation dependent and independent tools are required for revealing and characterizing unexplored bacterial diversity and understanding their interactions with biotic and abiotic environmental factors. Castro et al. (2010) showed that precipitation influences the abundance of *Proteobacteria* and *Acidobacteria* in the soil environment. pH has been one of best studied abiotic factors affecting soil microbiota composition and function, both at local and continental scales (Lauber et al., 2009; Rousk et al., 2010; Chu et al., 2010). Research on soils from the Peruvian Amazon showed that both diversity and composition of soil bacteria were strongly affected by a diverse range of ecosystem parameters including mean average temperature, potential evapotranspiration, latitude and soil pH. They concluded that pH was the best predictor of both soil bacterial diversity and richness in acidic soils (Fierer & Jackson, 2006). On the other hand, more recently Faoro et al. (2010) showed that the bacterial diversity in southern Brazilian Atlantic forest soils was influenced by altitude, phosphorus and $\text{Ca}^{+2}/\text{Mg}^{+2}$, whereas pH had no significant effect. Lauber et al. (2009) indicated that the bacterial diversity is affected by the pH and soil texture, whereas the fungal diversity was strongly influenced by nutrient availability.

Measurement of membrane phospholipid fatty acid (PLFA) profiles is a useful tool for the characterization of microbial composition, and it has been widely used to assess the effect of various physical-chemical parameters such as, pH, soil texture, aeration and the availability of nutrients, on the microbial diversity in soil (Frostegård et al., 1993; Bååth et al., 1995; Bååth & Anderson, 2003; Pettersson and Bååth, 2003; Frostegård & Bååth, 1996; Jia et al., 2006; Ramsey et al., 2006; Wu et al., 2010). It was already known that higher temperatures strongly influenced both PLFA patterns and soil microbial dynamics (Pettersson & Bååth, 2003; Wu et al., 2010). In addition, it was shown that, rather than the total PLFA amount, the PLFA composition was strongly affected by soil pH (Nilsson et al., 2007; Rousk et al., 2009; Rousk et al., 2010). Nevertheless, although PLFA methods are appropriate for many studies, rRNA-based techniques provide more information.

Branched glycerol dialkyl glycerol tetraether membrane lipids

Branched glycerol dialkyl glycerol tetraether (branched GDGT) membrane lipids have recently been introduced as a paleoenvironmental proxy for sedimentary records. Previous research has indicated that branched GDGT membrane lipids encountered in peat, soils, lake sediments and other habitats are biosynthesized by microorganisms in the bacterial domain. Branched GDGT membrane lipids were first identified from peat soil by Sinninghe Damsté et al. (2000). These lipids consist of two alkyl chains ether-bound to glycerol units, whereby the alkyl moieties contain 4 to 6 methyl groups and up to 2 cyclopentane moieties (Weijers et al., 2006). Furthermore, it could be shown that soil pH and temperature influence the distribution and the composition of branched GDGT membrane lipids (Weijers et al., 2007; Peterse et al., 2010; Sinninghe Damsté et al., 2011). Branched GDGTs are used to reconstruct the historic soil pH and temperature in sedimentary records. To this end, two indices, cyclisation of branched tetraethers (CBT) and methylation of branched tetraethers (MBT), have been defined to characterize the distribution of branched GDGT membrane lipids. The CBT index correlates with soil pH, whereas the MBT index related with both mean annual air temperature (MAT) and soil pH (Weijers et al., 2007). The distribution of branched GDGT membrane lipids was mainly related with both MAT and soil pH in 130 soil samples, taken from all over the world. Remarkably, GDGT I was mainly present in soils from tropical places, whereas GDGTs II and III were most abundant in samples from higher latitudes and elevated mountain areas (Figure 1, Chapter 3). It was suggested that bacteria produce these lipids to adjust their membrane stability as a response to changes in ambient temperature and pH (Weijers et al., 2009). Furthermore, analysis of soil samples taken from a long term experimental field with a pH range of 4.5 to 7.5 showed that CBT indices significantly correlated with soil pH, and that abundances of branched GDGTs were negatively affected by soil pH (Peterse et al., 2009). Interestingly, membrane lipids could be found in historic soil samples till the late Jurassic, reinforcing their usefulness as paleoenvironmental proxies (Casteñeda & Schouten, 2011). Unfortunately, the producers of branched GDGT membrane lipids are not yet known. Based on molecular surveys of the microbial diversity in peat bogs and because of the strong relationship between the pH and the

composition and abundance of branched GDGTs, it was suggested that members of the *Acidobacteria* might be responsible for the production of these lipids (Weijers et al., 2007; Peterse et al., 2009). Furthermore, it was suggested that bacteria that produce these lipids, should have a heterotrophic life style, based on the natural ^{13}C abundance in branched GDGT membrane lipids (Oppermann et al., 2010; Weijers et al., 2010; Huguet et al., 2012). Besides analysing branched GDGTs as core lipids (CL), which represent the fossil pool without polar head groups, there is a possibility to analyse the corresponding intact polar lipids (IPL) that are derived from living microorganisms. Liu et al. (2010) and Peterse et al. (2010) investigated the intact polar lipids (IPL) derived from branched GDGT lipids, and they identified several head groups including glycuronic acid, hexose-glycuronic acid, and phosphohexose. It is interesting to note that it has previously been shown that glucuronosyl lipids were detected in members of the *Actinobacteria*, as well as in strains within the alphaproteobacterial genus *Beijerinckia* (Liu et al., 2010). Sinninghe Damsté et al. (2011) showed that 2 acidobacterial isolates, *Edaphobacter aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c, are able to synthesize branched GDGTs. However, based on the complexity of the lipid pattern observed in soils and peat bogs it was suggested that other (acido)bacteria might also synthesize branched GDGT membrane lipids. Even though *Acidobacteria* are commonly present in a broad range of habitats including soil, peat bogs, acid mine drainage and hot springs (Hugenholtz et al., 1998; Barns et al., 1999; Dedysh et al., 2006; Janssen, 2006; Bryant et al., 2007), only limited information about their properties is available, which is mainly due to difficulties to culture these bacteria in the laboratory. Furthermore, the distribution of *Acidobacteria* and especially those belonging to subdivision 1, is strongly influenced by pH and to a lesser extent by other environmental parameters including temperature, soil moisture and soil nutrient availability (Fierer et al., 2007a; Fierer et al., 2007b; Mannisto et al., 2007; Jones et al., 2009; Rousk et al., 2010).

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CHAPTER 2

Chapter 2

The Mysterious Phylum of *Acidobacteria*

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Submitted

ABSTRACT

The phylum of *Acidobacteria* is one of the most abundant bacterial groups in a broad range of different environments. Based on analyses of 16S rRNA gene clone libraries, members of the *Acidobacteria* represent a fraction of 10 to 50 % of typical soil bacterial communities, and relative abundances up to 80% have been reported. *Acidobacteria* have been detected in almost every clone library of soil but also in many other habitats including peat bogs, acid mine drainage, a contaminated aquifer, a hot spring, a freshwater lake, and the pelagic zone of oceans. This suggests that the phylum of *Acidobacteria* is genetically and metabolically diverse, and environmentally widespread. Thus, *Acidobacteria* are likely to play an important role in biogeochemical processes in soil. Recently, the number of subdivisions was extended to 26. In contrast to their abundance, members of the *Acidobacteria* have been very difficult to isolate and culture in the laboratory. Because of their abundance, there is an enormous interest in their functionality in the environment, including their ecology and physiology within the interactive networks that shape microbial communities. Novel high-throughput screening methods such as metagenomics and single-cell sequencing approaches hold a great promise in expanding our understanding of the genomic and hence functional diversity of *Acidobacteria*.

INTRODUCTION

The *Acidobacteria* is one of the most widely distributed and diverse phyla in a variety of natural environments (Hugenholtz et al., 1998; Tringe et al., 2005; Janssen, 2006;). Based on the analysis of 16S rRNA gene clone libraries, members of this phylum represent a fraction of 10 to 50 % of typical soil bacterial communities (e.g. Barns et al., 1999; Dunbar et al., 1999; Selenska-Pobell et al., 2001; Lopez-Garcia et al., 2003; Kanoktratana et al., 2004; Branco et al., 2005; Fracchia et al., 2006; Penn et al., 2006; Lee et al., 2008). It has been reported that Acidobacteria can contribute up to 80% of total bacteria (Chan et al., 2006). *Acidobacteria* have been detected in almost every clone library of soil, but also in many other habitats including peat bogs, acid mine drainages, contaminated aquifers, hot springs, rhizosphere of leeks, freshwater lakes, marine invertebrates including sponges and corals, the pelagic zone of the oceans, and deep sea hydrothermal vents (Hugenholtz et al., 1998, Barns et al., 1999; Dedysh et al., 2006; De Long et al., 2006; Bryant et al., 2007; da Rocha et al., 2010; Izumi et al., 2012; Simister et al., 2012). The phylogenetic diversity within the *Acidobacteria* is nearly as great as in the *Proteobacteria* (Hugenholtz et al., 1998; Barns et al., 1999). The number of subdivisions within this *Acidobacteria* increased from 4-5 in 1997 (Ludwig et al., 1997) to 26 in 2007 (Barns et al., 2007). However, the taxonomically described diversity of *Acidobacteria* remains limited, since these microorganisms are difficult to isolate and culture in the laboratory (Hugenholtz et al., 1998; Zimmermann et al., 2005; Barns et al., 2007). Currently, only subdivisions 1, 3, 8 and 10 have characterized representatives (Figure 1), though a number of isolates have also been obtained from subdivisions 2, 4 and 6. Most of these species are Gram-negative, slow growing, and chemo-organotrophic utilizing a wide range of carbon and nitrogen sources. They neither fix N₂ nor do they denitrify. Up to date, nine genome sequences of Acidobacteria have been determined, including those of *Acidobacterium capsulatum*, ‘*Koribacter versatilis*’ Ellin345, *Terriglobus saanensis*, *Terriglobus roseus*, *Granulicella mallensis* MP5ACTX8, *Granulicella tundricola* MP5ACTX9 from subdivision 1, ‘*Solibacter usitatus*’ Ellin6076 from subdivision 3, *Candidatus Chloracidobacterium thermophilum* B from subdivision 4, and *Holophaga foetida* as a representative of subdivision 8 (Ward et al., 2009; Garcia et al., 2012; Rawat et al., 2012) (Table 1; Figure 1).

Of 166,760 high quality 16S rRNA gene sequences with a length of at least 1200 nucleotides from bacterial isolates available in the most recent release of the Ribosomal Database Project (RDP II v10.29; June 2012; <http://rdp.cme.msu.edu/>), a total of 151 have been classified as *Acidobacteria*.

When considering also sequences retrieved from environmental samples, acidobacterial 16S rRNA gene sequences currently amount to 12,104 out of a total number

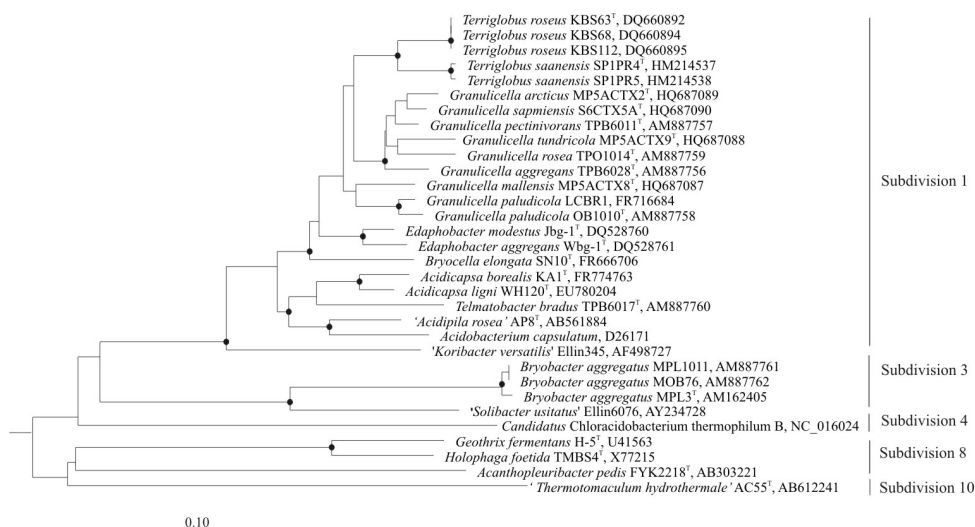


Figure 1. 16S rRNA gene-based neighbour-joining tree showing the characterized representatives of the phylum *Acidobacteria*. Filled circles indicate nodes that were also recovered in maximum-likelihood and maximum-parsimony trees. Six members of the *Planctomycetes*, i.e. *Isosphaera pallida* (AJ231193), *Gemmata obscuriglobus* (X54522), *Planctomyces brasiliensis* (AJ231190), *Planctomyces maris* (AJ231184), *Schlesneria paludicola* (AM162407) and *Singulisphaera acidiphila* (AM850678) were used as an outgroup (not shown). Bar, 0.1 substitutions per nucleotide position. GenBank accession numbers are given in the tree.

of 1.052.808 bacterial sequences. It has been demonstrated that representatives of the *Acidobacteria* can be cultured by using media with lowered pH (Sait et al., 2006); increased headspace CO₂ concentration (Stevenson et al., 2004); diffusion chambers (Bollmann et al., 2007), substrate amendments (Pankratov et al., 2008; Kulichevskaya et al., 2010; Pankratov & Dedysh, 2010) and extended incubation time (Davis et al., 2005). Despite this obvious success of the isolation of an increasing number of members of the *Acidobacteria*, most of the isolates are representatives of subdivision 1.

Members of the *Acidobacteria* play an important role in natural environments. They are capable of degrading different plant-derived polymers, e.g. pectin, laminarin, xylan, starch, and cellulose (Pankratov et al., 2008, 2011, 2012; Pankratov & Dedysh, 2010; Eichorst et al., 2011). Also metagenome data indicate that *Acidobacteria* play a key role in plant debris degradation (Kanokratana et al., 2011). In addition, it has been suggested that *Acidobacteria* can also play a role in the iron cycle, photosynthesis and, possibly, turnover of C1 compounds (Coates et al., 1999; Radajewski et al., 2002; Bryant et al., 2007; Kalyuzhnaya et al., 2008; Pankratov et

Table 1. General characteristics of completed acidobacterial genomes[†]

Genome name (subdivision)	GeneBank Project ID	Status ²	Bases (Mbp)	G+C (%)	Gene count	CDS count	16S rRNA	Plasmid	Pseudo	Orthologs	CRISPR
<i>A. capsulatum</i> (1)	28085	F	4.12	60	3425	3377	1	0	0	3006	2
<i>K. versatilis</i> Ellin345 (1)	15771	F	5.65	58	4837	4779	1	0	0	4474	0
<i>T. saarnensis</i> SP1PR4 (1)	48971	F	5.12	57	4400	4324	1	0	2	0	0
<i>T. roseus</i> DSM 18391 (1)	63509	F	5.23	60	4947	4898	2	0	0	0	0
<i>G. mallensis</i> MP5ACTX8 (1)	47903	F	6.21	58	4757	4898	1	0	0	0	0
<i>G. tundricola</i> MP5ACTX9 (1)	47621	F	5.44	60	8003	4666	1	1	0	0	0
<i>S. uzoniensis</i> Ellin6076 (3)	12638	F	9.96	62	3672	7940	2	0	114	7244	0
<i>H. foerida</i> DSM6591 (8)	53485	P	4.13	63	3425	3615		0			0
<i>Candidatus C. thermophilum</i>	73587	F	2.68/1.01	61	4837	3054	1	NR	NR	NR	

[†] Bases: genome size, G+C: DNA base composition, CDS count: number of CDS genes, NR: not reported. ¹ F: finished, P: permanent draft.

Data taken from [Jin et al., 2012](#), for *Candidatus C. thermophilum*.

al., 2008; Raymond et al., 2008; Lu et al., 2010). Furthermore, in soils that are polluted with polychlorinated biphenyls and parathion the abundance of *Acidobacteria* was found to be high (Nogales et al., 1999; Debatari et al., 2006). This suggests that some members of *Acidobacteria* are able to degrade specific soil pollutants. In young soils, the abundance of *Acidobacteria* was detected to be low compared to old soils, suggesting that they might utilize complex carbon substrates in more developed soils, possibly in interaction with members of other phyla, forming complex microbial food-webs (Nemergut et al., 2007; Tarlae et al., 2008).

The acidobacterial abundance and diversity in soil is strongly determined by pH (Fierer et al., 2007; Mannisto et al., 2007; Jones et al., 2009). The relationship between *Acidobacteria* and soil pH furthermore correlates with the occurrence of branched glycerol dialkyl glycerol tetraether (GDGT) membrane lipids. Branched GDGT membrane lipid abundances have been found to decrease with increasing soil pH, which led to the hypothesis that *Acidobacteria* may be a possible source of these orphan membrane lipids (Weijers et al., 2007; Peterse et al., 2010). Recently, it was shown that *Acidobacteria*, *Edaphobacter aggregans* Wbg-1T and *Acidobacteriaceae bacterium* A2-4c, are able to produce these membrane lipids (Sinninghe Damsté et al., 2011).

Taken together, *Acidobacteria* constitute a ubiquitous phylum, and evidence is accumulating that members of this mysterious clade provide key functions in a broad range of different environments. There is increasing interest in elucidating their functionality in the environment, including their ecology and physiology within the interactive networks that shape microbial communities.

Here we review the current knowledge about the ecological and physiological role of the *Acidobacteria*, and describe and evaluate the cultivation strategies used to isolate members of this mysterious phylum.

PHYLOGENY AND DIVERSITY

The phylum *Acidobacteria* was first defined in 1997 by Ludwig et al. (1997), and in 1999, it was proposed as a new bacterial kingdom by Barns et al. The first characterized member of this phylum, *Acidobacterium capsulatum*, was described by Kishimoto and co-authors (1991), who isolated eight novel strains of bacteria from an acidic mineral environment. This was also the first record of an isolation of an acidophilic heterotrophic bacterium from acidic mineral environments other than *Acidiphilum* (Kishimoto et al., 1991). Based on a 16S rRNA gene sequencing survey of different environments *Acidobacterium capsulatum* was recognized as a member of large and deeply branching, monophyletic lineage within the Bacteria (Hiraishi et al., 1995). *Holophaga foedita* was the second isolate within the *Acidobacteria*, and

was described as a strictly anaerobic, demethylating homoacetogen, which utilizes aromatic compounds leading to the production of acetate (Bak et al., 1992; Liesack et al., 1994). The strictly anaerobic *Geothrix fermentans* is capable of Fe (III) reduction with acetate and other simple organic acids as substrate. Because of considerable 16S rRNA sequence similarity with *Acidobacterium capsulatum*, *Holophaga foetida* and *Geothrix fermentans* were placed in the phylum *Acidobacteria*. Currently, the phylum *Acidobacteria* includes 21 validly described species in subdivisions 1, 3, and 8. The established genera of this phylum are, in addition to *Acidobacterium*, *Acanthopleuribacter*, *Acidicapsa*, *Bryobacter*, *Bryocella*, *Edaphobacter*, *Geothrix*, *Granulicella*, *Holophaga*, *Telmatobacter*, and *Terriglobus*. The genera ‘*Acidipila*’ (subdivision 1) and ‘*Thermotomaculum*’ (subdivision 10) have also been described, but these are not yet included in the list of valid names.

Based on 16S rRNA gene sequence analysis, the *Acidobacteria* is one of the most abundant phyla in a broad range of different environments. However, due to the lack of cultured representatives, evolutionary relationships to other phyla remain unclear. Genomic evidence showed that *Acidobacteria* share traits with *Cyanobacteria*, *Proteobacteria* and even Fungi (Ward et al., 2009). Studies of the evolutionary link between the *Acidobacteria* and the *Proteobacteria* suggested that *Acidobacteria* may be a sister group of the *Deltaproteobacteria* (Ciccarelli et al., 2006; Ward et al., 2009). There is also similarity between *Rhizobiales* and *Acidobacteria* which was explained by horizontal gene transfer (Quaiser et al., 2003). Phylogenetic analysis of 31 proteins predicted from the first available 3 acidobacterial genomes confirmed that acidobacterial sequences clustered adjacent to those of *Proteobacteria* in 87% of the trees generated from bootstrapped data sets (Ward et al., 2009).

STRATEGIES FOR ISOLATION

Significant breakthrough in the isolation of *Acidobacteria* was made during the last decade. It turned out that many representatives of this phylum can be cultured by using modified techniques. As a consequence, these elusive microorganisms were isolated from diverse soils (Janssen et al., 2002; Sait et al., 2002; Joseph et al., 2003; Eichorst et al., 2007, 2011; Koch et al., 2008; Stott et al., 2008; George et al., 2009), Sphagnum-dominated wetlands (Pankratov et al., 2008; Kulichevskaya et al., 2010; Pankratov & Dedysh, 2010; Pankratov et al., 2012; Kulichevskaya et al., 2012), termite hindgut (Eichorst et al., 2007), and decaying wood (Folman et al., 2008; Valas-kova et al., 2009). Most of these isolates are subdivision 1 acidobacteria. However, some strains belonging to subdivisions 2, 3, 4, 6, 8 and 10 have also been cultured. What are the “secret tips” for successful isolation and cultivation of acidobacteria?

The use of low-nutrient media and gellan as solidifying agent

It has been known for a long time that plating a sample onto dilute nutrient media yields higher numbers of bacterial colonies than plating onto the standard, non-diluted versions of the same media. The difference in the number of colony forming units recorded on high- and low-nutrient media may comprise 1-2 orders of magnitude. The colonies appearing on high-nutrient media are usually formed by fast-growing microorganisms, which affiliate with commonly isolated and well characterized bacterial groups. By contrast, a significant proportion of colonies that develop on low-nutrient media belong to poorly studied bacterial groups, including the *Acidobacteria* (Janssen et al., 2002; Davis et al., 2005). Most isolates of soil-inhabiting acidobacteria were obtained using either nutrient broth at 1/100 of its normal concentration or VL55 medium, which was formulated to mimic the low concentrations of inorganic ions in soils (Sait et al., 2002; Davis et al., 2005). Isolation of acidobacteria from Sphagnum-dominated peatlands, which are depleted of mineral nutrients, was also achieved using low-nutrient media MM, MM1 or 10-fold diluted R2A (Dedysh et al., 2006; Pankratov et al., 2008; Dedysh, 2011). One of these media, MM1, does not contain phosphate since growth of some peat-inhabiting acidobacteria was shown to be inhibited by phosphate (Pankratov and Dedysh, 2010).

The use of gellan gum instead of agar is also one of the strategies to culture different novel bacteria (Janssen et al., 2002; Stott et al., 2008; Dedysh, 2011). This polysaccharide is free of contaminants and results in clear media that allows very small colonies to be readily seen. Interestingly, the very first set of acidobacterial isolates, which were later described as a novel genus and species *Acidobacterium capsulatum*, was also obtained on a gellan-solidified medium (Kishimoto et al., 1991).

Extended incubation time and selection of mini-colonies

Hunting for difficult-to-culture microbes requires patience. As has been shown in several studies, most acidobacteria form visible colonies only after incubation for 12 to 24 weeks (Davis et al., 2005; Sangwan et al., 2005; Stott et al., 2008). This is due to the low specific growth rates, which for many acidobacteria do not exceed 0.01 h⁻¹. At the stage of isolation, these bacteria produce very small (25-200 µm to 500 µm in diameter) colonies, which can be observed and picked with the use of a dissecting microscope only. It has been shown that acidobacteria and several other rarely cultured groups of soil bacteria are most abundant among the mini-colonies that developed after > 12 weeks of incubation (Davis et al., 2011). In many cases, these isolates grow slowly upon subculture as well. Interestingly, similar incubation times of up to 3 months recently also allowed for the first time isolation of representatives of subdivision 6 from alkaline soils (George et al., 2011). Some pink- and orange-pigmented subdivision 1 acidobacteria, however, form visible colonies relatively fast, within 2 weeks of incubation. This is characteristic of the genera *Terri-*

globus, *Granulicella* and *Acidobacterium* which may show growth rates as high as 0.10-0.15 h⁻¹.

Plant polymers as growth substrates

The use of plant-derived polymers as growth substrates has proved to be highly effective for isolation of acidobacteria (Sait et al., 2002; Koch et al., 2008; Pankratov and Dedysh, 2010; Eichorst et al., 2011). Since many members of this phylum are slow-growing oligotrophs, they are easily outcompeted by fast-growing bacteria on media containing readily oxidizable carbon substrates or may be inhibited by high substrate concentrations, which are uncommon for their natural habitat. Polysaccharides xylan, pectin and starch are among the most useful substrates for isolation of acidobacteria. The recent study of Eichorst and co-authors (2011) addressed the influence of plant polymers on the cultivation of acidobacteria from agricultural and managed grassland soils. The use of a medium with a mixture of plant polymers (xylan, xanthan, pectin and methyl cellulose) reduced the total recovery of bacteria from studied soils by 6-fold compared to the medium containing readily oxidizable carbon (yeast extract, Bacto-peptone, Casamino Acids, and dextrose). However, the plates amended with plant polymers selected for more diverse acidobacteria from subdivisions 1, 2 and 4 than those containing readily oxidizable carbon, where only subdivision 1 sequences were identified.

Effect of medium pH

Among the 26 currently recognized subdivisions, only members of subdivision 1 display a clear preference for growth in acidic media. The cultivation experiments carried out on a medium with a pH of 5.5 yielded significantly more colonies of subdivision 1 acidobacteria than did the same medium with a pH of 7.0 (Sait et al., 2006). The colonies obtained at pH 5.5 were formed by bacteria that spanned almost the entire phylogenetic breadth of this subdivision. Mildly acidic media of pH 4.0-5.0 were later successfully applied for isolation of subdivision 1 acidobacteria from Sphagnum-derived peat of pH 3.8-4.5 (Pankratov et al., 2008; Pankratov & Dedysh, 2010; Dedysh, 2011) and from decaying wood of pH 3.3-3.6 (Folman et al., 2008; Valaskova et al., 2009). This agrees well with the observation that the proportion of subdivision 1 acidobacteria in clone libraries of 16S rRNA and 16S rRNA genes from different soils is negatively correlated to soil pH (Sait et al., 2006) and with the fact that all currently characterized subdivision 1 acidobacteria are moderate acidophiles. The pH preference of acidobacteria from other subdivisions is not yet established since only a very few (if ever) isolates from these groups are currently available.

Co-cultures with other bacteria

Many colonies that develop on a surface of a solid medium represent not a single bacterium, but rather consortia of two or more different microorganisms. Examination of such mixed colonies obtained from acidic peat revealed that many of them contained cells of acidobacteria as well as some other rarely cultured organisms (Dedysh et al., 2006). This observation suggested that an isolation approach based on enrichment of mixed cultures or microbial biofilms might in some cases be more efficient for isolating acidobacteria than a routine “single-colony pick-up” strategy. Indeed, application of this biofilm-mediated enrichment approach enabled isolation of peat-inhabiting subdivision 3 acidobacteria, which were later described as *Bryobacter aggregatus* (Kulichevskaya et al., 2010). It is interesting to note that the photoheterotrophic *Candidatus Chloracidobacterium thermophilum* cannot be maintained in axenic culture, and its propagation requires the presence of additional heterotrophs (Bryant et al., 2007). Recent analysis of the genome indeed confirmed the inability of this organism to synthesize a number of essential nutrients (Garcia Costas et al., 2012).

None of the above listed approaches look very special. They give, however, a desirable result and show that acidobacteria can be cultivated. The use of different molecular techniques greatly facilitates surveillance of isolation and purification procedures. However, isolation is only the first step towards obtaining knowledge about novel microorganisms. Detailed physiological, taxonomic and genetic studies are essential. Here, the results are less impressive since characterization of slow-growing bacteria requires a big deal of time and patience. As a consequence, the taxonomically and physiologically described diversity within the *Acidobacteria* remains limited.

TAXONOMICALLY CHARACTERIZED DIVERSITY OF ACIDOBACTERIA

At present, only subdivisions 1, 3 and 8 include taxonomically characterized representatives of the *Acidobacteria*. The list of validly described taxa from subdivision 1 includes the genera *Acidobacterium* (Kishimoto et al., 1991), *Terriglobus* (Eichorst et al., 2007; Männistö et al., 2011), *Edaphobacter* (Koch et al., 2008), *Granulicella* (Pankratov & Dedysh, 2010), *Bryocella* (Dedysh et al., 2012), *Acidicapsa* (Kulichevskaya et al., 2011) and *Telmatobacter* (Pankratov et al., 2012). The only described genus in subdivision 3 is *Bryobacter* (Kulichevskaya et al., 2010), while subdivision 8 includes the genera *Holophaga* (Liesack et al., 1994), *Geothrix* (Coates et al., 1999) and *Acanthopleuribacter* (Fukunaga et al., 2008) (Table 1). Two acidobacteria, ‘*Acidipila rosea*’ from subdivision 1 (Okamura et al., 2011) and ‘*Ther-*

motomaculum hydrothermalis' from subdivision 10 (Izumi et al., 2012) were also described but not yet included in the list of valid names. Members of all these genera are represented as Gram-negative, non-spore-forming cells of rod-like morphology that divide by binary fission and occur singly, in pairs, in short chains or in shapeless aggregates. Cell length varies from 1 µm in *Terriglobus* spp. to 15 µm in *Granulicella* spp. Old cultures of some acidobacteria may contain strongly elongated cells (up to 50 µm in length) as well as spherical bodies of varying sizes (0.5-3 µm), which represent starvation forms. Motility is a characteristic of *Acidobacterium capsulatum*, *Edaphobacter modestus*, *Telmatobacter bradus* and *Acanthopleuribacter pedis*. Production of amorphous extracellular polymeric substance is typical for *Terriglobus* spp. and *Granulicella* spp. Pigmentation due to production of carotenoids occurs in several subdivision 1 acidobacteria and in *Acanthopleuribacter pedis*. Colonies formed by members of the *Acidicapsa*, '*Acidipilia*', *Terriglobus* and *Granulicella* are pink-pigmented, whereas *Acidobacterium capsulatum* and *Acanthopleuribacter pedis* are orange and yellow, respectively.

All validly described organisms in subdivision 1 are mesophilic and moderately acidophilic chemo-organotrophs that grow at pH values between 3.0 and 7.0-7.5 (Table 2). Optimal growth temperature for subdivision 1 acidobacteria is between 20 and 30°C. *Terriglobus saanensis*, *Telmatobacter bradus* and *Granulicella* spp. are psychrotolerant bacteria growing at temperatures as low as 2-4°C. Though *Acidobacterium capsulatum* was originally described as a strict aerobe (Kishimoto et al., 1991), it appears to grow in micro-oxic and anoxic conditions by means of fermentation (Blöthe et al., 2008; Coupland & Johnson, 2008; Pankratov et al., 2012). *Telmatobacter bradus* is a facultative anaerobe that grows under reduced oxygen tension and under anoxic conditions. *Terriglobus* spp., *Edaphobacter* spp., and *Granulicella* spp. are strict aerobes. The preferred growth substrates are sugars though several organic acids and polyalcohols can also be utilized by some strains.

For example, glucuronic acid is utilized by nearly all described subdivision 1 acidobacteria, succinate is used by *Terriglobus roseus* and *Granulicella pectinivorans*, while acetate and lactate are poorly utilized only by *Granulicella pectinivorans*. Hydrolytic capabilities are present in all described subdivision 1 acidobacteria but are most strongly pronounced in *Granulicella* spp. and *Telmatobacter bradus* (Pankratov & Dedysh, 2010; Pankratov et al., 2012). The ability to degrade pectin, xylan, starch, laminarin, lichenan, aesculin and gelatin varies between different species, but only *Telmatobacter bradus* is capable of hydrolysing cellulose. The respiratory quinones in subdivision 1 acidobacteria are represented by menaquinone MK-8. The genomic DNA G+C content varies between 56 and 61 mol%.

Bryobacter aggregatus is the only currently described organism in subdivision 3 of the Acidobacteria (Kulichevskaya et al., 2010). It is an aerobic, acid- and psychrotolerant chemo-organotroph, which utilizes various sugars as well as galacturonic

and glucuronic acids. It is also capable of hydrolysing pectin, starch, gelatin, chondroitin sulphate, casein and aesculin. The major respiratory quinones are MK-9 and MK-10; the DNA G+C content is 55-56 mol%.

Subdivision 8 acidobacteria with validly published names are represented by three neutrophilic and mesophilic bacteria that display highly contrasting characteristics. *Holophaga foetida* is a strictly anaerobic, homoacetogenic bacterium that degrades methoxylated aromatic compounds to acetate and is capable of transferring methyl groups from phenylmethylethers to sulphide, forming methanethiol and dimethyl sulphide (Liesack et al., 1994). Substrates used by this bacterium are almost exclusively aromatic compounds, especially a range of methylated and non-methylated derivatives of trihydroxybenzenes, and pyruvate. Cells contain c-type cytochromes. The DNA G+C content is 62.5 mol%. *Geothrix fermentans* is also a strictly anaerobic, mesophilic bacterium that oxidizes acetate as well as several other simple organic and long-chain fatty acids with Fe(III) as the electron acceptor (Coates et al., 1999). Nitrate, Mn(IV), fumarate and the humic acid analogue 2,6-antraquinone disulfonate can also be used as alternative electron acceptors. In addition to anaerobic respiration, *Geothrix fermentans* can also grow by fermentation of citrate or fumarate. Acetate and succinate are the primary end products of citrate fermentation. Cells grown with Fe(III) or fumarate as electron acceptors contain a c-type cytochrome. By contrast, *Acanthopleuribacter pedis* is a strictly aerobic chemo-organotroph that utilizes only a very limited number of growth substrates including glucose and several amino-acids (Fukunaga et al., 2008). The respiratory quinones are menaquinones MK-6 and MK-7; the DNA G+C content is 56.7 mol%.

Only recently, '*Thermotomaculum hydrothermale*' has been described as the first cultured representative of subdivision 10 (Izumi et al., 2012). The organism has been isolated from a deep sea hydrothermal vent chimney, is strictly anaerobic, neutrophilic and moderately thermophilic. Main respiratory quinones are MK-7 and MK-8, the DNA G+C content is 51.6 mol%, and the organism requires salt (1.5 – 4.5% NaCl) for fermentative growth with yeast extract or tryptone peptone as carbon source.

In summary, only subdivision 1 acidobacteria look more or less uniform with regard to their physiology. The characteristics of *Bryobacter aggregatus* may not be representative of other subdivision 3 acidobacteria, while three currently known members of subdivision 8 possess completely different lifestyles. Therefore, increasing the described diversity within the phylum *Acidobacteria* remains one of the high-priority tasks for further studies.

GENOMIC TRAITS

Nine genome sequences of *Acidobacteria* are currently available in public databases,

Table 2. Characterized members of the phylum *Acidobacteria*

S.division	Genus (species)	Relation to O ₂	pH growth range (optimum)	Preferred growth substrates	Isolation source	Reference
1	<i>Acidobacterium</i> (<i>A. capsulatum</i>)	facultative aerobe*	3.0 – 6.0 (ND)	Various sugars	Acidic mineral environment	Kishimoto et al., 1991
	<i>Terriglobus</i> (<i>T. roseus</i> , <i>T. saanensis</i>)	aerobe	4.5 - 7.5 (5.0 - 6.0)	Various sugars	Boreal and tundra soil, termite hindgut	Eichorst et al., 2007; Mannistö et al., 2011
	<i>Edaphobacter</i> (<i>E. modestus</i> , <i>E. aggregans</i>)	aerobe	4.0 - 7.0 (5.5)	Various sugars	Alpine and forest soil	Koch et al., 2008
	<i>Granulicella</i> (<i>G. paludicola</i> , <i>G. pectinivorans</i> , <i>G. rosea</i> , <i>G. aggregans</i> , <i>G. arctica</i> , <i>G. mallensis</i> , <i>G. sapmiensis</i> , <i>G. tundricola</i>)	aerobe	3.0 - 7.5 (3.8 - 4.5)	Various sugars and polysaccharides	<i>Sphagnum</i> peat and <i>Cladonia</i> sp.; Tundra soil	Pankratov & Dedysh, 2010 ; Mannistö et al., 2012
	<i>'Acidipla'</i> (<i>'A. rosea'</i>)	aerobe	3.0-6.0 (4.5)	Sugars, gluconate, some amino acids	Acid mine drainage and acidic soil	Okamura et al., 2011

	<i>Bryocella</i> (<i>B. elongata</i>)	aerobe	3.2-6.6 (4.7-5.2)	Sugars and some polysaccharides	<i>Sphagnum</i> peat	Dedysh et al., 2012
	<i>Acidicapsa</i> (<i>A. borealis</i> ; <i>A. ligni</i>)	aerobe	3.5-7.3 (4-5.5)	Sugars, organic acids and polysaccharides	<i>Sphagnum</i> peat and wood decay	Kulichevskaya et al., 2012
	<i>Telmatobacter</i> (<i>T. bradius</i>)	facultative anaerobe	3.0 - 7.5 (4.5 – 5.0)	Sugars and some polysaccharides including cellulose	<i>Sphagnum</i> peat	Pankratov et al., 2012
3	<i>Bryobacter</i> (<i>B. aggregatus</i>)	aerobe	4.5 - 7.2 (5.5 - 6.5)	Sugars, some polysaccharides, galacturonic and glucuronic acids	<i>Sphagnum</i> peat	Kulichevskaya et al., 2010
8	<i>Holophaga</i> (<i>H. foetida</i>)	anaerobe	5.5 - 8.0 (6.8 - 7.5)	Aromatic compounds	Anoxic freshwater mud	Liesack et al., 1994
	<i>Geothrix</i> (<i>G. fermentans</i>)	anaerobe	ND	Organic acids, long-chain fatty acids	Petroleum- contaminated aquifer	Coates et al., 1999
	<i>Acanthopleuribacter</i> (<i>A. pedis</i>)	aerobe	5.0 - 9.0 (7.0 - 8.0)	Glucose and several amino acids	Chiton <i>Acanthopleura</i> <i>japonica</i>	Fukunaga et al., 2008
10	<i>'Thermotomaculum'</i> (<i>'T. hydrothermale'</i>)	anaerobe	5.5-8.5 (6.6)	Proteinaceous substances	Deep sea hydrothermal vent chimney	Izumi et al., 2012

* Originally described as a strict aerobe by Kishimoto et al. (1991). Later it was shown that *A. capsulatum* is a facultative aerobe capable of fermentation (Blöthe et al. (2008); Coupland & Johnson (2008) and Pankratov et al. (2012). ND – not determined.

eight of which comprise complete sequences. Three of these genomes have been studied in more detail in a first comparative genomics effort, namely *Acidobacterium capsulatum*, ‘*Koribacter versatilis*’ Ellin345 from subdivision 1 and ‘*Solibacter usitatus*’ Ellin6076 from subdivision 3 (Ward et al., 2009). More recently, the complete genome sequence of the chlorophyll-based photoheterotroph *Candidatus Chloracidobacterium thermophilum* (subdivision 4) was determined from a highly enriched population (95%) of *C. thermophilum* cells, as this organism can so far only be grown as a defined co-culture with two heterotrophic microorganisms, *Anoxybacillus* sp. and *Meiothermus* sp. (Garcia Costas et al., 2012). Furthermore, genomes of *Terriglobus saanensis* and *T. roseus*, *Granulicella mallensis* MP5ACTX8 and *G. tundricola* MP5ACTX9 have recently been completed and compared with other acidobacterial genomes (Rawat et al., 2012). The publicly available genomes differ largely in size, and plasmids were only identified for *Granulicella tundricola* MP5ACTX9 (Table 1). The largest genome is that of the subdivision 3 isolate Ellin 6076 with almost 10Mbp (Table 1). Estimation of genome sizes of additional isolates from subdivisions 1 and 3 using pulse field electrophoresis confirmed that indeed genomes of subdivision 3 representatives occur larger than those of subdivision 1 (Challacombe et al., 2011). Interestingly, the genome of *Candidatus Chloracidobacterium thermophilum* was shown to comprise of two chromosomes, which both harbor essential genes, and thus are true chromosomes rather than megaplasms (Garcia Costas et al., 2012). Genome analysis revealed that all acidobacterial genomes analyzed to date have only one or two 16S rRNA gene copies (Table 1), in line with the slow growth rates observed for cultured isolates in nutrient limited environments (Klappenbach et al., 2000; Stevenson et al., 2004; Ward et al., 2009). Furthermore, the limited number of genomes available to date reflects the ability of *Acidobacteria* to utilize a broad range of complex carbon substrates and reveal *Acidobacteria* as decomposers that potentially participate in the cycling of plant, fungal, and insect organic matter. The genomes all harbor numerous genes that can be linked to the usage of sugars, alcohols and amino acids, but also of complex substrates such as pectin, xylan, starch, hemicellulose and chitin. The genomes contain an impressive number of glycosyl hydrolases, being within the top 5% of all annotated genomes in 2009 (Ward et al., 2009). Interestingly, the recent elucidation and comparative analysis of three genomes from arctic tundra isolates has revealed that, in comparison with previously characterized genomes of mesophilic acidobacteria, their genomic content of genes coding for members of the carbohydrate-active enzyme (CAZyme) family is highly enlarged (Rawat et al., 2012). In addition to their ability to scavenge organic polymers as carbon sources from otherwise nutrient poor environments, genome analysis also indicated that *Acidobacteria* should be able to oxidize carbon monoxide as evidenced by the presence of an aerobic CO dehydrogenase. This was also the case for the genome of the phototrophic *Candidatus*

Chloracidobacterium thermophilum, which further lacks the necessary machinery for carbon fixation, confirming its lifestyle as photoheterotroph (Garcia Costas et al., 2012). Genomic evidence suggested that *Acidobacteria* might obtain iron via interaction with other microorganisms, as a number of siderophore receptor encoding genes was identified, whereas complete gene sets encoding siderophore production could not be found. Furthermore, there are indications for the ability to uptake and oxidize ferrous iron, whereas *Acidobacteria* don't have the full gene set for dissimilatory ferric iron reduction found in iron-reducing bacteria such as *Shewanella* and *Geobacter* spp (Ward et al., 2009). The presence of genes predicted to encode macrolide glycosylases and polyketide synthases suggest that they might also be producers of yet-uncharacterized antimicrobial compounds. This was further confirmed by the analysis of metagenomic libraries derived from agricultural soil (Parsley et al., 2011). The three well-characterized genomes of *Acidobacterium capsulatum*, '*Koribacter versatilis*' Ellin345 and '*Solibacter usitatus*' Ellin6076 contained candidate genes from the acid tolerance AR3 system, suggesting that they have a tolerance for acidic conditions. It should be noted, however, that genes encoding components of other known AR systems were lacking, and that differences in acid tolerance between members of different acidobacterial subdivisions were not reflected in coding sequences (Ward et al., 2009). The ability to synthesize extracellular cellulose indicates that at least these three members of the *Acidobacteria* might have the ability to survive repeated cycles of rehydration and drying. The genome of the *Candidatus Chloracidobacterium thermophilum* further supports its ability to derive its energy by photosynthesis. Genes predicted to code e.g. for bacteriochlorophylls as well as chlorophyll, type-1 reaction centres and carotenoids were identified, some of which are located on the smaller chromosome 2 (Garcia Costas et al., 2012). These findings further support recent biochemical studies that identified and characterized these components (Bryant et al., 2007; Tsukatani et al. 2010; Garcia Costas et al., 2011; Wen et al., 2011; Garcia Costas et al., 2012b; Tsukatani et al, 2012). Interestingly, genome comparison with other acidobacteria, and members of other phyla, using best Blast P hit analysis, indicated that only 19% of all genes of *Candidatus Chloracidobacterium thermophilum* have closest neighbors in other acidobacterial genomes, but were in turn more related to those found in genomes of representatives of other chlorophototrophic groups including *Proteobacteria*, *Cyanobacteria* and *Chloroflexi* (Garcia Costas et al., 2012). According to the currently available genome information (Table 1), only the genomes of *Acidobacterium capsulatum* and *Candidatus Chloracidobacterium thermophilum* contain clustered regularly interspaced short palindromic repeats (CRISPR) sequences, whereas several CRISPR-associated sequences were also found in other genomes. CRISPR sequences guide the degradation of either DNA or RNA from invading viruses or plasmids (van der Oost et al., 2009). Interestingly, the CRISPR sequences found in *Candidatus Chloracidobacte-*

rium thermophilum seem to reflect its ecological niche, i.e. microbial mats in alkaline hot springs based on sequence similarity to viruses found in those environments (Garcia Costas et al., 2012).

Recently, application of metagenomics approaches has provided the first insight in the genomic characteristics of yet uncultured acidobacterial subdivisions. The first description of acidobacterial genome fragments obtained from a metagenome library was provided by Liles et al. (2003). In total 24,400 BAC clones from a soil metagenome library were screened, twelve of which were found to be derived from members of the phylum *Acidobacteria* (9 clones from subdivision 6, 2 from subdivision 4, and 1 from subdivision 5). A single BAC insert from subdivision 5, for which still no cultured representative is available, was selected for full-length sequencing, giving the first glimpse into the genome of an uncultured bacterium from the phylum of *Acidobacteria*. The authors 20 ORFs, including several predicted genes that are responsible for the biosynthesis of secondary metabolites, cell division and cell cycling and substrates metabolism. The first metagenome study specifically targeting the *Acidobacteria* was done by Quaiser et al. (2003). These authors constructed a large insert library to gain more insight into the phylum *Acidobacteria*. Comparative sequence analysis indicated a specific evolutionary relationship of *Acidobacteria* with *Proteobacteria* based on the observation that orthologous protein-encoding sequences shared a common root. More recently, Quaiser and co-workers recovered six acidobacterial fosmid clone fragments from a sandy ecosystem, followed by an additional 11 clones from deep-sea environments (Quaiser et al., 2008). Using rRNA operons as phylogenetic anchor sequences, a number of clones was found to be affiliated with subdivision 6, and contained genes predicted to encode proteins involved in purine biosynthesis. Based on elevated sequence similarities, data furthermore suggested events of horizontal gene transfer between Rhizobiales and Acidobacteria. Recently, Kielak et al. (2009) constructed a large insert fosmid library (28,800 clones) to recover additional genomic information from *Acidobacteria* in soil. Seventeen positive clones were selected for full-length sequencing, followed by annotation of ORFs. The majority of clones was found to be derived from subdivision 6 (10 clones). These authors also showed that the diversity of sequences of subdivision 6 derived from soil environments is much larger than of those recovered so far from aquatic environments (Kielak et al., 2009).

The analysis of fosmid-end sequences from a metagenomic library of Mediterranean plankton sampled at a depth of 3000-m revealed a high percentage of *Acidobacteria*-related sequences, suggesting that members of this phylum could be important players in the microbial community at these depths (Martin-Cuadrado et al., 2007). A high percentage of genes encoding carbon monoxide dehydrogenases, cox genes, were found, suggesting that aerobic carbon monoxide oxidation may be important in the deep ocean. Interestingly, cox genes are also present in the genome sequence of

the acidobacterial isolates ‘*Solibacter usitatus*’ and ‘*Koribacter versatilis*’ Ellin345 (Ward et al., 2009). In an additional study, Quaiser et al. (2008), screened three metagenomic deep-sea libraries from the Mediterranean, showing an unexpectedly high relative proportion of acidobacterial fosmids, which were affiliated to the *Solibacterales* (subdivision 3), to subdivision 11 and, most frequently, to subdivision 6. These results suggest that *Acidobacteria* are abundant members of the communities in the deep-sea ecosystem (Quaiser et al., 2008).

Taken together, these data suggest that *Acidobacteria* are well adapted to deep oceanic waters, increasing their relative abundance in the deepest part of the water column to values that come close to those observed in soils. It is tempting to speculate that the increase of *Acidobacteria* with depth might be related to their metabolic capacities. They may be specialized in the degradation of specific recalcitrant compounds, which are the only organic carbon sources available at very high depths.

MOLECULAR ECOLOGY TECHNIQUES FOR SPECIFIC DETECTION OF ACIDOBACTERIA

Despite some recent progress in culturing acidobacteria, commonly used cultivation-based techniques are not appropriate for assessing their diversity, distribution and abundance in the environment. Cultivation-independent, rRNA- and rRNA gene-based molecular surveys that were developed for specific detection of acidobacteria are reviewed below.

PCR-based approaches

The first PCR-based *Acidobacteria*-specific assay was developed by Barns et al. (1999). Based on available 16S rRNA gene sequences of acidobacteria, the forward primer 31F (5'- GAT CCT GGC TCA GAA TC-3') that specifically amplified rRNA genes from members of this phylum when used with a conserved reverse bacterial primer was designed. These primers have been used i) to generate clone libraries that specifically target acidobacterial diversity in various environments (Barns et al., 1999; Barns et al., 2007; Bryant et al., 2008; Jones et al., 2009; Kielak et al., 2009; Eichorst et al., 2011); ii) to screen isolation plates for the presence of acidobacteria (Stevenson et al., 2004), iii) to develop group-specific denaturing gradient gel electrophoresis (Boon et al., 2002), and iv) to quantify acidobacterial abundances in environmental samples (Felske et al., 2000; Fierer et al., 2005; Fierer et al., 2007). Barns et al. (1999) noted, however, that the primer 31F is not suitable for detection of the *Holophaga-Geothrix* group (subdivision 8 according to Hugenholtz et al., 1998). This was further confirmed by Jones and co-authors (2009), who compared the relative abundances of *Acidobacteria* subdivisions detected using pyrosequenc-

ing with universal bacterial primers and clone libraries obtained with the acidobacteria-specific primer set (31F + 1492r). This comparison showed that acidobacterial subdivisions 2, 7, 8, 10, 16, 22 and 25 were detected using pyrosequencing but were missing in the clone libraries. Similar results were obtained by Kielak et al. (2009), who compared the acidobacterial diversity in clone libraries obtained with universal bacterial primers and the acidobacteria-specific primer set (31F + 1378r). In this case, sequences belonging to subdivisions 2, 7 and 10 were also found only in the general bacterial libraries. The examination of the 31F primer-binding sites within the various *Acidobacteria* subdivisions revealed several mismatches in a majority of sequences from subdivisions 2, 7, 8 and 10 (Kielak et al., 2009). Therefore, the acidobacteria-specific primer 31F appears to be not as universal as thought before and detection of several subgroups within the *Acidobacteria* might require development of alternative PCR primer systems. One of the alternative primer sets, Acg8f and Acg8r, was recently designed for specific detection and quantification of a subset of acidobacteria within subdivision 8 (da Rocha et al., 2010).

FISH-based detection and enumeration

The oligonucleotide probe HoAc1402 (5'-CTT TCG TGA TGT GAC GGG-3') was originally designed and applied for specific detection of acidobacteria in an activated sludge from industrial sewage treatment plant using fluorescence in situ hybridization (FISH) (Juretschko et al., 2002). Further probe-match checks revealed that the target specificity of this probe is quite wide and includes different subdivisions of the *Acidobacteria*. For example, most of the acidobacterial clone sequences retrieved from acidic Sphagnum peat bog (subdivisions 1, 3, 4 and 8) had no mismatches in the target region of probe HoAc1402 (Dedysh et al., 2006). This finding allowed enumerating acidobacteria by FISH in native peat sampled from nine different Sphagnum-dominated wetlands of Northern Russia (Pankratov et al., 2008). The number of cells detected by HoAc1402 ranged from 0.4×10^6 to 1.3×10^7 cells per g of wet peat, comprising up to 4% of total bacterial cells. The same FISH-based approach was successfully used to screen isolation plates for the presence of acidobacteria, which greatly simplified the isolation procedure (Dedysh et al., 2006, 2011; Pankratov et al., 2008).

Two alternative phylum-specific oligonucleotide probes, 23S rRNA-targeted LS_HOL189 (5'-CTG AGA TGG TTC ACT TCC-3') and 16S rRNA-targeted SS_HOL1400 (5'-TTC GTG ATG TGA CGG GC-3'), were applied for detection of acidobacteria in microbial mats from a chemolithoautotrophic cave ecosystem (Meisinger et al., 2007). The target region of SS_HOL1400 is nearly the same as for HoAc1402. Several additional probes were designed in this study to specifically target particular groups of sequences within acidobacterial subdivisions 7 and 8 that were detected in this unique cave ecosystem. Application of these probes showed

that acidobacteria constituted approximately 5-10% of all bacteria in the samples that were suitable for FISH. Application of FISH, therefore, allows visualizing and enumerating difficult-to-culture acidobacteria in complex microbial communities. It should be noted, however, that the applicability of the phylum-specific probe(s) may depend on the ecosystem type and the diversity range of indigenous acidobacteria. In addition, this approach does not detect dormant cells, so that the population size of acidobacteria might remain underestimated by FISH.

ROLES IN NATURAL ENVIRONMENTS

Degradation of plant-derived polymers

Decomposition of plant debris is one of the key processes mediated by microorganisms in the global carbon cycle. Until recently, the major research interest was focused on fast-growing hydrolytic bacteria from well-characterized phyla, such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. As we start realizing now, the possible role of other organisms, particularly, the *Acidobacteria*, in degrading plant polymers may have remained hidden for a long time due to their low growth rates.

Many characterized subdivision 1 and 3 acidobacteria appear to be capable of degrading various plant-derived polymers, such as pectin, xylan, laminarin, lichenan and starch (Eichorst et al., 2007; Koch et al., 2008; Kulichevskaya et al., 2010; Pankratov and Dedysh, 2010; Männistö et al., 2011). The experiments with native Sphagnum-derived peat also showed substrate-induced growth of acidobacteria after amendment of the sample with xylan, pectin and starch (Pankratov et al., 2008).

Telmatobacter bradus is the only currently characterized cellulolytic acidobacterium (Pankratov et al., 2012). By degrading cellulose under micro-oxic or anoxic conditions, this peat-inhabiting bacterium avoids competition with cellulolytic fungi, since their activity strongly depends on oxygen availability. The products of cellulose degradation by *Telmatobacter bradus* in anoxic conditions are acetate and hydrogen. Two uncharacterised subdivision 1 acidobacteria, strains KBS 83 and CCO287, and subdivision 3 acidobacterium KBS 96, were also recently reported to possess cellulolytic potential (Eichorst et al., 2011; Pankratov et al., 2011). The actual rates of cellulose decomposition by these bacteria are very low and are not comparable to those in well-characterized cellulose degraders. The quantitative contribution of the acidobacteria to cellulose degradation in the environment, therefore, is difficult to assess. In cold and acidic northern wetlands where well-known cellulose degraders are absent, acidobacteria seem to represent important members of the indigenous hydrolytic microbial community (Pankratov et al., 2011). A similar observation was made with regard to another type of acidic water-logged habitat,

such as a primary tropical peat swamp forest in southern Thailand (Kanokratana et al., 2011). Metagenomic analysis of the microbial community in the surface peat layer revealed a variety of putative genes encoding a range of cellulolytic, hemicellulolytic, and amilolytic enzymes from the *Acidobacteria*, suggesting a key role of these microbes in plant debris degradation.

Iron cycling

Acidobacteria appear to be particularly abundant in Fe-rich acidic environments (Barns et al., 2007; Rowe et al., 2007; Blöthe et al., 2008; Kleinstauben et al., 2008; Lu et al., 2010). Among taxonomically characterized members of this phylum, the ability to use Fe(III) as the electron acceptor in anaerobic respiration was demonstrated only for subdivision 8 acidobacterium *Geothrix fermentans* (Coates et al., 1999). Recently, however, *Acidobacterium capsulatum* DSM 11244T and several related subdivision 1 isolates were shown to be capable of dissimilatory Fe(III) reduction under strict anoxic or micro-oxic conditions (Blöthe et al., 2008; Coupland & Johnson, 2008; Lu et al., 2010). *Acidobacterium capsulatum* DSM 11244T formed Fe(II) at pHs ranging from 2.2 to 5 during glucose fermentation, but the dissimilatory reduction of Fe(III) was not coupled with growth (Blöthe et al., 2008). Thus, *Acidobacteria* seem to play a role in the cycling of iron in various ecosystems as it is documented (Lu et al., 2010).

Photosynthesis

An impressive discovery of chlorophyll-based phototrophy in a member of the *Acidobacteria* was made in the course of a metagenomic analysis of the microbial mats of alkaline siliceous hot springs in Yellowstone National Park (Bryant et al., 2007). This analysis revealed an unusual *pscA* gene sequence encoding the BChl *a*-binding apoprotein of type 1 reaction center, which was only distantly related to homologous sequences in known phototrophs with type 1 reaction centers, i.e. *Cyanobacteria*, *Chlorobi* and *Heliobacteria*. This suggested the existence of a previously unrecognized chlorophototroph in the mat community, which was further supported by the presence of the gene for the Fenna-Matthews-Olson (FMO) protein, a signature photon energy transfer protein found only in the photosynthetic complex of green sulfur bacteria. Unexpectedly, the 16S rRNA gene sequence derived from the same BAC clone affiliated with the phylum *Acidobacteria*, and several other genes encoded the NADH oxidizing and oxygen-reducing cytochrome oxidase complexes, which are characteristic of bacteria that respire with oxygen. This unusual bacteriochlorophyll-synthesizing acidobacterium, which was named *Candidatus Chloracidobacterium thermophilum*, was further enriched in a co-culture with a non-phototrophic *Anoxybacillus* sp. Isolation of a light-harvesting organelle from *Chloracidobacterium*, the chlorosome, and identification of the dominant pigments produced by this bacterium

confirmed its phototrophic nature (see genomic trait section above for additional details and references). The mosaic features of the *Candidatus* Chloracidobacterium thermophilum genome seem to be a result of horizontal gene transfer, since hot springs present an ideal habitat for gene swapping to occur (Raymond, 2008). Interestingly, the two chromosomes of the laboratory enrichment culture differ significantly in sequence and organization from the metagenome-derived assembly, indicating considerable genomic and functional diversity within this group (Garcia Costas et al., 2012). How widely Chloracidobacterium-like organisms are distributed in non-thermal environments remains an open question.

Evidence for C1-metabolism

None of the currently characterized acidobacteria is able to grow on C1 compounds and no direct evidence for the occurrence of a methylotrophic metabolism in members of this phylum is available. However, a clear response of acidobacteria to amendments with methane and methanol was noticed in several cultivation-independent studies (Radajewski et al., 2002; Kalyuzhnaya et al., 2008; Pankratov et al., 2008). In one of these studies, several 16S rRNA gene sequences affiliated with subdivision 1 of the *Acidobacteria* were retrieved from a ^{13}C -labelled DNA fraction obtained after amendment of acidic soil with ^{13}C -methanol (Radajewski et al., 2002). Another study reported activation of uncultured *Acidobacteria* in lake Washington sediment in response to methane (Kalyuzhnaya et al., 2008). Finally, experiments with acidic Sphagnum peat showed an increase in cell number of *Acidobacteria* after amendment of peat soil with methanol (Pankratov et al., 2008). All these results suggest that members of the *Acidobacteria* might be involved in the turnover of C1 compounds in natural environments.

Concluding Remarks

Currently, more than 12000 16S rRNA gene sequences of >1200bp have been deposited in public databases making up 26 subdivisions within the phylum of *Acidobacteria*. This is still in stark contrast to the relatively small number of characterized representatives, which so far come from only 4 subdivisions, with a majority of isolates coming from subdivision 1. Therefore, our knowledge about ecophysiology and genomics within the *Acidobacteria* remains limited. This discrepancy can in part be attributed to the fact that acidobacteria have been difficult to cultivate in the laboratory, and few molecular investigations have focused exclusively on this group.

To better understand their ecology, physiology and also their life history, culture-independent approaches were increasingly used during the last 20 years. Often, more

than 50% of the sequences obtained from clone libraries from different environmental samples belong to the *Acidobacteria*. This raises important questions regarding their ecological role and metabolic activities in the environment, as well as their interaction with other microbial groups residing in these ecosystems. The increasing application of functional (meta)genomics approaches offer the chance to study different subdivisions of *Acidobacteria* that previously remained unknown and uncharacterized. Up to date, there are only 9 genomes, covering representatives from subdivision 1, 3, 4 and 8. More functional studies will be needed in the future to fully exploit this genomic information.

Novel high-throughput screening methods such as metagenomics and single-cell sequencing approaches hold a great promise in expanding our understanding of the genetic and functional diversity of *Acidobacteria*, which can be applied towards the design of tailored high-throughput isolation and screening methods, especially focusing on those subdivisions for which to date neither culture representatives, nor genome sequence information, is available.

Acknowledgement

This is publication number DW-2012-1004 of the Darwin Center for Biogeosciences, which partially funded this project. S.N.D. was supported by the Program MCB RAS.

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CHAPTER 3

Chapter 3

Effect of Temperature Gradients on the Microbial Community Structure of Soils Near Hot Springs

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To be submitted

Abstract

Hot springs are excellent habitats to study the influence of environmental factors, including temperature and pH on the microbial community composition. We analysed soil samples taken along temperature gradients associated with two different hot springs in Surprise Valley, California, using barcoded pyrosequencing analysis of PCR-amplified 16S rRNA gene fragments. Diverse bacterial communities were obtained from Leonard's and Ray's hot springs. Most abundant phyla included *Proteobacteria* followed by *Chloroflexi*, *Cyanobacteria*, *Acidobacteria* and *Actinobacteria*. Using multivariate analysis, we showed that temperature ($p=0.022$) and distance ($p=0.032$) from the springs most strongly contributed to explaining observed variation in microbial community composition, whereas pH had only a minor additional effect ($p=0.116$). Furthermore, the relative abundance of several microbial groups correlated with the concentration of branched glycerol dialkyl glycerol tetraether membrane lipids, the main bacterial sources of which have yet to be identified.

Introduction

Soil is a complex ecosystem in which microorganisms play an important role (Coleman & Crossley, 1996). Soil contains up to one billion bacterial cells per gram. They are the key players that drive many soil functions, including the conversion of soil organic carbon to CO₂, fuelling the global carbon cycle. The rate of microbial CO₂ production depends on abiotic factors including temperature, soil moisture, pH and soil depth. Furthermore, an increase in soil temperature results in a shift in the composition of soil microbial communities towards populations better adapted to higher temperatures (Neidhardt et al., 1990; Ranneklev & Bååth, 2001; Pietikäinen et al., 2005).

Terrestrial hot springs are excellent natural environments to study the effect of temperature gradients on bacterial community composition. Hot springs are inhabited by a broad range of different microorganisms including photoautotrophic and chemolithoautotrophic guilds, and temperature has been shown to affect the diversity of microorganisms (Ward et al., 1998). Moreover, terrestrial hot springs contain microbial mats which harbour a highly diverse community including cyanobacteria, thermophilic denitrifying bacteria and *Chloroflexi* (Ward et al., 1987; Hollocher & Kristjansson, 1992; Ward et al., 1997).

Soils surrounding terrestrial hot springs decrease in temperature with increasing distance from the hot spring, and they are as such appropriate models to study microbial adaptation to changes in ambient temperature. Furthermore, these soils can form suitable environments to investigate the adaptation of the membrane composition of bacteria in response to environmental changes such as temperature and pH. For example, it has been shown that the distributions of branched glycerol dialkyl glycerol tetraether (GDGT) membrane lipids (Figure 1) are strongly related to soil temperature and pH. Therefore, these types of lipids are used to reconstruct historic temperatures and pH (Weijers et al., 2007; Peterse et al., 2009). This relationship was tested by analyzing soils along hot springs, which have a strong temperature gradient, and indeed a strong correlation was found between the methylation of branched tetraethers (MBT) and soil temperature (Peterse et al., 2009). However, neither the source of these compounds nor the exact mechanisms

of membrane adaptation have yet been fully established.

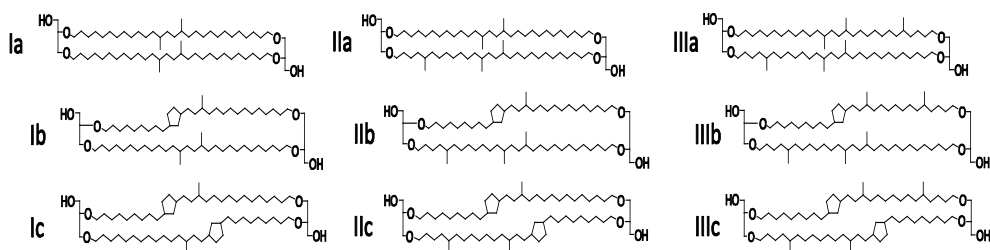


Figure 1. Molecular structures of branched GDGTs.

The purposes of this study i) to investigate the influence of temperature on the bacterial community composition, and ii) to identify bacterial groups potentially responsible for the observed production of branched GDGTs. Soil samples were taken along a temperature gradient adjacent to two different Californian hot springs, which were previously shown to contain different amounts of these lipids (Peterse et al., 2009). Barcoded pyrosequencing analysis of PCR-amplified 16S rRNA gene fragments was performed to characterize the microbial communities from the two different hot springs.

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Materials and Methods

Field site and sample collection

Samples were collected from two hot springs, Leonard's hot spring (41°36.086 N, 120°05.135W) and Ray's hot spring (41°31.855 N, 120°04.966 W), located at Surprise Valley in California, USA (Table 1; partly from Peterse et al., 2009). Soil temperature was measured with an Ama-Digit ad 20th digital thermometer in

the field. The upper 10 cm were taken for each sample, and they were stored in geochemical bags. Samples were immediately deep-frozen with dry ice in the field,

Table 1. Location, soil temperature, pH and branched GDGT concentration of two hot springs.

Location	Sample ID code	Distance (cm)*	Temperature (°C) [#]	pH	SUM branched GDGTs (µg/g dry wt soil)
Leonards's hot spring	D	sediment	58	8.4	0.01
	A	10	35.5	8.6	2.4
	B	30	28	8.4	0.39
	C	60	19.6	8.6	0.23
Ray's hot spring	E	10	41.6	7	0.23
	F	20	33	6.1	0.76
	G	40	25.2	6.9	0.15
	H	80	19.5	7	0.2
	I	250	12.4	7.7	0.01

*Distance from the spring, [#]temperature at 10 cm depth.

Branched GDGT analysis

Soil samples, freeze-dried and homogenized, were extracted (3 times for 5 min) with a solvent mixture of dichloromethane (DCM): methanol (MeOH) 9:1 (v/v) with an accelerated solvent extractor (ASE 200, Dionex) at 7.6×10^6 Pa and 100°C. It was dried under near-vacuum using a rotary evaporator. Each extract was dissolved in DCM and passed over a Na₂SO₄ column to remove all remaining water, dried again under a N₂ flow, and weighed before adding a known amount of C₄₆ GDGT standard (Huguet et al. 2006). They were then separated by passing them over an activated Al₂O₃ column using hexane:DCM 9:1 (v/v) and DCM:MeOH 1:1 (v/v) to obtain an apolar and polar fraction, respectively.

The polar fractions, containing the branched GDGTs, were dried under N₂, ultrasonically dissolved in hexane: isopropanol 99:1 (v/v) at a concentration of about 3 mg/ml, and filtered over a 0.45 µm PTFE filter prior to analysis by high performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (HPLC/APCI-MS) on an Agilent 1100 series LC/MSD SL according to Schouten et al. (2007), with minor modifications. Separation of the branched GDGTs was achieved on an Alltech Prevail Cyano column (150 mm x 2.1 mm; 3 µm). The

compounds were eluted isocratically at a flow rate of 0.2 ml/min with 90%A and 10%B for 5 min and then with a linear gradient to 16% B in 34 min, where A=hexane and B=hexane: isopropanol 9:1 (v/v). The injection volume was 10 µl for all extracts. Selective ion monitoring of the $[M+H]^+$ was used to detect and quantify the different branched GDGTs, and absolute quantification of each compound was achieved by calculating the area of its corresponding peak in the chromatogram, comparing it with that of the internal standard, and correcting it for the different response factors (Huguet et al. 2006).

Nucleic acid extraction

DNA was isolated directly from frozen soil samples (0.5 g) using FastDNA®SPIN kit for soil (MP Biomedicals, Solon, OH, USA). The DNA yield was measured with a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

16S rRNA gene amplicon pyrosequencing

Barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated by PCR using the 27F-DegS primer (van den Bogert et al., 2011) that was appended with the titanium sequencing adaptor A and an 8 nt sample-specific barcode (Hamady et al., 2009) at the 5' end, and an equimolar mix of two reverse primers (338R I and II (Daims et al., 1999); Table 2), that carried the titanium adaptor B at the 5' end.

Table 2. Oligonucleotide primers and sequence adapters used for this study

Primer ^a	Oligonucleotide sequence (5'-3') ^b	Reference
Adaptor A	CCATCTCATCCCTGCGTGTCTCCGACTCAG	Provided by GATC-Biotech
Adaptor B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	Provided by GATC-Biotech
27F-DegS	GTTYGATYMTGGCTCAG	Van den Bogert et al., 2011
338R-I	GCWGCCTCCCGTAGGAGT	Daims et al., 1999
338R-II	GCWGCCACCCGTAGGTGT	Daims et al., 1999

^a Primer names may not correspond to original publication. ^b M = A or C; R = A or G; W = A or T; Y = C or T

After DNA extraction, DNA was diluted to a concentration of 20 ng/µl based on Nanodrop readings. PCR was performed using a GS0001 thermocycler (Gene

Technologies, Braintree, United Kingdom). The PCR mix (100 μ l final volume) contained 20 μ l of 5 \times HF buffer (Finnzymes, Vantaa, Finland), 2 μ l PCR Grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 1 μ l of Phusion hot start II High-Fidelity DNA polymerase (2U/ μ l) (Finnzymes), 500 nM of the reverse primer mix and the forward primer (Biolegio BV, Nijmegen, The Netherlands) (Table 2), 2 μ l (i.e. 40 ng) template DNA, and 65 μ l nuclease free water. PCR was run under the following conditions: 98 °C for 30 s to activate the polymerase, followed by 30 cycles consisting of denaturation at 98 °C for 10 s and annealing at 56 °C for 20 s and elongation at 72 °C for 20 s, and a final extension step at 72 °C for 10 min. Five μ l of the PCR products, approximately 375 bp in length, were analyzed by 1% (w/v) agarose gel electrophoresis, containing 1 \times SYBR® Safe (Invitrogen, Carlsbad, CA, USA) purified from gel using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to manufacturer's instructions. DNA concentration of gel-purified amplicons was measured by Nanodrop® ND-1000 spectrophotometer, and purified PCR products were mixed in equimolar amounts and run again on an agarose gel with following excision and purification by using a second DNA gel extraction kit (Milipore, Billerica, MA, USA). A mixture of 44 samples, including those described here, was analyzed by pyrosequencing on half a plate using a FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

Analysis of the pyrosequencing data

Pyrosequencing data was analyzed using the QIIME 1.3.0 pipeline (Caporaso et al., 2010). Low quality sequences were removed using default parameters, and operational taxonomic units (OTUs) were defined at a 97% identity level. A representative sequence from each OTU was aligned using PyNAST (DeSantis et al., 2006). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% (Wang et al., 2007). Possible chimeric OTUs were identified using QIIME's ChimeraSlayer (Haas et al., 2011) and subtracted from the previously generated OTU list, producing a non-chimeric OTU list.

PAST software (version 1.82b) (Hammer et al., 2001) was used for the calculation of statistical indexes of diversity and richness of bacterial sequences in each data set.

Multivariate analysis

A multivariate analysis was applied to assess to what extent environmental parameters influence the bacterial community composition. Redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) (Lepš & Šmilauer, 2003). The environmental variables tested were temperature, distance from the springs, pH and the concentration of the branched GDGTs. A Monte-Carlo permutation procedure (MCP) based on 999 random permutations was used to test significance of observations. Community structure was visualized via ordination triplots with scaling focused on inter-sample differences.

Results

The soils surrounding two different hot springs that are fed by geothermally heated water were studied. The soils were more or less similar in structure except for the samples taken closest to the springs which had a greyish blue color and sulfurous smell, indicating anoxic conditions. As previously reported by Peterse et al. (2009), soil temperature was found to decrease with increasing distance from the respective hot spring (Table 1). Soil temperature in Leonard's hot spring ranged from 35.5°C to 19.6°C at distances between 10 and 60 cm from the spring, and a temperature of 58°C was observed in the spring sediment. The water temperature was 89°C in Ray's hot spring, and the soil temperature ranged from 41.6°C to 12.4°C at distances ranging from 10 to 250 cm. The pH of Leonard's hot spring soils was relatively constant along the gradient, whereas this was not the case for samples taken adjacent to Ray's hot spring (Table 1; Peterse et al., 2009). Samples taken from Leonard's hot spring were more alkaline, whereas the samples from Ray's hot spring had a close to neutral pH. Peterse et al. (2009) previously reported the concentrations and distribution of branched GDGT (Table 1). They found the highest concentration close to the hot spring, at 10 cm distance from Leonard's hot spring and at 20 cm from Ray's hot spring.

Pyrosequencing analysis of 16S rRNA gene fragments

To obtain a general overview of the bacterial community composition, 16S rRNA gene amplicons pyrosequencing was performed. After removal of chimeric sequences, in total 29526 high quality sequences were obtained from the nine different samples studied here, and 8028 operational taxonomic units (OTUs) were identified. Good's coverage value, an estimation of the proportion of the community represented by retrieved bacterial sequences indicated Dominance (D) values close to zero, suggesting that no OTUs were predominant in the bacterial community, which was confirmed by the high values of the Shannon-Wiener index (Table 3). Good's coverage ranged between 42 and 88%. The lowest Good's coverage was found for the sample taken at 10cm distance from Leonard's hot spring (42%) whereas the highest value was found for the corresponding hot spring sediment sample (88%).

Table 3. Comparison of the richness, diversity and Good's coverage values of the samples

	A*	B*	C*	D*	E*	F*	G*	H*	I*
Taxa S	1112	667	996	457	814	749	949	1593	1107
Individuals	1920	1864	2842	3669	4298	5398	3737	3267	2531
Dominance D	0.003	0.009	0.008	0.153	0.052	0.079	0.019	0.004	0.005
Shanon H	6.529	5.59	5.891	3.44	4.543	4.134	5.393	6.676	6.237
Good's Coverage	42	64	65	88	81	87	75	52	57

*A= pH:8.6-35.5°C, B= pH:8.4-28°C, C= pH:8.6-19.6°C, D= pH:8.4-58°C, E= pH:7-41.6°C, F= pH:6.1-33°C, G= pH:6.9-25.2°C, H= pH:7-19.5°C, I= pH:7-12.4°C

Redundancy analysis showed that temperature ($p=0.022$; Figure 2) and distance from the springs ($p=0.032$) significantly contributed to explaining the observed variation in bacterial community composition. Eigen values of the first and second RDA axes constrained to the environmental variables were 0.435 and 0.222, respectively, indicating that the set of environmental variables used in the analysis was able to explain 65,7% of the variation in the sequence data at phylum level. The relative abundance of the phyla *Bacteroidetes*, *Chlorobi*, *Chloroflexi*, and *Armatimonadetes* was positively correlated with temperature and decreasing distance from the spring (Figures 2, 3). Sequences belonging to the *Actinobacteria*, *Gemmatimonadetes*, *SPAM*, *Planctomycetes* and *Acidobacteria* were positively correlated with the

distance from the springs and negatively with temperature (Figure 2).

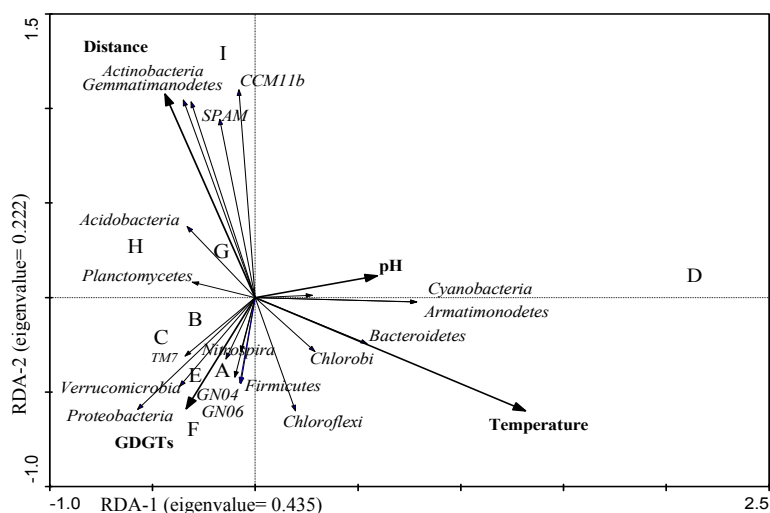


Figure 2. Ordination triplots for RDA analysis, using relative abundance of pyrosequencing reads classified into bacterial phyla. Arrows represent environmental parameters. Capital letters represent samples included in the analysis (A=pH 8.6-35.5°C; B=pH 8.4-28°C; C=pH 8.6-19.6°C; D=pH 8.4-58°C; E= pH 7.0-41.6°C; F=pH 6.1-33°C; G= pH 6.9-25.2°C; H=pH 7.0-19.5°C;; I=pH 7.7-12.4°C).

The relative abundance of the phyla *Cyanobacteria* and *Armatimonadetes* correlated most strongly with pH. Although the branched GDGT membrane lipids concentration could not be linked to the observed variation in microbial composition, the relative abundance of the phyla *Proteobacteria*, *Verrucomicrobia*, GN06, GN04, *Firmicutes*, and *TM7* showed some correlation with these lipids (Supplementary Tables S1 and S2).

Sample 5 clustered separately from the others, which could be expected based on the fact that it was taken from the sediment inside of Leonard's spring, rather than from surface soil adjacent to the spring.

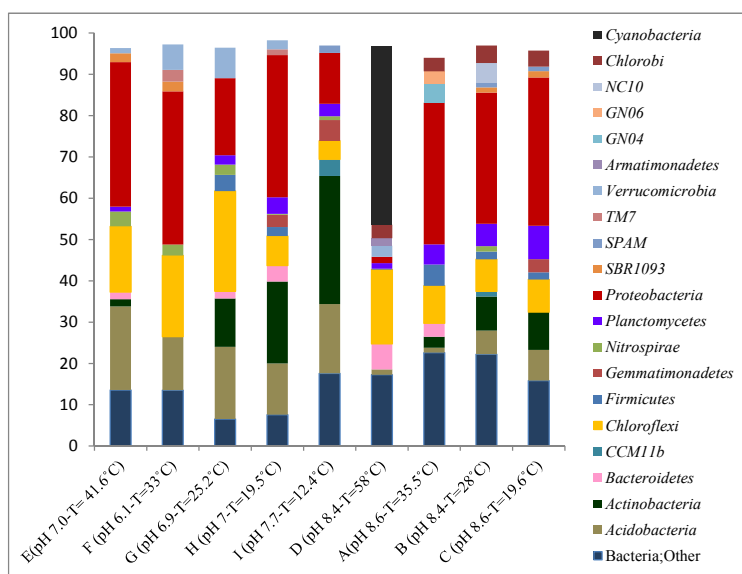


Figure 3. Relative abundance of bacterial phyla observed in samples collected from soils surrounding two hot springs. Samples F, G, E, H and I belonged to “Ray’s” spring, whereas samples D, A, B and C were taken from Leonard’s spring. Phyla represented by less than 1% of sequences are not shown. Detailed information can be found in the supplementary tables.

Leonard’s hot spring

In total four samples from Leonard’s hot spring were included in the analysis, one of which was taken directly from spring sediment, whereas the other 3 were soil samples collected at increasing distance from the spring (Table 1). 53 phyla were detected from sediment and soils associated with Leonard’s hot spring. The vast majority of sequences were classified into the phylum of *Proteobacteria* (26%) followed by *Cyanobacteria* (11%), *Chloroflexi* (10%), *Planctomycetes* (5%), *Actinobacteria* (5%), *Acidobacteria* (4%), *Chlorobi* (3.6%) and several unclassified bacteria (Table S1). The rest of sequences clustered within other phyla that represented only a minor proportion (Figure 3; Table S1). The highest branched GDGT content was determined in the sample at 10 cm distance from the spring (pH 8.6, 35.5°C; Peterse et al., 2009), which was dominated by deltaproteobacterial sequences (18%). Within the *Deltaproteobacteria*, we detected members of the

Desulfobacteraceae, *Synthophaceae* and *Synthrophobacteraceae* (Table S1). The microbial community associated with the hot spring sediment (pH 8.4 and 58°C) was less diverse compared to other samples (Figure 3). The result corroborates previous studies in which hot spring sediments were dominated by *Cyanobacteria* (43%) and *Chloroflexi* (18%) and several unclassified bacteria (Castenholz, 1969; War et al., 1998; Miller and Castenholz, 2000). We detected the phylum of *Proteobacteria* in a high proportion (35%) in all samples except for the sediment sample from spring itself, where only 1.5% of the sequences belonged to the phylum of *Proteobacteria*. Sequences belonging to certain groups within the *Deltaproteobacteria*, GN04, GN06, *Firmicutes*, and *Bacteroidetes*, *Caulobacterales* were only present in sample A (pH 8.6 and 35.5°C; Figure 3; Figure 4).

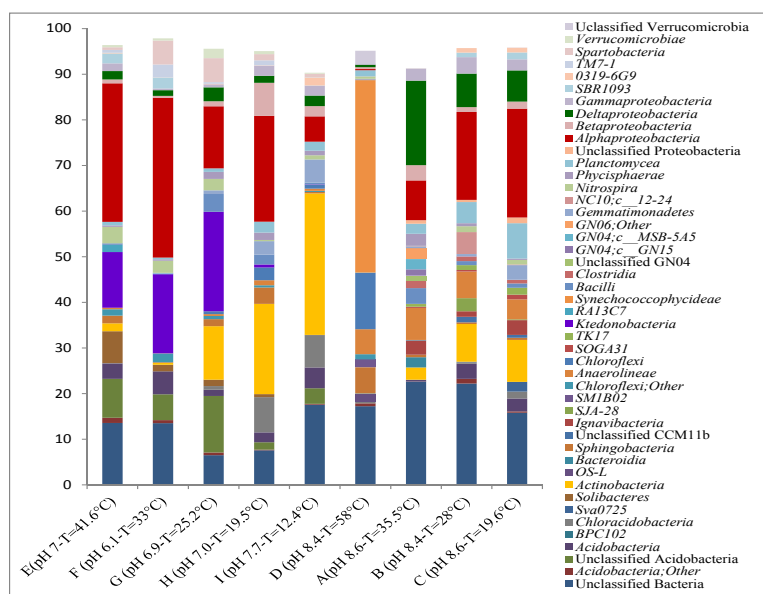


Figure 4. Relative abundance of bacterial classes observed in samples collected from soils surrounding two hot springs. Samples D, A, B and C were taken from Leonard's spring, whereas samples F, G, E, H and I belonged to Ray's spring. Class represented by less than 1% of sequences were removed. Detailed information can be found in the supplementary tables.

Ray's hot spring

In total five samples were analyzed at different distance from the spring, covering a range of temperatures and pH values (Table 1). 38 phyla were detected from Ray's hot spring soil samples. The majority of sequences were related to the *Proteobacteria* (27%) followed by sequences related to *Chloroflexi* (14%), *Acidobacteria* (12.4%), *Actinobacteria* (13%) and *Verrucomicrobia* (3.5%) and several unclassified bacteria (Figure 3, 4). The highest GDGT content was found in the sample taken at 20 cm distance from the spring. This sample has the lowest pH (pH 6.1) within the entire dataset and 3 major bacterial groups were detected, including *Proteobacteria* (37%), *Chloroflexi* (19%) and *Acidobacteria* (13%).

Discussion

Barcoded pyrosequencing of PCR-amplified 16S rRNA gene fragments was conducted to assess the effect of distance from hot springs, soil temperature and pH on microbial community composition in soils taken around two hot springs. As gradients of temperature and pH have previously been shown to correlate with the composition and concentration of bacterial branched GDGT membrane lipids (Peterse et al., 2009), we further aimed to obtain new leads regarding the bacterial groups potentially responsible for the production of these lipids. This study showed that temperature and distance from the spring most strongly contributed to explaining the observed variation in bacterial community composition.

The abundance of *Bacteroidetes*, *Actinobacteria*, *Acidobacteria* and *Planctomycetes* was negatively correlated with temperature in Leonard's hot spring (mildly alkaline), whereas *Acidobacteria* were positively correlated with temperature at Ray's hot spring (acidic to neutral in pH). The phylum of *Bacteroidetes* was most strongly affected by temperature, albeit to a different extent in the two springs studied here (Figure 2). Member of the *Bacteroidetes* are widely distributed in marine and terrestrial environments including soils, hydrothermal vents, and they are the most dominant populations in gastrointestinal microbiota (Bowman et al, 1997; Whitman et al., 1998; Cotrell & Kirchman, 2000; Ramsak et al., 2000; Buckley & Schmidt, 2003; Bäckhed et al., 2005; Reichenbach, 2006). The phylum of *Bacteroidetes*

contribute to carbon cycling especially in cold habitats, which was supported by the observation that abundances of *Bacteroidetes* decrease with increasing ambient temperature (Kirchman, 2002; Junge et al., 2004).

The phylum of *Chloroflexi* showed similar behavior in the two hot springs. The highest numbers of *Chloroflexi* sequences were detected in the sediment of Leonard's hot spring (18%), and their numbers were higher at increasing temperature and decreased distance from the spring. The highest numbers of *Chloroflexi* sequences were detected at pH 6.9 and 25.2°C at Ray's hot spring and were lower in the lower temperature samples. The results of the *Chloroflexi* sequences are in line with previous studies showing that hot spring sediments are dominated by *Chloroflexi* and *Cyanobacteria* (Ward et al., 1998; Roeselers et al., 2007; Schouten et al., 2009; Finkelstein et al., 2010).

Based on RDA analysis, we found that the relative abundance of sequences belonging to a number of phyla including *Proteobacteria*, GN04, GN06, *Nitrospira*, *Firmicutes* were correlated with the content of branched GDGT lipids. However, lipid concentrations only marginally contributed to explaining variance in microbial composition in addition to other environmental factors ($p = 0.332$ based on MCPP with forward selection). Oppermann et al. (2010) and Weijers et al. (2010) suggested that bacterial producers of branched GDGT membrane lipids have a heterotrophic lifestyle. This is in line with data presented here as we detected mainly heterotrophic phyla within the *Alpha*-, *Beta*-, *Deltaproteobacteria*, *Bacteroidetes* and *Acidobacteria*. In the present study, several bacterial groups were only detected in the soil with the highest branched GDGT concentration, at relative abundances larger than 1%, including *Bacteroidales* (1.6%), *Ignavibacteriaceae* (2.8%), unclassified-*Anaerolineae* (3.7%), *Bacillaceae* (2.7%), unclassified GNO4 (1.0%), unclassified GN15 (1.4%), unclassified *MSB-5A5* (2.2%), unclassified GN06 (2.4), unclassified *Phycisphaerae* (2.8%), unclassified- *Alphaproteobacteria* (1.2%), unclassified *Deltaproteobacteria* (5.3%), *Desulfobulbaceae* (1.5%), *Desulfobacteraceae* (4.8%), and *Syntrophaceae* (1.9%) (Table S1, S2). Among these are a number of only recently proposed taxa, such as the phyla GN04 and GN06, for most of which no information is available regarding their physiological properties and membrane lipid composition.

Surprisingly, a high proportion of deltaproteobacterial sequences (18%) were detected in the sample containing the highest amount of branched GDGT at pH 8.6 and 35.5°C. It is known that *Alpha*-, *Beta*- and *Gammaproteobacteria* are generally more dominant than the class of *Deltaproteobacteria* in soils (Castro et al., 2009). However, their function in specific soils is often unknown. The class of *Deltaproteobacteria* includes Gram-negative, sulfate-reducing bacteria (SRB) and syntrophic fatty acid-oxidizing anaerobes (Harmsen et al., 1998; Van Kuijk and Stams, 1995; Plugge et al., 2011). Certain long chain alcohols and straight chain and methyl-branched fatty acids are biomarkers of these sulfate-reducing bacteria (Hinrichs et al., 2000; Pancost et al., 2000; Zhang et al., 2002). In addition, mono and dialkyl glycerol ethers are also well-known biomarkers of sulfate-reducing bacteria (Orphan et al., 2001; Werne et al., 2002). Members of the family of *Syntrophobacteraceae* produce both ester- and ether- bound alkyl chains within one glycerol membrane lipid (Rütters et al., 2001). Moreover, previous studies suggested that members of the *Syntrophobacteraceae* are able to synthesize monoalkyl and dialkyl ether lipids (Hinrichs et al., 2000; Pancost et al., 2001). Weijers et al. (2009) detected in Swedish peat bogs one sequence from the class of *Deltaproteobacteria*, while the remaining 13 sequences belonged to the phylum of *Acidobacteria*. Potentially, species within the broad class of *Deltaproteobacteria* might be additional candidates as a source for these orphan lipids.

We further zoomed in to the phylum of *Acidobacteria* to follow up on previous indications for their involvement in synthesis of branched GDGT (Weijers et al., 2009; Peterse et al., 2010; Sinninghe Damsté et al., 2011). The average abundance of *Acidobacteria* was much higher in Ray's hot spring (15.9%) compared to Leonard's spring (3.9%), most probably due to the fact that members of the *Acidobacteria* prefer slightly acidic or neutral conditions rather than alkaline pH (Figure 3). In total 13 classes within the phylum of *Acidobacteria* were detected, namely *Acidobacteria*, *Acidobacteria-5*, BPC102, *Chloracidobacteria*, *Holophagae*, *MVS-40*, *OS-K*, *PAUC37f*, *RB25*, *Solibacteres*, *Sva0725*, *iii1-8*, unclassified *Acidobacteria*. Interestingly, within the *Acidobacteria*, chloracidobacterial sequences were negatively correlated with temperature, whereas sequences retrieved from *Solibacterales* showed a positive correlation (Supplementary Tables S1 and S2). Our study showed

that besides pH, also temperature affected the acidobacterial composition. Further research is needed to get insight into how bacteria of the order of *Solibacterales* and *Chloracidobacterales* are influenced by temperature changes. Overall, the results obtained from Leonard Hot spring, sequences assigned to the different acidobacterial classes did not show a significant correlation with the concentration of branched GDGT membrane lipids, and as such could not confirm previous studies (Weijers et al., 2009; Peterse et al., 2009, Sinninghe Damsté et al., 2011). Since peat bogs have specific physical-chemical characteristics as compared to other habitats, including acidity and low nutrient contents, it is plausible to assume that observed differences in microbial composition are largely related to the differences in habitat characteristics. Hence other microbial groups might be responsible for the production of branched GDGTs in the hot spring environment studied here. Leonard's hot spring is alkaline and Ray's hot spring is slightly acidic. Thus, it is tempting to speculate that also the two different hot springs studied here might be different in terms of branched GDGT producing bacteria. This is further corroborated by previous observations that showed differences in the composition of branched GDGTs between both hot springs (Peterse et al., 2009). GDGT IIa was the most dominant class in the sample from Leonard's hot spring with the highest GDGT content, followed by GDGT Ib, IIb and Ia. In contrast, for Ray's hot spring, the sample with the highest GDGT content was enriched in GDGT Ia and IIa.

It is interesting to note that both hot springs differed in their composition of the associated acidobacterial communities. Members of the families of *Acidobacteriaceae* (5%) and *Koribacteraceae* (3.5%), *Solibacteraceae* (1.44%) that belong to subdivisions 1 and 3 were detected at pH 6.1 and 33°C. Sinninghe Damsté et al. (2011) showed that 2 heterotrophic strains from subdivision 1 and 3, *Edaphobacter aggregans* Wbg-1^T and *Acidobacteriaceae* strain A2-4c, produce branched GDGT membrane lipids. The results obtained from Ray's hot spring, the abundances of *Acidobacteria*, and detection of subdivisions 1 and 3, support the previous findings obtained with acidic soils and peats (Weijers et al., 2009; Peterse et al., 2010; Sinninghe Damsté et al., 2011).

In a study described in Chapter 4 of this thesis it was shown that certain species belonging to the *Acidobacteria*, *Alphaproteobacteria* (*Rhizobiales*) and

Deltaproteobacteria were only detected in high proportions in surface soils taken from the Têt watershed that were characterized by high concentrations of branched GDGT membrane lipids (2.9 – 41.7 µg/g dry wt soil). In line with these findings, certain groups of *Alpha* (*Rhizobiales*) - and *Deltaproteobacteria*, and *Bacteroidetes* (*Bacteroidales*), were only represented in the high content branched GDGT samples studied here, suggesting that they might contribute to the production of branched GDGT membrane lipids.

To conclude the present study showed that temperature as well as distance from hot springs is important abiotic factors affecting the microbial community composition in soils. To this end, this is the first report regarding the extent to which the distribution of specific classes of *Acidobacteria* is influenced by ambient temperature. Although the concentration of branched GDGT membrane lipids could not be directly linked to the observed variation in bacterial composition, members of the phyla *Proteobacteria*, *Verrucomicrobia*, GN06, GN04, *Firmicutes*, and TM7 showed stronger correlation with branched GDGT levels than other microbial groups. Future research should focus on selecting or isolating representatives of these groups to test for their ability to produce branched GDGT membrane lipids.

Acknowledgments

We thank the Darwin Center for Biogeosciences (142.16.2051) for financial support, and T. van den Bogert for providing help with technical aspects related to the analysis of pyrosequencing data.

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CHAPTER 3

Chapter 3

Supplemental Materials

Table 1. Detailed taxa summary from Leonard's hot spring. A*: 35.5°C, pH 8.6; B: 28°C, pH 8.4; C: 19.6°C, pH 8.6; D: 58°C, pH 8.4. Taxa represented by less than 1% of the sequences were removed.

Family level -Relative distribution (%) – Leonard's hot spring samples	A*	B	C	D
Bacteria;Other;Other;Other;Other	22.6	22.2	15.8	17.2
Acidobacteria;Other;Other;Other	0	1.0	0.3	0.6
Acidobacteria; Sva0725; Sva0725	-	0.1	2.1	-
Acidobacteria; Acidobacteria; Acidobacteriales	0.3	3.3	2.8	-
Acidobacteria; Chloracidobacteria	0.1	0.3	1.5	0.3
Actinobacteria; Actinobacteria;Other;Other	0.2	0.7	1.8	-
Actinobacteria; Actinobacteria; 0319-7L14	0.2	2.5	1.9	0
Actinobacteria; Actinobacteria; Actinomycetales;Other	0	1.5	1.5	-
Actinobacteria; Actinobacteria; Solirubrobacterales;	0	1.2	1.0	-
Armatimonadetes; OS-L	-	-	-	1.6
Bacteroidetes; Bacteroidia; Bacteroidales	1.6	-	-	1.1
Bacteroidetes; Sphingobacteria; Sphingobacteriales; Saprospiraceae	0.2	-	-	4.9
CM11b	-	1.2	0.6	-
Chlorobi; Ignavibacteria; Ignavibacteriales; Ignavibacteriaceae	2.8	1.2	3.2	0
Chlorobi; SJA-28	-	2.9	0.2	0.1
Chlorobi; SM1B02	-	-	-	1.6
Chloroflexi; Anaerolineae; Other; Other	3.7	2.8	0.9	0.2
Chloroflexi; Anaerolineae; BPC110	0	0.6	1.7	0
Chloroflexi; Anaerolineae; Caldilineales; Caldilineaceae	0.2	0.8	1.0	1.0
Chloroflexi; Anaerolineae; H39	-	0.2	-	1.9
Chloroflexi; Anaerolineae; WCHB1-50	-	-	-	1.7
Chloroflexi; Anaerolineae; envOPS12	1.3	0.2	0	0
Chloroflexi; Chloroflexi; Chloroflexales; Chloroflexaceae	-	-	-	12.3
Chloroflexi; SOGA31	0.2	0.3	1.0	-
Cyanobacteria; Other; Other	0.1	-	-	1.1
Cyanobacteria; Synechococcophycideae; Pseudanabaenales; Other	0	-	-	36.6
Cyanobacteria; Synechococcophycideae; Synechococcales; Other	-	-	-	5.5
Firmicutes; Bacilli; Bacillales; Bacillaceae	2.7	0	0.5	0
GN04; Other; Other; Other	1	-	-	-
GN04; GN15	1.4	0	0	-
GN04; MSB-5A5	2.2	-	-	0
GN06; Other; Other; Other	2.4	-	-	-
Gemmatimonadetes; Gemmatimonadetes	0.4	0.4	2.9	-
NC10; 12-24; Methylospirillales; Methylospirillaceae	-	4.5	0	-
Planctomycetes; Phycisphaerae	2.0	0.5	0.2	0
Planctomycetes; Planctomycea; Gemmatales; Gemmataceae	-	0.7	1.6	1.3
Planctomycetes; Planctomycea; Pirellulales	0.5	1.7	4.3	-
Proteobacteria; Other; Other; Other	0.8	0.5	1.2	-
Proteobacteria; Alphaproteobacteria; Other; Other	1.2	2.6	4.0	-
Proteobacteria; Alphaproteobacteria; Rhizobiales; Other	2.7	4.6	6.3	0
Proteobacteria; Alphaproteobacteria; Rhizobiales	0.4	4.4	4.9	-
Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyhomicrobiaceae	1.2	1.2	1.7	-
Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	1.0	3.0	3.2	0
Proteobacteria; Alphaproteobacteria; Rhodobacterales	-	1.1	0.2	-
Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae	0.4	1.8	2.0	-
Proteobacteria; Betaproteobacteria; Rhodocyclales	1.2	0.5	0.4	0.3
Proteobacteria; Deltaproteobacteria; Other; Other	5.4	0.5	0.3	0
Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae	1.5	0.3	0.8	0
Proteobacteria; Deltaproteobacteria; Syntrophobacterales; Desulfobacteraceae	4.8	1.0	1.2	0
Proteobacteria; Deltaproteobacteria; Syntrophobacterales; Syntrophaceae	1.9	0.5	0.4	0
Proteobacteria; Deltaproteobacteria; Syntrophobacterales; Syntrophobacteraceae	1.4	3.5	1.9	0.2
Proteobacteria; Gammaproteobacteria; Chromatiales; Other	0.5	2.1	0.7	0.2
SBR1093	-	1.0	1.5	0
SPAM; 0319-6G9	0	0.9	1	-
Verrucomicrobia; Other; Other; Other	0.1	-	0	2.7

Table 1. Detailed taxa summary from Ray's hot spring. E: 41.6°C, pH 7.0; F: 33°C, pH 6.1; G: 25.2°C, pH 6.9; H: 19.5°C, pH 7.0; I: 12.4°C, pH 7.7. Taxa represented by less than 1% of the sequences were removed.

Family level -Relative distribution (%) – Ray's hot spring samples	E*	F	G	H	I
Bacteria;Other;Other;Other;Other	13.6	13.5	6.5	7.6	17.6
Bacteria; Acidobacteria;Other;Other;Other	1.1	0.7	0.6	0.2	0.2
Acidobacteria;Other	1.0	1.8	3.3	0.3	0.2
Acidobacteria;	0.1	0.4	3.7	-	-
Acidobacteria; Koribacteraceae	7.5	3.6	5.4	1.3	3.2
Acidobacteria; Acidobacteria; Acidobacteriales	0.1	-	0.7	2.0	4.6
Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae	3.3	5.0	0.7	0.2	-
Acidobacteria; Chloracidobacteria	0.1	0.0	0.8	7.7	7.1
Acidobacteria; Solibacteres; Solibacterales; Solibacteraceae	7.0	1.4	1.4	0.7	-
Actinobacteria; Actinobacteria;Other;Other	-	-	0.2	0.7	2.0
Actinobacteria; Actinobacteria; 0319-7L14	0.1	0.1	0.2	0.4	2.3
Actinobacteria; Actinobacteria; Actinomycetales;Other	0.2	0.1	1.0	3.0	1.0
Actinobacteria; Actinobacteria; Solirubrobacteriales;	0.1	-	0.9	3.8	8.8
Actinobacteria; Actinobacteria; Acidimicrobiales; EB1017	-	0.1	1.4	0.2	0.2
Actinobacteria; Actinobacteria; Actinomycetales; Micromonosporaceae	-	-	0.3	1.5	0.1
Actinobacteria; Actinobacteria; Actinomycetales; Streptomycetaceae	0.4	-	0.7	1.0	0.5
Actinobacteria; Actinobacteria; MC47	0.6	0.1	5.9	2.7	13.0
Actinobacteria; Actinobacteria; Solirubrobacteriales;Solirubrobacteraceae	-	-	0.1	1.0	0.5
Bacteroidetes; Sphingobacteria; Sphingobacteriales	1.6	0.1	1.4	3.5	0.3
CM11b	-	-	-	-	4.0
Chloroflexi; Other; Other; Other	1.4	1.9	0.7	0.5	0.2
Chloroflexi; Chloroflexi; Roseiflaxales; Kouleothrixaceae	-	-	0.4	1.7	0.5
Chloroflexi; Ktedonobacteria	12.2	17.3	21.8	0.6	0.1
Chloroflexi; RA13C7	1.6	0.1	-	-	-
Firmicutes; Bacilli; Bacillales; Bacillaceae	0.1	-	-	-	-
Gemmatimonadetes; Gemmatimonadetes	-	-	0.2	0.4	1.9
Gemmatimonadetes; Gemmatimonadetes; Gemmatimonadales	-	0.1	0.1	0.4	1.9
Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae	3.2	0.2	2.5	0.2	0.9
Planctomycetes; Planctomycea; Gemmatales; Gemmataceae	0.6	0.2	0.6	1.1	0.7
Proteobacteria; Alphaproteobacteria; Rhizobiales;Other	23.6	29.2	2.3	4.9	1.5
Proteobacteria; Alphaproteobacteria; Rhizobiales; Beijerinckiaceae	2.9	2.0	0.3	-	-
Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae	0.3	0.5	1.5	9.0	0.8
Proteobacteria; Alphaproteobacteria; Rhizobiales;Hyphomicrobiaceae	0.6	1.1	4.3	1.9	1.0
Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	1.3	1.1	0.5	0.2	-
Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae	0.4	0.6	1.6	1.9	1.0
Proteobacteria; Betaproteobacteria	-	-	0.3	2.0	0.2
Proteobacteria; Betaproteobacteria; Burkholderiales; Commamonadaceae	-	-	0.2	1.6	0.1
Proteobacteria; Betaproteobacteria; Rhodocyclales	0.1	0.2	0.2	1.7	1.4
Proteobacteria; Deltaproteobacteria; Syntrophobacteriales; Syntrophaceae	0.4	0.4	1.2	0.4	1.5
Proteobacteria; Gammaproteobacteria; Chromatiales;Sinobacteraceae	0.2	-	0.3	1.8	0.2
SBR1093	2.2	2.4	-	-	-
SPAM; 0319-6G9	0.1	-	0.1	0.1	1.8
Verrucomicrobia; Spartobacteria; Spartobacteriales; Spartobacteriaceae	0.6	5.3	5.3	1.4	0.7
Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales	0.3	0.1	1.3	0.5	0.2

CHAPTER 4

Investigation of Branched GDGT Lipids Producing Bacteria in Soil

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Abstract*To be submitted*

Bacterial branched glycerol dialkyl glycerol tetraether (GDGT) membrane lipids are used as a paleoenvironmental proxy to reconstruct past soil pH and continental air temperatures. The identity of the bacteria that produce branched GDGT membrane lipids is not yet fully known, however, two species belonging to *Acidobacteria* subdivisions 1 and 3 were recently shown to synthesize GDGT-Ia. Here, we analyzed the bacterial community structure using DGGE fingerprinting and clone library analysis of PCR-amplified bacterial 16S rRNA gene fragments, of four representative soils, two with a high and two with a low branched GDGT content. In total 827 bacterial clones were obtained, representing 16 different phyla. *Proteobacteria* and *Acidobacteria* were the most dominant phyla and some classes within the *Acidobacteria*, and within the *Alpha*- and *Deltaproteobacteria*, were only detected in the soils with a high content of branched GDGTs. Thus, branched GDGT-producing bacteria might be found in these taxa. Specific interactions among bacteria belonging to different phyla may be important for the synthesis of branched GDGTs. Also, such interactions potentially contribute to ongoing difficulties to isolate and identify the target microorganisms.

Introduction

Branched glycerol dialkyl glycerol tetraether (GDGT; see Chapter 3, Figure 1 for detailed structures) lipids are ubiquitously present in soils and peat bogs (e.g. Schouten et al., 2000; Sinninghe Damsté et al., 2000; Weijers et al., 2006; Weijers et al., 2007a), lakes (e.g. Blaga et al., 2009; Tierney et al., 2009), and coastal and marine environments (e.g. Hopmans et al., 2004; Kim et al., 2007). Branched GDGTs were first discovered and identified by Sinninghe Damsté et al. (2000), and are, based on their branched carbon skeleton and stereochemistry of their glycerol moieties, thought to be of bacterial origin (Schouten et al., 2000; Sinninghe Damsté et al., 2000; Weijers et al., 2006). Branched GDGTs contain 4 to 6 methyl groups and can have up to two cyclopentane moieties in their carbon skeleton (Weijers et al., 2006). A study of a large number of soils showed that the number of cyclopentane moieties relates to soil pH, whereas the number of methyl branches relates to mean annual temperature (MAT), and to a lesser extent also to soil pH (Weijers et al., 2007a). Two indices were proposed to quantify these relations, namely the Cyclisation of Branched Tetraethers (CBT) and the Methylation of Branched Tetraethers (MBT). The combination of both indices into the MBT-CBT proxy enables the reconstruction of the temperature of the living environment of the branched GDGT producers (Weijers et al., 2007a). The MBT-CBT proxy has been applied to sediments from various areas and geological ages, which has generated a considerable number of paleotemperature records (e.g. Weijers et al., 2007b; Ballentyne et al., 2010; Schouten et al., 2008; Fawcett et al., 2011; Peterse et al., 2011).

It should be noted, however, that the relationship of branched GDGTs with MAT and pH has been empirically determined, and so far only tested in environmental studies, e.g. hot springs (Peterse et al., 2009) and soils sampled along a pH transect (Peterse et al., 2010). For true validation of the proxy, a microbiological approach with cultures of branched GDGT producers is needed. It has been suggested that bacteria synthesize these membrane lipids to regulate membrane fluidity in response to changes in temperature and pH (Weijers et al., 2007a). Nevertheless, the most important producers have not yet been unequivocally identified, although it has been suggested that members of the phylum *Acidobacteria* are the most likely

candidates based amongst others on the higher abundance of branched GDGTs with lower pH, similar as observed for *Acidobacteria* (Weijers et al, 2009; Peterse et al., 2010). Recently, Sinninghe Damsté et al. (2011) showed that two isolates from *Acidobacteria* subdivisions 1 and 3 are able to produce branched GDGT-Ia membrane lipids, confirming suggestions made based on previous studies. Nevertheless, since environmental distribution of these membrane lipids is much more diverse, it was not excluded that members of other subdivisions of *Acidobacteria* or yet other phyla are also able to produce branched GDGTs. Furthermore, Huguet et al. (2012) showed that root associated microorganisms might be involved in the production of branched GDGT membrane lipids observed in rhizoliths. Based on the natural ^{13}C abundance in the lipids, these and other authors suggested that branched GDGT-producing bacteria likely have a heterotrophic life style (Oppermann et al., 2011; Weijers et al., 2011; Huguet et al., 2012).

The aim of this study was to identify the bacteria able to synthesize branched GDGTs by applying culture independent techniques to a selected set of soil samples from the Têt watershed (France), with varying amounts of branched GDGTs. To this end, 16S ribosomal RNA (rRNA) gene clone library analysis and DGGE fingerprinting of 16S rRNA gene fragments were done to link shifts in microbial community composition to differences in soil properties and to branched GDGT levels.

Material and Methods

Soil collection

The surface soils (approximately 0-10 cm) used in this study were collected in the catchment area of the Têt River (France) in June and July 2007, and were selected from a larger set, based on their properties, location, and branched GDGT concentration (Kim et al., 2010). Teso 1 and Teso 2 are acidic, peat-like soils, and were sampled at high elevation in the Pyrenees in the source area of the river. Teso 15 and Teso 36 were sampled close to the river mouth at the Gulf of Lions, and are more alkaline and relatively undeveloped (Table 1). All soils were under natural vegetation and were not influenced by agriculture. Mean annual air temperature

(MAT) and precipitation data were obtained from the meteorological bulletin book of Meteo-France 1980-2000. At all sites, samples were collected in geochemical bags and immediately deep-frozen with dry ice in the field, and stored frozen at -20°C until further processing for molecular analyses. The samples for lipid analysis were freeze-dried and sieved with a 2-mm mesh to remove gravel and roots at the Royal Netherlands Institute for Sea Research. The sieved soils were ground with a grinding machine to obtain a homogenized sample. Soil pH was measured in a 10:25 (w/v) soil: distilled water suspension, after 1 min vigorous shaking and settling for 30 min.

Table 1. Location, environmental parameters, pH, and branched GDGT concentration of the soils from the Têt watershed used in this study. Masl=meters above sea level.

Soil ID	Longitude (°E)	Latitude (°N)	Sampling depth (cm)	Soil pH	MAT (°C)	MAP (mm)	Altitude (masl)	SUM brGDGTs (µg/g)
Teso 1	1.97633	42.5593	2-7	4.2	4	1009	2128	2.
Teso 2	1.97533	42.5675	0-10	4.7	3.9	1053	2136	41.7
Teso 15	2.48691	42.6452	1-5	7.2	13.8	624	271	0.21
Teso 36	3.03795	42.7140	0-5	7.5	15.4	577	0	0.31

Branched GDGT analysis

The freeze-dried and homogenized soils were extracted (3 times for 5 min) with a solvent mixture of dichloromethane (DCM): methanol (MeOH) 9:1 (v/v) with an accelerated solvent extractor (ASE 200, Dionex) at 7.6×10^6 Pa and 100°C. Each extract was dried under near vacuum using a rotary evaporator. Each extract was dissolved in DCM and passed over a Na_2SO_4 column to remove all remaining water, dried again under a N_2 flow, and weighed before adding a known amount of C_{46} GDGT standard (Huguet et al. 2006). The extracts were then separated by passing them over an activated Al_2O_3 column using hexane: DCM 9:1 (v/v) and DCM:MeOH 1:1 (v/v) to obtain an apolar and polar fraction, respectively.

The polar fractions, containing the branched GDGTs, were dried under N_2 , ultrasonically dissolved in hexane: isopropanol 99:1 (v/v) at a concentration of about 3mg/ml, and filtered over a 0.45 µm PTFE filter prior to analysis by high performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (HPLC/APCI-MS) on an Agilent 1100 series LC/MSD SL according to Schouten et al. (2007), with minor modifications. Separation of the branched GDGTs

was achieved on an Alltech Prevail Cyano column (150 mm x 2.1 mm; 3 μ m). The compounds were eluted isocratically at a flow rate of 0.2 ml/min with 90%A and 10% B for 5 min, and then with a linear gradient to 16%B in 34 min, where A= hexane, and B= hexane: isopropanol 9:1 (v/v). The injection volume was 10 μ l for all extracts. Selective ion monitoring of the $[M+H]^+$ was used to detect and quantify the different branched GDGTs, and absolute quantification of each compound was achieved by calculating the area of its corresponding peak in the chromatogram, comparing it with that of the internal standard, and correcting it for the different response factors (cf. Huguet et al. 2006).

Nucleic acid extraction and biomolecular analysis of microbial composition

DNA was extracted using the FastDNA®SPIN kit for soil according to manufacturer's instructions (MP Biomedicals, Solon, OH). The DNA was quantified with a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR was performed using the GoTaq DNA Polymerase Kit (Promega, Leiden, The Netherlands). PCR targeting the V6-V8 regions of the bacterial 16S rRNA gene was performed using primers 968-GC-F and 1401-R (Muyzer et al., 1993). Denaturing gradient gel electrophoresis (DGGE) was performed according to the protocol of Muyzer et al. (1993) using the Bio-Rad Dcode system (Bio-Rad, Hercules, CA), with 8% (v/v) polyacrylamide gels and a denaturing gradient ranging from 30% to 60% (100% denaturing solution was defined as 7 M urea and 40% formamide). The gel was stained with $AgNO_3$ (Sanguinetti et al., 2004), scanned using a GS-800 Calibrated Densitometer (Bio- Rad) and analyzed using Bionumerics version 4.6 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The Pearson product-moment correlation was used to determine the similarity between DGGE fingerprints by calculating the similarity indices of the densitometric curves of the profiles.

For the construction of clone libraries, full length bacterial 16S rRNA gene fragments were amplified by PCR with primers 27F and 1492R (Park et al., 2006). PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. Purified PCR product were cloned by

using pGEM-T Easy plasmid vector (Promega, Madison, WI), and *E. coli* XL1 blue cells (Stratagene, La Jolla, CA) according to the manufacturers' instructions.

Sequence analysis

A total of 827 clones were obtained from the Têt watershed soils, and were sequenced from one side, yielding partial sequences of 600-850 bp. Operational taxonomic units (OTUs) were defined on the basis of 97% sequence identity by using ContigExpress program of the *Vector NTI* software (Invitrogen, Carlsbad, CA). The phylum composition was determined by taxonomic assignment performed using the RDP Classifier (Wang et al., 2007) with default parameters through the RDP II website (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). PAST software (version 1.82b) (Hammer et al., 2001) was used to compute the statistical indexes for the bacterial sequences in each data set.

Nucleotide accession numbers

The sequences retrieved in this study were deposited in the GenBank database under accession numbers JQ695953 - JQ696773.

Results

General soil properties and branched GDGT abundance

The MAT at the locations of the two peat-like soils from the source area, Teso 1 and Teso 2, is about 4°C, and the annual precipitation reaches just over 1000 mm. Teso 15 and Teso 36 are situated at lower elevation, close to the mouth of the Têt River, where MAT is 13.8 and 15.4°C, respectively, and the annual precipitation is 624 and 577 mm (Table 1). The pH of the Teso 1 and Teso 2 soils is low (4.7 and 4.9), compared to the values of Teso 15 and Teso 36 (7.2 and 7.5; Table 1).

Branched GDGTs were detected in all soils, although not in every soil all nine different types could be detected (Figure 1). The total abundance of the branched GDGTs varied between 0.21 and 0.31 µg/g dry wt soil for the more alkaline Teso 15 and Teso 36 soils, to 2.9 and 41.7 µg/g dry wt soil for the acidic Teso 1 and

Teso 2 samples (Table 1). The distribution of the branched GDGTs changed from predominantly branched GDGTs of type a, i.e. without cyclopentane moieties, in Teso 1 and Teso 2, to more branched GDGTs of type b and c, i.e. with one or two cyclopentane moieties, in Teso 15 and Teso 36 (Figure 1).

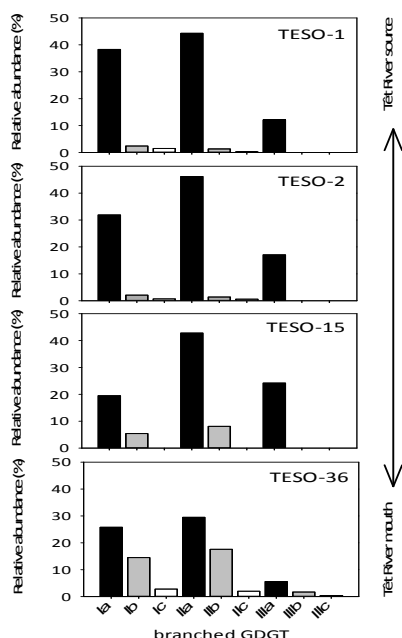


Figure 1. Distribution of branched GDGTs in the surface soils in the Têt watershed used in this study. Branched GDGT numbers refer to structures in Figure 1, Chapter 3.

Soil bacterial community composition

DGGE analysis of PCR-amplified 16S rRNA gene fragments was performed to obtain a first overview of the bacterial community structure in the four different Têt soils. A comparison based on DGGE band patterns using Pearson Correlation analysis showed more than 80% similarity between the soils, with Teso 1 clustering separately from Teso 2, Teso 15 and Teso 36 (Figure 2). To obtain a more detailed insight in the microbial composition of the different soils, 16S rRNA gene clone libraries were constructed, and a total of 162 to 232 clones per soil were analyzed by partial rRNA gene sequencing (Table 2).

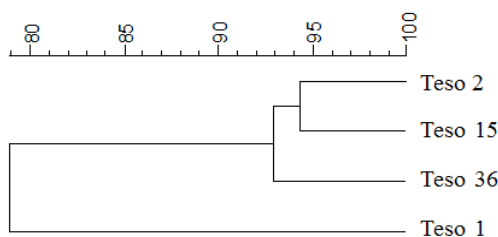


Figure 2. Dendrogram based on Pearson correlations of denaturing gradient gel electrophoresis (DGGE) profiles of bacterial 16S rRNA gene fragments retrieved from Teso 1, Teso 2, Teso 15, and Teso 36. The scale bar indicates Pearson correlations (%) between profiles.

The taxonomic affiliation of the cloned sequences revealed the presence of members of 16 different phyla. In total 372 OTUs were detected from the four clone libraries. Good's coverage value, an estimation of the proportion of the community represented by retrieved sequences, indicated a relatively low coverage of the true bacterial richness in the soils (Table 2). Low dominance (D) values indicated that no OTUs were predominant in the community, which was confirmed by values observed for the Shannon-Weaver index of diversity.

Table 2. Estimated OTU (97% sequence identity) richness, diversity indices and estimated sample coverage for 16S rRNA gene libraries of Têt watershed soil samples.

Library	No. of seq.	No. of OTUs	Good's coverage value (%)	Shannon-Wievers index	Dominance (D) value	Evenness e^H/S
Teso 1	203	126	38	4.606	0.014	0.788
Teso 2	162	131	20	4.827	0.009	0.911
Teso 15	224	191	15	5.173	0.006	0.923
Teso 36	232	186	20	5.103	0.007	0.884

The *Proteobacteria* and *Acidobacteria* were the most abundant phyla in the analyzed soils, followed by *Bacteroidetes* and *Actinobacteria*.

The Teso 1 soil was dominated by clones that grouped within the *Proteobacteria* (37%, Figure 3a), followed by the phyla *Actinobacteria* (18%), *Acidobacteria* (16%) and *Verrucomicrobia* (9%). Sequences belonging to unclassified bacteria (4%), *Bacteroidetes* (5%), *Bacteria_incertain_sedis* (5%), *Firmicutes* (4%) and

Gemmatimonadetes (2%) were represented as minor components in the Teso 1 library (Figure 3a; Table S1).

The bulk of the sequences (40%) of the Teso 2 soil, which contained the highest amount of branched GDGTs, were affiliated with the *Proteobacteria* (Figure 3a). The second most abundant phylum was *Acidobacteria* (15%), and *Bacteroidetes* and *Actinobacteria* were each represented by 8% of clones. Sequences related to *Verrucomicrobia* (3.6%) and *Chloroflexi* (2%) were found to constitute minor components in the library. Certain families and certain orders within the *Acidobacteria*, *Deltaproteobacteria* and *Alphaproteobacteria* were only represented in the Teso 2 library (Figure 4a-b, Table S1). A total of 68 sequences were affiliated with the *Proteobacteria*. The most dominant class was *Deltaproteobacteria* (33%) followed by *Alphaproteobacteria* (30%), *Gammaproteobacteria* (14%) and *Betaproteobacteria* (16%) (Figure 3b). Within the *Deltaproteobacteria* the genera *Syntrophorhabdus*, *Syntrophus*, *Syntrobacter* and *Desulfomonile* were only detected in the Teso 2 library (data not shown). From the *Alphaproteobacteria* the family of *Beijerinckiaceae* was only detected in Teso 2 (Figure 4c). In total 24 sequences were detected that grouped within the phylum of *Acidobacteria*. In the phylum of *Acidobacteria* most of the sequences came from subdivisions 1 (25%), 2 and 3 (21% each). *Acidobacteria* subdivision 23 was only represented in the Teso 2 library, albeit only with one sequence (Figure 3c). Thirteen sequences were affiliated with the *Bacteroidetes*, nine of which (69%) could not be classified beyond the phylum level.

Clone library analysis of the Teso 15 soil identified representatives of 13 phyla. The majority of sequences came from the phylum of *Proteobacteria* (51%), within which the *Betaproteobacteria* were most abundant (48%), followed by *Alphaproteobacteria* (30%), *Gammaproteobacteria* (11%), *Deltaproteobacteria* (7%) and unclassified *proteobacteria* (3.5%). Besides *Proteobacteria*, we detected *Bacteroidetes* (13%), unclassified *bacteria* (8%), *Actinobacteria* (9%) and *Acidobacteria* (8%). Sequences belonging to the phyla of *Planctomycetes* (3%), *Verrucomicrobia* (3%), *Chloroflexi* (1.3%), *Gemmatimonadetes* (0.9%), *Firmicutes* (0.4%), *Cyanobacteria* (0.9%), *Nitrospira* (0.8%), and *WS3* (0.4%) were represented in minor proportions (Figure 3a).

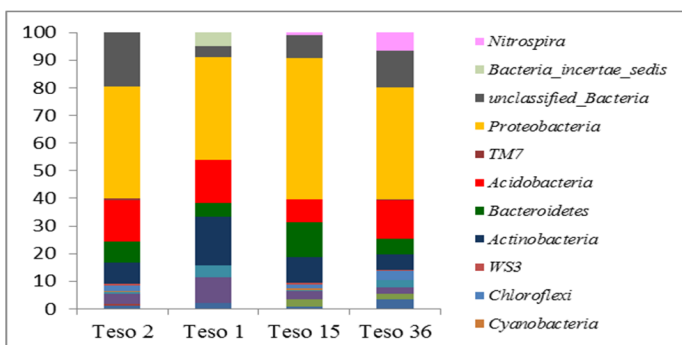


Figure 3a.

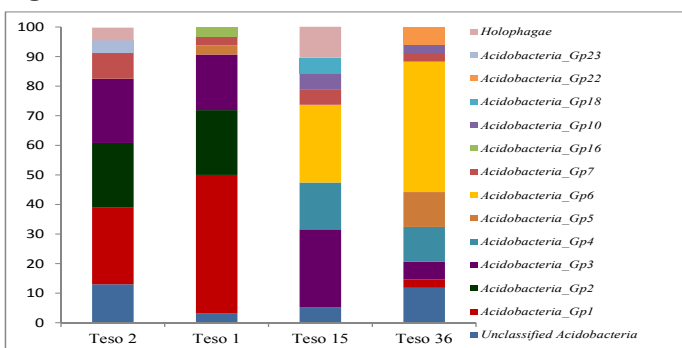


Figure 3b.

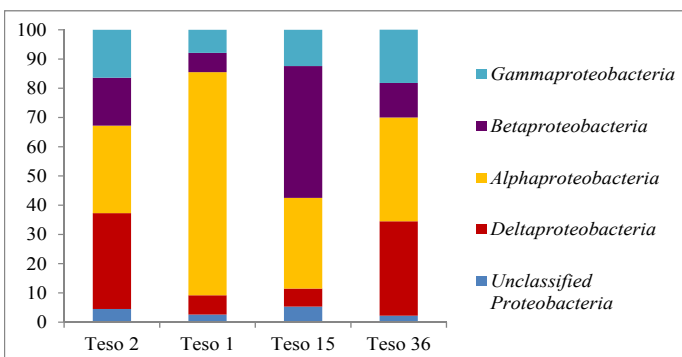


Figure 3c.

Figure 3. The distribution of partial sequences of bacterial 16S rRNA genes obtained from the Têt watershed samples; Teso 1, Teso 2, Teso 15, Teso 36. Classification was done using Ribosomal Database Project (RDP) Classifier, and is shown at phylum level (a), and at class level for the two most abundant phyla, the *Acidobacteria* (b) and the *Proteobacteria* (c).

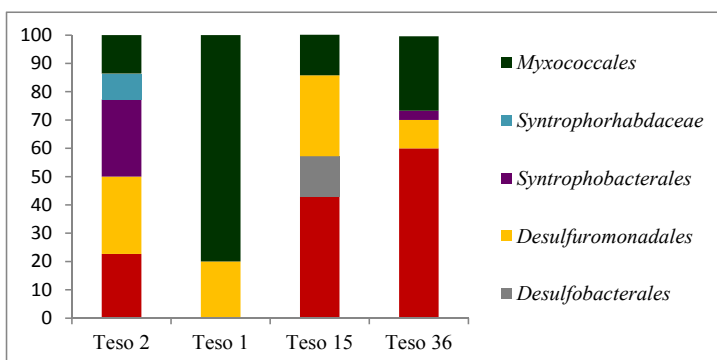


Figure 4a.

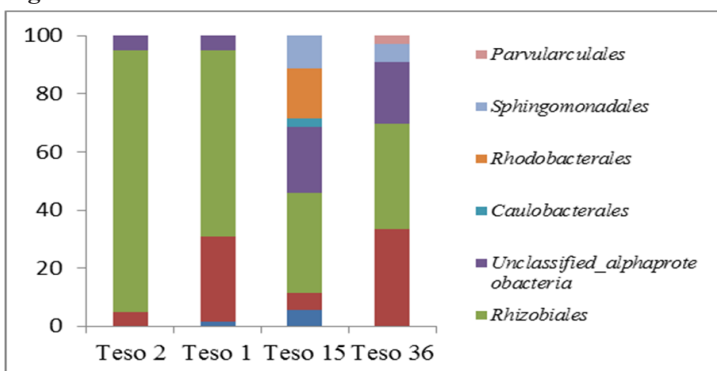


Figure 4b

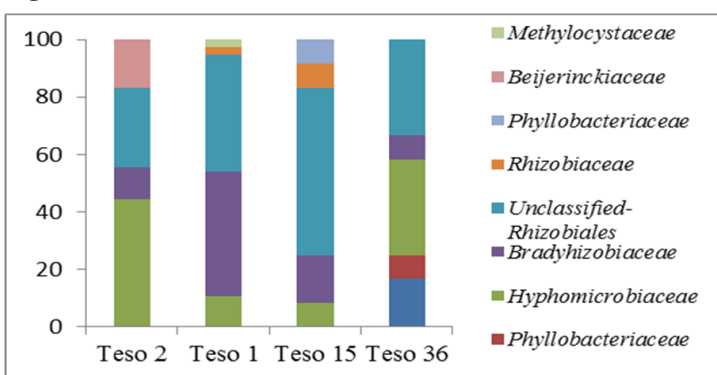


Figure 4c.

Figure 4. The distribution of partial sequences of bacterial 16S rRNA genes obtained from the Têt watershed samples; Teso 1, Teso 2, Teso 15, Teso 36. Classification was done by using Ribosomal Database Project (RDP) Classifier, and is shown at order level for *Deltaproteobacteria* (a) and *Alphaproteobacteria* (b), and at family level for *Rhizobiales* (c).

The Teso 36 soil harboured *Proteobacteria* (40%), *Acidobacteria* and unclassified bacteria (14%), *Nitrospira* (7%), *Actinobacteria* and *Bacteroidetes* (6%). Sequences related to *Chloroflexi* and *Gemmatimonades* (3%), *Firmicutes* (2.6%), *Verrucomicrobia* and *Planctomycetes* (2%), *WS3* and *TM7* (0.4%) were only represented in small proportions (Figure 3a; Table S1).

Discussion

Based on their general properties, the soils can be divided into two sets; Teso 1 and 2 in the source area of the Têt River, with low soil pH and MAT, and a high abundance of branched GDGT membrane lipids, and Teso 15 and 36 near the mouth of the river, with a high soil pH and MAT, and a low concentration of branched GDGTs (Table 1). This separation is also reflected in the distribution of branched GDGTs. Teso 1 and Teso 2 are dominated by branched GDGTs of GDGT type a, i.e. without cyclopentane moieties, whereas the branched GDGTs in Teso 15 and Teso 36 do contain GDGTs with cyclopentane moieties (Figure 1). The increase in cyclopentane moieties in the more alkaline soils towards the river mouth is in line with the empirical relation between the amount of rings and soil pH observed in soils worldwide (Weijers et al., 2007a, Peterse et al., 2010). Also the high branched GDGT concentrations in the soils with low soil pH (and vice versa) is consistent with earlier studies (Weijers et al., 2007a; Peterse et al., 2010), and soil pH has indeed been shown to be the major control on branched GDGT concentrations in the Têt watershed (Kim et al., 2010). The two distinct sets of soils provide an excellent base for the comparison of microbial communities, and to link possible differences to the branched GDGT concentrations and general properties of the soils.

Soils with a high content of branched GDGTs, Teso 2 and Teso 1, were found to be dominated by bacteria belonging to the *Proteobacteria* (Figure 3a; Table S1), which is often the most dominant bacterial phylum in soils (Janssen et al., 2006). Within the phylum of *Proteobacteria*, *Alphaproteobacteria* represented the most dominant class in Teso 2 and Teso 1, followed by *Deltaproteobacteria* in Teso 2. *Deltaproteobacteria* were less abundant in Teso 1 (Figure 3c). We observed a similar

trend, i.e. dominance of the classes of *Delta*- and *Alphaproteobacteria* in high branched GDGTs content samples, in almost all samples that we analyzed in similar studies (Chapter 3 and 5 of this thesis). More specifically, sequences belonging to the orders *Rhizobiales*, *Desulfuromonadales*, and *Syntrophobacterales* were detected in high proportion compared to other orders that were detected in this study. These findings led us also speculate that a complex relationship such as syntrophy might be associated with some of the bacteria that produce branched GDGTs, potentially including other microbial groups besides *Acidobacteria* that might produce branched GDGTs. Since branched GDGT have been detected in a broad range of soil samples, it might be plausible to assume that representatives of major phyla present in soils, such as *Proteobacteria* and *Acidobacteria*, are the main branched GDGT producers. To this end, it is interesting to note that based on the phylogenetic analyses, *Deltaproteobacteria* have been suggested to be a sister group of *Acidobacteria* (Ciccarelli et al., 2006; Ward et al., 2009; Kielak et al., 2010). Taken together, besides *Acidobacteria*, *Deltaproteobacteria* might also be responsible for branched GDGT production. *Deltaproteobacteria* contain major groups of Gram-negative sulfate reducing bacteria, and also comprise well-known species of syntrophic fatty acid-oxidizing anaerobes such as the genera *Syntrophobacter* and *Syntrophus* (Harmsen et al., 1998; Van Kuijk and Stams, 1995; Chen et al., 2005; Plugge et al., 2011). *Deltaproteobacterial* sequences recovered from the Teso 2 clone library represented mainly members of the orders of *Desulfuromonadales*, *Myxococcales* and *Syntrophobacterales* and unclassified *Deltaproteobacteria*. Interestingly, one sequence affiliated with the genus *Syntrophobacter* was also detected from the Saxnäs Mosse peat bog in Sweden, studied by Weijers et al. (2009). It has been shown that some species from the family of *Syntrophobacteraceae*, namely *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, are able to produce both ester- and ether-bound alkyl chains within one glycerol membrane lipid (Rütters et al., 2001; Weijers et al 2007). Also, mesophilic SRB are responsible for the biosynthesis of monoalkyl and dialkyl ether lipids (Hinrichs et al., 2000; Pancost et al., 2001). It has, however, not yet been shown that members of the *Syntrophobacteraceae* are able to produce branched GDGT membrane lipids. This is further complicated by the fact that branched GDGTs are not in the list of fatty acids/lipids that are checked

routinely when describing new isolates. In the Saxnäs Mosse peat bog, microbial communities were dominated by members of subdivisions 1, 3, 4 and 8 of the phylum *Acidobacteria*, and unclassified *Acidobacteria* (Table 3). Interestingly, a recent study by Sinninghe Damsté et al. (2011) showed that some species from *Acidobacteria* subdivisions 1 and 3, namely *Acidobacteriaceae* strain A2-4c and *Edaphobacter aggregans* Wbg-1^T, are able to produce branched GDGT membrane lipids, though the amount of branched GDGTs in these cultures was low compared to values found in e.g. peat bog environments. It was, therefore, suggested that other yet uncultured (acido)bacteria may also produce branched GDGT membrane lipids.

Table 3. Comparison of data obtained from the study of Weijers et al. (2009) and this study.

	Number of sequences This study	Number of sequences Weijers et al., 2009
Phylum <i>Acidobacteria</i>		
Class <i>Acidobacteria</i> _Gp1	6	4
Class <i>Acidobacteria</i> _Gp2	5	
Class <i>Acidobacteria</i> _Gp3	5	1
Class <i>Acidobacteria</i> _Gp4		3
Class <i>Acidobacteria</i> _Gp7	2	
Class <i>Acidobacteria</i> _Gp23	1	
Class <i>Holophagae</i>	2	
Unclassified <i>Acidobacteria</i>	3	5
Class <i>Deltaproteobacteria</i>		
Order <i>Myxococcales</i>	3	
Order <i>Syntrophobacterales</i>	6	1
Order <i>Desulfuromonadales</i>	6	
Unclassified- <i>Deltaproteobacteria</i>	6	
Total number of sequences	162	14

Besides the amount and distribution of branched GDGTs, also the acidobacterial composition in a soil is strongly determined by pH (Männistö et al., 2007; Hartman et al., 2008; Jones et al., 2009). Jones et al. (2009) compared the relative abundances of *Acidobacteria* subdivisions in a broad range of soil samples using pyrosequencing and clone library analysis techniques. This showed that the abundance of subdivisions 1, 2, 3, 12 and 13 correlates with a low pH, whereas members of subdivisions 4, 6, 7, 10, 11, 16, 17, 18, 22 and 25 are more abundant in higher pH soils. This trend was

later confirmed by Rousk et al. (2010). Furthermore, members of subdivision 1 are more readily isolated at slightly acidic pH (Sait et al., 2006; Eichorst et al., 2007). With the exception of subdivision 3, our results corroborate these previous studies. We found that sequences that cluster with members of *Acidobacteria* subdivisions 1, 2, 16 and 23 were only detected in the samples with low pH and high content of branched GDGTs (Teso 2 and Teso 1), with *Acidobacteria* group 23 being only detected in Teso 2, the sample with the highest branched GDGT content. In contrast, samples with a higher pH and low concentrations of branched GDGTs, Teso 15 and Teso 36, were dominated by members of subdivisions 4, 5, 6, 10, 18 and 22. In order to further allow identifying the potential of specific subgroups within the *Acidobacteria* to produce branched GDGT membrane lipids, future studies might aim at obtaining a range of additional representative isolates of the various subdivisions, using high throughput cultivation approaches such as those used previously for cultivation of soil bacteria (Joseph et al., 2003), in combination with innovative microscale cultivation technology (Ingham et al., 2007).

In the present study, members of the family of *Syntrophorhabdaceae* within the *Deltaproteobacteria* and the family of *Beijerinckiaceae* within the *Alphaproteobacteria* were only recovered from the high GDGT sample, Teso 2 (Figure 4a). The family *Beijerinckiaceae* is metabolically diverse and includes methanotrophs, methylotrophs and chemoheterotrophs. Two genera belonging to this family, *Methylocella* and *Methylocapsa*, are very abundant in acidic soils and peats (Dedysh et al., 2001).

In a study on the polar lipid precursors of branched GDGT membrane lipids two IPLs (intact polar lipids), namely glucosyl- and glucuronosyl lipids, were identified (Liu et al., 2010). It was reported that some cultured species of *Actinobacteria* and *Alphaproteobacteria* produce glucuronosyl lipids (Liu et al., 2010). It was also shown that branched GDGT amounts were higher in rhizoliths compared to loess, which is an indication for the involvement of root-associated microorganisms (Huguet et al., 2012). These observations are in line with our findings, as we found mostly *Rhizobiales* within the *Alphaproteobacteria* in our clone libraries (Figure 4b).

In summary, comparison of 16S rRNA gene clone libraries of 4 soils with

contrasting pH, MAT, precipitation and branched GDGT lipid content showed that certain bacterial populations belonging to the *Acidobacteria*, *Deltaproteobacteria* and *Alphaproteobacteria* were only detected in the soil with the highest GDGT content (Teso 2), which originated from a peat bog like site characterized by low ambient temperature and low pH. The low amount of branched GDGT membrane lipids observed in some acidobacterial pure cultures suggest that additional (acido) bacterial members may also produce these specific membrane lipids. More detailed research is needed to isolate and characterize in particular populations associated with the high branched GDGT content samples studied here, namely members of the *Acidobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*.

Acknowledgments

We thank the Darwin Center for Biogeosciences (142.16.2051) and the Chemical Science division (CW; project 700.55.343) of the Netherlands Organization for Scientific Research (NWO) for financial support. We thank J.-H. Kim and B. Zarzycka for help in the field and the GDGT analysis, and H. Heilig for providing help with technical aspects of 16S rRNA gene analyses.

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CHAPTER 4

Supplemental Materials

Table S1. Bacterial community composition of soil samples from the Têt watershed; Teso 1, Teso 2, Teso 15, Teso 36.

Taxonomy	Teso 1	Teso 2	Teso 15	Teso 36
Phylum Gemmatimonadetes	4	2	2	9
class <i>Gemmatimonadetes</i>	4	2	2	9
Phylum Firmicutes	9	1	1	6
class <i>Clostridia</i>	2	1	1	2
class <i>Bacilli</i>	7			4
Phylum Verrucomicrobia	18	6	6	5
class <i>Opitutae</i>			1	1
class <i>Subdivision3</i>		4	2	4
class <i>Spartobacteria</i>	16	1	1	
class <i>Verrucomicrobiae</i>			1	
class <i>Subdivision5</i>			1	
unclassified_ <i>Verrucomicrobia</i>	2	1		
Phylum Chloroflexi	1	3	3	8
class <i>Caldilineae</i>			1	2
class <i>Anaerolineae</i>		2	1	1
unclassified_ <i>Chloroflexi</i>	1	1	1	5
Phylum WS3		1	1	1
genus		1	1	1
<i>WS3_genera_incertaine_sedis</i>				
Phylum Actinobacteria	36	13	20	13
class <i>Actinobacteria</i>	36	13	20	13
Phylum Bacteroidetes	10	13	29	13
class <i>Bacteroidia</i>		1		
class <i>Flavobacteria</i>				1
class <i>Sphingobacteria</i>	9	2	22	11
family		1	2	
<i>Bacteroidetes_incertaine_sedis</i>		9	5	1
unclassified_ <i>Bacteroidetes</i>				
Phylum Acidobacteria	32	24	19	32
class <i>Acidobacteria_Gp1</i>	15	6		1
class <i>Acidobacteria_Gp2</i>	7	5		
class <i>Acidobacteria_Gp3</i>	6	5	5	2
class <i>Acidobacteria_Gp4</i>			3	4
class <i>Acidobacteria_Gp5</i>	1			4
class <i>Acidobacteria_Gp6</i>			5	15
class <i>Acidobacteria_Gp7</i>	1	2	1	1
class <i>Acidobacteria_Gp10</i>			1	1
class <i>Acidobacteria_Gp16</i>	1			
class <i>Acidobacteria_Gp22</i>				2
class <i>Acidobacteria_Gp23</i>		1		
class <i>Acidobacteria_Gp18</i>			1	

class <i>Holophagae</i>		2	2	
unclassified_ <i>Acidobacteria</i>	1	3	1	2
Phylum <i>TM7</i>		1		1
genus		1		1
<i>TM7 genera incertae sedis</i>				
Phylum <i>Proteobacteria</i>	76	68	115	94
class <i>Alphaproteobacteria</i>	58	20	35	33
class <i>Gammaproteobacteria</i>	6	11	13	15
class <i>Deltaproteobacteria</i>	5	23	8	31
class <i>Betaproteobacteria</i>	5	11	55	12
unclassified_ <i>Proteobacteria</i>	2	3	4	3
phylum <i>Planctomycetes</i>			7	6
class <i>Planctomycetacia</i>			7	6
phylum <i>Nitrospira</i>			2	15
class <i>Nitrospira</i>			2	15
phylum	10			
<i>Bacteria incertae sedis</i>	10			
class <i>Ktedonobacteria</i>	10			
Phylum <i>Cyanobacteria</i>			2	
class <i>Cyanobacteria</i>			2	
Unclassified_ <i>Bacteria</i>	8	34	19	29
Total number of sequences	203	162	224	232

CHAPTER 5

The Microbial Community Structure of Soil Samples Enriched with Methanol at Different pH and Temperature

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To be submitted

ABSTRACT

Methylotrophs are important players in the global carbon cycle. In order to assess the effect of environmental factors on the composition of methylotrophic communities, microcosms from a soil collected in the source area of the Têt River in southeast France were incubated in the presence of methanol at a range of different temperatures and pH. Soil microbial community dynamics was evaluated by pyrosequencing of PCR-amplified 16S rRNA gene fragments as well as by analysis of membrane phospholipid fatty acids and branched glycerol dialkyl glycerol tetraethers (branched GDGT). Clear differences could be observed in the microbial community composition of the enriched samples. Besides well-known methylotrophs including *Alpha*-, *Beta*- and *Gammaproteobacteria*, we also detected members of other taxa, including *Pedobacter* and *Acidobacteria* subdivision 1, which were not known to be able to grow with methanol. The selected soil had previously been shown to harbour high levels of branched GDGT membrane lipids, but their producers are not yet fully identified. Among the different microcosm incubations performed here, branched GDGTs were detected at 15°C and pH 5.5, suggesting that specific methylotrophic bacteria might be potential sources. However, further enrichment attempts of the candidate bacteria were not successful.

INTRODUCTION

Methylophilic bacteria use reduced one-carbon compounds, including methane, methanol, methylated amines and other methylated compounds, as a sole source of carbon and energy (Anthony et al., 1982; Lidstrom, 1990). They play an important role in the global carbon cycle, (Galbally and Kirstine, 2002). Most methylophilic bacteria are able to use methanol as a substrate (Kolb, 2009). Methanol is formed from methylated organic compounds, such as lignin derivatives and pectin, and by chemical oxidation of methane, which is produced by anaerobic degradation of organic matter (Anthony et al., 1982). Most of the known methylophilic bacterial species are either strictly aerobic or facultative anaerobes, but some strictly anaerobic bacteria are also able to utilize methanol (Kerby et al., 1983; Jiang et al., 2010). The first described methanol-utilizing bacterium was *Bacillus methylicus*, a Gram-positive, aerobic, facultative methylophilic (Loew, 1892). Currently known aerobic methanol-utilizing bacteria are distributed over more than 50 genera, and belong to a range of different phyla, namely the *Alpha*-, *Beta*- and *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Bacteroidetes*, and *Cytophagales* (Lidstrom, 2006; Kolb et al., 2009). In addition, recent studies showed that some species from the *Acidobacteria* and *Chloroflexi* are also capable of using methanol as a sole carbon source (Radajewski et al., 2002; Lueders et al., 2004; Kalyuzhnaya, 2009). In this study we investigated how differences in environmental conditions affect the composition of a methylophilic enrichment culture. To this end, we specifically focused on variations in pH and temperature, and compared community changes with lipid composition. We characterized the dynamics of the microbial composition, membrane phospholipid fatty acid (PLFA) profiles. The community structure was analyzed by pyrosequencing of barcoded PCR-amplified 16S rRNA gene fragments. We were particularly interested to assess whether differences in methylophilic community composition would be reflected in changes in the lipid composition. As inoculum for the enrichments, a surface soil collected from the source area of the Têt River (Pyrenees, southeast France) was used. Additionally, we further investigated potential producers of branched glycerol dialkyl glycerol tetraether (GDGT) membrane lipids. Since the original soil was shown to contain high amounts of branched GDGTs (Chapter 4), it was a good opportunity to test the influence of pH and temperature on the abundance of branched GDGTs. Branched GDGTs are membrane lipids derived from bacteria, and they are widely distributed in environments such as hot springs, sediments, peat bogs and lakes (Weijers et al., 2006; Schouten et al., 2007; Peterse et al., 2009; Blaga et al., 2009). Branched GDGTs are used as a paleoenvironmental proxy, and their distribution is associated with temperature and pH (Weijers et al., 2007; Schouten et al., 2007; Peterse et al., 2010).

The correlation between abundance of several groups within the Acidobacteria and soil pH furthermore coincides with the occurrence of branched GDGT membrane lipids. Branched GDGT membrane lipid abundances decrease with increasing soil pH, which led to the hypothesis that Acidobacteria may be a possible source of these orphan membrane lipids (Peterse et al., 2010; Weijers et al., 2007). Sinninghe Damsté et al. (2011) showed that some species from the Acidobacteria subdivisions 1 and 3 produce branched GDGT membrane lipids.

MATERIALS and METHODS

Growth medium and culture conditions

The surface soil used in this study, further referred to as Teso 2, was collected in the source area of the Têt River, France, in June 2007 (see Chapter 4 for details on sampling location). The peat-like soil is acidic (pH 4.7), and the mean annual air temperature at the sampling location is 3.9 °C and the in situ temperature at the time of sampling was 11.2°C. It was previously shown that this soil has a high branched GDGT content (Chapter 4). A slightly acidic VL55 medium (Sait et al., 2002) was used to enrich bacteria from the soil sample. Medium VL55 contained 1.95 g 2-[N-morpholino]ethanesulfonic acid, 0.2 mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM (NH₄)₂HPO₄, 1 ml of selenite/tungstate solution (Tschech and Pfennig, 1984) and 1 ml of trace element solution SL-10 (Widdel et al., 1983) per liter, and the pH was adjusted to 5.5 with a mixture of 200 mM NaOH plus 100 mM KOH. For routine cultivation and subcultivation 120-ml serum bottles were used. Serum bottles containing 48 ml of VL55 medium were sealed with rubber stoppers and autoclaved at 121°C for 20 min. At the time of inoculation, the headspace was filled with air. One gram of Teso 2 soil sample was added to 48 ml VL55 medium without growth substrate and vitamins, and homogenized on a rotary shaker for 24 h. This soil slurry was used as inoculum for enrichment cultivation. Two percent of the initial soil suspension was taken for inoculation, and 20 mM methanol was used as the sole carbon source and incubated without shaking at 15°C. All subsequent transfers were done using an inoculum size of 2% (v/v). The initial enrichment culture was incubated for 2 weeks in the dark at 15°C, and used to inoculate bigger (1200ml) bottles, containing approximately 500 ml of VL55 medium, to obtain sufficient biomass for lipid analyses. A third transfer was done, incubated at pH 5.5 and 15°C for two weeks, and used as inoculum of cultures that were then incubated a) at different temperatures ranging from 4 to 30°C, at pH 5.5, and b) at pH values ranging from 2 to 7, at 15°C.

Lipid analysis

Biomass material was freeze dried and saponified by refluxing the freeze dried ma-

terial for 1 h with 2 ml 1N KOH in methanol (MeOH) (96%). After cooling down, the solution was adjusted to pH 5 with 2N HCl:MeOH (1:1). Subsequently, 2 ml bidistilled water and 3 ml dichloromethane (DCM) were added, and the MeOH/water layer was washed twice with 2 ml DCM. The combined DCM layers were dried over Na₂SO₄. Extracts were methylated using diazomethane in ether and eluted with ethyl acetate (EtOAc) over a small silica column (pipette; Merck 60, 0.063-0.2 mm, 70-230 mesh) to remove polar constituents. Alcohols were silylated by adding BSTFA in pyridine and heated at 60°C for 20 min. The extracts were dissolved in EtOAc in a concentration of 1 mg/ml prior and analyzed by gas chromatography (GC) and GC/mass spectrometry (MS). GC analysis was performed on a Hewlett Packard HP6890 Series GC System, fitted with on column injection and a flame ionization detector. Helium was used as carrier gas, the column was a fused silica capillary column (25 m long, 0.32 mm Ø), coated with CP Sil5 (film thickness 0.12 µm). The samples were injected on the GC at 70°C, with the temperature rising to 130°C at a rate of 20°C/min and then to 320°C at a rate of 4°C/min, where temperature was held constant for 10 min. GC/MS analysis was carried out using a Finnigan Trace GC Ultra, Thermo Election Corporation, interfaced with a Finnigan Trace DSQ mass spectrometer, using a mass range of *m/z* 40-800. GC conditions for GC/MS were similar to the conditions described for GC earlier. Fatty acid methyl esters were identified according to their mass spectra and retention times. To determine the position of the double bonds, an aliquot of the saponified material was derivatized with dimethyldisulfide (DMDS) by adding 100 µl hexane, 100 µl DMDS, and 10 µl I₂ in ether (60 mg/ml) and heated overnight at 40 °C. After cooling down, 400 µl hexane and 200 µl 5% -S₂O₃ solution was added, and the sample was washed with hexane (3x). The sample was silylated, dissolved in EtOAc, and analyzed by GC and GC/MS.

Nucleic acid extraction

10 ml of liquid was taken from cultures and centrifuged for 15 min at 4600×*g*. DNA was extracted from the resulting pellet using the FastDNA® SPIN kit for soil (MP Biomedicals, Solon, OH, USA), and quantified with a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies®, Wilmington, DE).

16S rRNA gene amplicon pyrosequencing

Barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated by PCR using the 27F-DegS primer (van den Bogert et al., 2011), appended with the titanium sequencing adaptor A and an 8 nt sample specific barcode (Hamady et al., 2009) at the 5' end, and an equimolar mix of two reverse primers, 338R I and II (Daims et al., 1999; Table 2, Chapter 3), that carried the titanium adaptor B at the 5' end.

After DNA extraction, DNA was diluted to a concentration of 20 ng/μl based on Nanodrop readings. PCR was performed using a GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom). The PCR mix (100 μl final volume) contained 20 μl of 5× HF buffer (Finnzymes, Vantaa, Finland), 2 μl PCR Grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 1 μl of Phusion hot start II High-Fidelity DNA polymerase (2U/μl) (Finnzymes), 500 nM of the reverse primer mix and the forward primer (Biolegio BV, Nijmegen, The Netherlands) (see Chapter 3, Table 2), 2 μl (i.e. 40 ng) template DNA, and 65 μl nuclease free water. PCR was run under the following conditions: 98°C for 30 s to activate the polymerase, followed by 30 cycles consisting of denaturation at 98 °C for 10 s and annealing at 56 °C for 20 s and elongation at 72 °C for 20 s, and a final extension step at 72 °C for 10 min. Five μl of the PCR products, approximately 375 bp in length, were analyzed by 1% (w/v) agarose gel electrophoresis, containing 1× SYBR® Safe (Invitrogen, Carlsbad, CA, USA) and purified from gel using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to manufacturer's instructions. DNA concentration of gel-purified amplicons was measured by Nanodrop® ND-1000 spectrophotometer, and purified PCR products were mixed in equimolar amounts and run again on an agarose gel with following excision and purification by using a DNA gel extraction kit (Milipore, Billerica, MA, USA). A mixture of 44 samples was analyzed by pyrosequencing on half a plate using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

Analysis of the pyrosequencing data

Pyrosequencing data was analyzed using the QIIME 1.3.0 pipeline (Caporaso et al., 2010). Low quality sequences were removed using default parameters, and operational taxonomic units (OTUs) were defined at a 97% identity level. A representative sequence from each OTU was aligned using PyNAST (DeSantis et al., 2006). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% (Wang et al., 2007). Possible chimeric OTUs were identified using QIIME's Chimera Slayer (Haas et al., 2011) and subtracted from the previously generated OTU list, producing a non-chimeric OTU list.

RESULTS

Enrichment and growth

The initial culture, incubated at pH 5.5 and 15°C, was slightly pinkish and formed small flocs. Growth was observed after 3 days in cultures incubated at 15, 20, and 30°C at pH 5.5, whereas at 4°C growth was only observed after two weeks of incubation. Cultures incubated under different conditions showed a different appearance.

Cultures incubated at 15° and 30°C formed layers of biofilm both at the surface and in the liquid, whereas at 4° and 20°C cultures had a more homogenous appearance. The cultures incubated at 30°C showed formation of flocs (Figure S1-S2). The pinkish color observed for some of the enrichments is most probably attributed to pink-pigmented facultative methylotrophic bacteria. Growth was observed within three days in cultures incubated at 15°C at pH values above 3, whereas after three weeks, no growth was observed at pH 2 and 3.

Bacterial community structure revealed by pyrosequencing analysis of barcoded PCR amplified 16S rRNA gene fragments

Microbial community composition of the initial culture that was incubated at 15°C/pH 5.5, as well as the subsequent enrichment cultures after incubation at 4°C/pH5.5, 20°C/pH5.5, 30°C/pH5.5, 15°C/pH4.5 and 15°C/pH6 was analyzed by pyrosequencing of partial 16S rRNA gene fragments. In total 42895 sequences were obtained from 6 samples, with 4215, 7265, 6021, 7860, 8047, and 9087 reads for individual samples, respectively (Table 1). In total 7 phyla were retrieved from the initial enrichment, including *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Spirochetes* and *Tenericutes*, and also several unclassified bacteria were detected (Table 2, Figure 1). Sequences related to *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria* and *Actinobacteria* were found in the subsequent enriched samples, however, sequences belonging to the *Tenericutes* and *Spirochetes* were not detected in further enriched samples. *Proteobacteria* was the dominant phylum in all samples and *Alphaproteobacteria* was the most abundant class within the *Proteobacteria*.

Table 1. Total number of reads obtained per bacterial phyla based on classification using the RDP classifier tool at a confidence threshold of 80% (Wang et al., 2007).

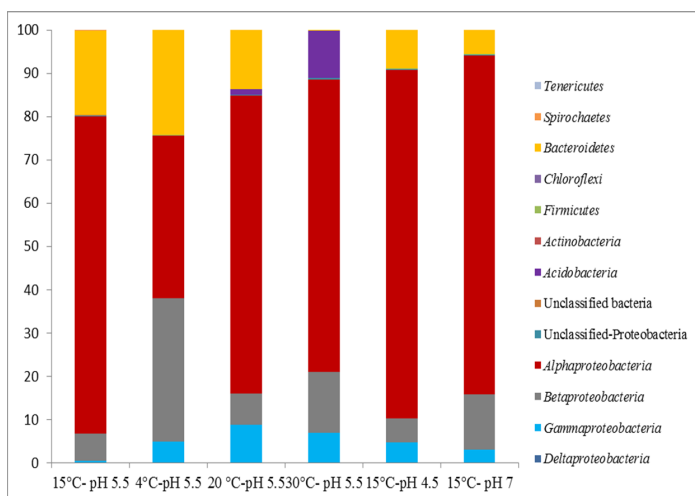
Phylum	15°C pH 5.5 A	A (%) [*]	4°C pH 5.5 B	B (%)	20°C pH 5.5 C	C (%)	30°C pH 5.5 D	D (%)	15°C pH 4.5 E	E (%)	15°C pH 7 F	F (%)
<i>Deltaproteobacteria</i>	0		1		1				1		1	
<i>Gammaproteobacteria</i>	21		362	5	530	9	552	7	375	5	280	3
<i>Betaproteobacteria</i>	261	6	2401	33	433	7	1098	14	444	6	1161	13
<i>Alphaproteobacteria</i>	3088	73	2726	38	4143	69	5317	68	6483	81	7110	78
Unclass-Proteobacteria	8		3		11		19		10		10	
Unclass-Bacteria	8				1		5		6		1	
<i>Acidobacteria</i>	1		4		82	1	854	11	1		9	
<i>Actinobacteria</i>			1									
<i>Firmicutes</i>	4		3				5		8		6	
<i>Chloroflexi</i>	1						1					
<i>Bacteroidetes</i>	820	19	1764	24	820	14	9		719	9	509	6
<i>Spirochaetes</i>	2											
<i>Tenericutes</i>	1											
Total	4215		7265		6021		7860		8047		9087	

^{*}Phyla represented by less than 1% of the sequences were not included

Table 2. Phospholipid fatty acid profiles of original and further enrichment cultures.

Compound (%)	15°C pH 5.5	4°C pH 5.5	20°C pH 5.5	30°C pH 5.5	15°C pH 4.5	15°C pH 7
(ω -7)C16 fatty acid cis	6	18	1	8	6	12
(ω -7)C16 fatty acid trans	2	2	0	2	0	0
n-C16:0 fatty acid	7	2	6	4	1	3
β -C14 hydroxy fatty acid	1	0	1	0	0	3
C17 cyclopropyl	1	1	1	1	1	2
(ω -7)C18 fatty acid	61	61	69	66	44	60
n-C18:0 fatty acid	1	0	4	1	1	1
C19 cyclopropyl	6	2	3	2	0	0
Diplopterol	11	11	15	11	43	19
Unknown	5	3	1	4	4	0
Branched GDGTs	*	ND	ND	ND	ND	ND

*Branched GDGT production was detected.

**Figure 1.** Relative abundance of bacterial phyla in the pyrosequencing data set of analyzed samples. Initial enrichment was performed at 15°C/pH 5.5. Further enrichments were done at 4, 20 and 30°C at pH 5.5, and at pH 4.5 and 7 at 15°C.

Effect of temperature on the community composition

We found that the vast majority of sequences came from *Alpha*- and *Betaproteobacteria* (Figure 1), followed by *Bacteroidetes*, *Acidobacteria* and *Gammaproteobacteria*. Sequences belonging to the *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Spirochetes* and several unclassified bacteria were less represented. The alphaproteobacterial sequences were further classified as *Rhodospirales*, *Caulobacteriales*, *Sphingomonadales*, *Rhizobiales*, *Rhodobacterales* and several unclassified *Alphaproteobacteria*.

The bulk of the sequences belonged to the class of *Rhizobiales* (Figure 2a). In general, the relative abundance of sequences from this group was highest at 4°C (91%) compared to cultures incubated at 15, 20 and 30°C, (about 64%). Interestingly, the composition within the *Rhizobiales* observed at 30°C was different from that in the cultures incubated at 4, 15 and 20°C (Figure 2b). Members of the genus *Methylobacterium* were detected in high proportion at 30°C, whereas sequences in the family of *Beijerinckiaceae* were most abundant at lower temperature (Figure 2b). Similar to the *Methylobacteriaceae*, also members of the family of *Xanthobacteraceae* were enriched at 30°C (Figure 2b). Besides the *Rhizobiales*, bacteria belonging to the *Sphingomonadales*, *Rhodospirales* and *Caulobacterales* were mainly enriched at 4°C (Figure 2a).

Sequences belonging to the *Betaproteobacteria* were mainly observed in cultures enriched at 4°C (30%) (Figure 1). The betaproteobacterial fraction mainly consisted of the order *Burkholderiales* (99%) (data not shown). Within the *Burkholderiales*, *Oxalobacteraceae* and *Burkholderiaceae* were detected in high proportion (Figure 2c). The family of *Oxalobacteraceae* was mainly enriched at 4, 15 and 20°C, whereas *Burkholderiaceae* was more enriched at 30°C (Figure 2c). The phylum *Bacteroidetes* was mostly enriched at lower temperature, i.e. at 4 and 15°C, whereas its relative abundance decreased with increasing temperature (Figure 1). Within the phylum *Bacteroidetes* more than 90% of the sequences belonged to the genus of *Pedobacter*. In contrast, acidobacterial sequences were highest in the enrichments obtained at 20°C (1.36%) and 30°C (10.8%) (Figure 1, Table 1)

Effect of pH on the community composition

The effect of pH was tested by incubating the enrichment cultures at 15°C and pH 4.5, 5.5, and 7. In all samples, the most dominant group was *Alphaproteobacteria* followed by *Bacteroidetes*, *Beta*- and *Gammaproteobacteria* (Figure 1). Sequences belonging to the *Firmicutes*, *Acidobacteria*, and several unclassified-bacteria were not very abundant. Incubation at different pH caused some marked changes in the alphaproteobacterial composition. Remarkably, sequences of *Beijerinckiaceae* were most abundant in enrichments obtained at pH 5.5, whereas sequences belonging to the *Methylobacteriaceae* dominated enrichments both at pH 4.5 and pH 7 (data not shown). The relative abundance of *Rhodospirillales* was negatively correlated with increasing pH, which is in line with previous studies that indicated pH as the best predictor for the abundance of this order (Fierer and Jackson, 2006; Fierer et al., 2009). On the other hand, sequences belonging to the class of *Caulobacterales* were mainly found at pH 5.5 (Figure 3). Sequences affiliated with the *Oxalobacteraceae*, mostly classified within the genus *Janthinobacterium*, were mainly detected in the pH 7 enrichment, whereas the opposite trend was observed for sequences belonging to the family of *Burkholderiaceae* (data not shown).

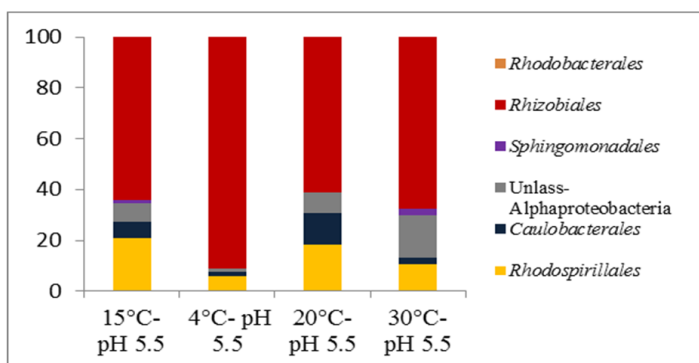


Figure 2a.

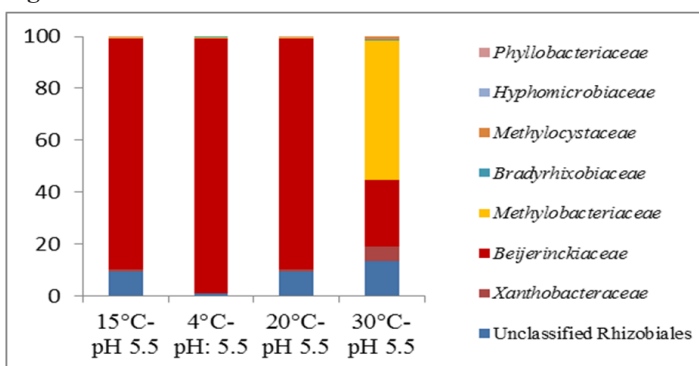


Figure 2b.

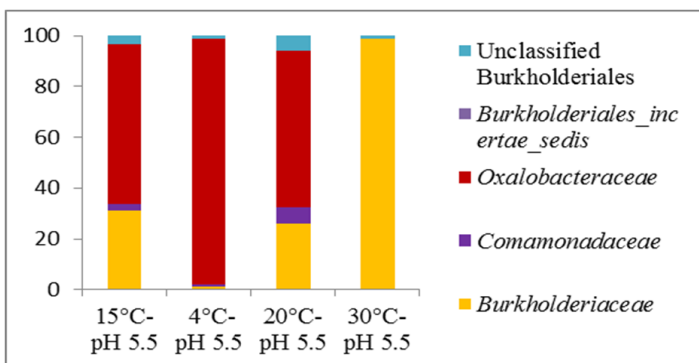


Figure 2c.

Figure 2. Relative abundance of microbial groups in all samples in the temperature data set. **a.** Orders within the class of *Alphaproteobacteria*. **b.** Families in the order of *Rhizobiales*. **c.** Families in the order of *Burkholderiales*

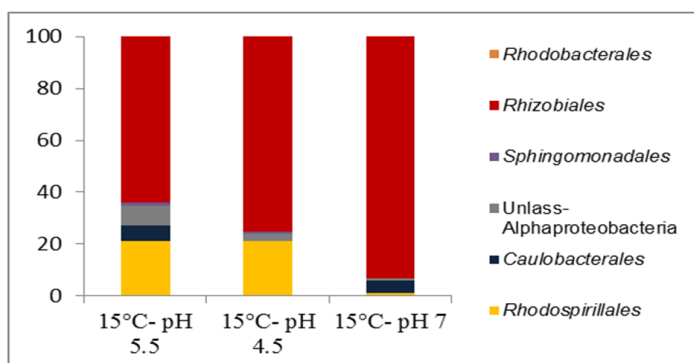


Figure 3. Relative abundance of orders within the class of Alphaproteobacteria in all samples in the pH data set.

Membrane lipid composition

All enrichment cultures were analyzed for PLFAs. In total, 11 compounds were detected, 7 of which were found in all cultures (Table 2). The most abundant fatty acid was (ω -7)C18, which was present in all samples, followed by (ω -7)C16 cis, (ω -5)C16 trans, n-C16:0, β -C14, n-C18:0. Diplopterol and C19 cyclopyl and some unknown compounds were also detected. The amount of diplopterol was highest in the sample enriched at 15°C and pH 4.5. In turn, the proportion of (ω -7) C18 in that sample was low compared to enrichments incubated at pH 5.5 and pH 7. The highest proportion of (ω -7)C16 fatty acids was observed in the sample enriched at 4°C and pH 5.5. We were not able to detect C19 cyclopropyl at pH 4.5 and pH 7. Branched GDGTs were only detected in the original culture at 15°C and pH 5.5.

Discussion

In our enrichments, we observed members of bacterial groups not previously reported as methylotrophs, namely *Acidobacteria* subdivision 1 and *Pedobacter*, and it is tempting to speculate that these might comprise yet unknown methanol-utilizing bacteria. We also found well-known methylotrophic groups belonging to the *Alpha*-, *Beta*- and *Gammaproteobacteria*. It was not shown before that environmental parameters, pH and temperature, had a strong effect on the differential enrichment of specific groups of methylotrophs.

The relative abundance of sequences retrieved from the phylum of *Acidobacteria* subdivision 1 was affected by both pH and temperature. Highest numbers of acidobacterial sequences were detected at 30°C and pH 5.5 (10.8%), whereas only a low proportion of acidobacterial sequences (0-1%) was obtained from the rest of the samples. The *Acidobacteria* constitute a diverse phylum reported to occur in a vari-

ety of natural environments, including peat bogs, acid mine drainages, contaminated aquifers, hot springs, freshwater lakes, marine invertebrates including sponges and corals, the pelagic zone of the oceans, and deep sea hydrothermal vents (Hugenholtz et al., 1998; Tringe et al., 2005; Janssen et al., 2006; Barns et al., 2007). However, information on the function of the taxonomically described diversity of *Acidobacteria* is limited (Barns et al., 2007; Hugenholtz et al., 1998). Increased numbers of *Acidobacteria* after amendments with methane and methanol were noticed in several studies (Radajewski et al., 2002; Nercessian et al., 2005; Kalyuzhnaya et al., 2008; Pankratov et al., 2008). Radajewski et al. (2002) showed that several 16S rRNA gene sequences belonging to *Acidobacteria* subdivision 1 were retrieved from a ^{13}C -labelled DNA fraction obtained after amendment of acidic soil with ^{13}C -methanol. These findings suggested that members of the *Acidobacteria* might be involved C1 turnover, and support our observations that they might be methylotrophs.

More than 90% of the Bacteroidetes sequences belonged to the genus of *Pedobacter*. Members of the genus of *Pedobacter* were most strongly enriched at the lowest temperature, 4°C, and at pH 5.5, whereas they were less abundant at higher temperature. *Pedobacter* species are Gram negative, non-motile, aerobic and they grow optimally between pH 6 and 8, and are generally known to be psychrophilic (Steyn et al., 1998). Up till now, there is not much information related to degradation of C1 compounds by members of this genus. Eyice and Schäfer (2010) showed that some *Pedobacter* species are able to use methylated sulphur compounds. However, there are no reports related with methanol utilization.

In the methylotrophic microcosms described here, we detected a broad range of bacteria including members of *Xanthobacter*, *Acidobacteria* subdivision 1, *Sphingomonas*, *Caulobacter*, *Oxalobacter*, *Methylobacter*, *Beijerinckia*, *Burkholderia* and *Pedobacter* (Table 3). Some of these include well-known methylotrophic genera such as *Hyphomicrobium*, *Xanthobacter*, *Beijerinckia*, *Burkholderia*, *Methylobacter* and *Janthinobacterium* (Dijkhuizen et al., 1988; Alves et al., 2001; Radajewski et al., 2002; Dedysh et al., 2005; Lidstrom, 2006; Chistoserdova et al., 2009; Boden et al., 2010). Sequences retrieved from *Alphaproteobacteria* dominated almost all samples with a relative abundance of 65 to 80% with an exception of the microcosm incubated at 4°C, where *Alphaproteobacteria* represented only 38% of the sequences. The relative abundance of sequences belonging to the *Beijerinckiaceae* and *Methylobacteriaceae* was affected more by changes in pH and temperature than the other families within the order of *Rhizobiales*. *Beijerinckiaceae* were mainly enriched at low temperatures from 4 to 20°C, confirming previous reports on the general characteristics of this genus (Kennedy, 2005). The genus of *Beijerinckia* is known to comprise aerobic, chemotrophic and acid tolerant bacteria, and have the ability to fix dinitrogen under aerobic or microaerobic conditions (Becking, 1999). Members

of *Beijerinckia* utilize a wide range of compounds. *B. mobilis* uses one carbon compounds via the ribulose biphosphate (RuBP) pathway (Dedysh et al., 2005). The relative abundance of *Methylobacteriaceae* was positively correlated with temperature, and their members showed a broad range of pH tolerance between 4.5 and 7 in our enrichments. Members of the genus *Methylobacterium* are able to degrade methanol and methylamine, via the serine pathway. They are well known pink-pigmented facultative methylotrophs and are widespread in a broad range of different environments including soil, water, plants, leaf and heavy metal contaminated areas (Green and Bousfield, 1982; Balachandar et al., 2008; Dourado et al., 2012). We observed a pinkish color in enrichments incubated at 30°C/pH 5.5 and at 15°C/pH 7, where the *Methylobacteriaceae* was the most abundant family.

The *Betaproteobacteria* represented the second most dominant class in all samples. The bulk of the sequences belonged to the order of *Burkholderiales* followed by *Neisseriales*, *Rhodocyclales* and several unclassified *Betaproteobacteria*. The vast majority of the sequences belonged to the family of *Oxalobacteraceae*, except for the enrichment incubated at 30°C (Figure 2c). Although the members of *Oxalobacteraceae* are acid tolerant (Garritty et al., 2005), this group was most abundant at pH 7 in our study. Nakatsu et al (2006) showed that some other families from the order of *Burkholderiales* are also able to utilize one carbon compounds. Members of the *Burkholderiales* are metabolically and ecologically diverse. Some species from the *Burkholderiales* degrade methylated compounds including methanol via the serine cycle (Kane et al., 2007). Within the *Burkholderiales*, the families of *Comamonadaceae* and *Rhodocyclaceae* are well known methylotrophs (Nakatsu et al., 2006; Lidstrom, 2006; Kalyuzhnaya et al., 2008). Vishnivetskaya et al. (2010) showed that members of the *Betaproteobacteria* including *Burkholderiaceae*, *Comamonadaceae*, *Oxalobacteraceae* and *Rhodocyclaceae* played an important role in ethanol and methanol conversion in microcosms that were set up to investigate U(VI) reduction. We detected a high proportion of sequences belonging to the genus *Janthinobacterium* within the *Oxalobacteraceae* which are known to produce violacein (a violet pigment), are Gram negative and motile. *Janthinobacterium* are widespread in different environments, including soil, lakes and permafrost regions (Sneath et al., 1984; Garritty et al., 2005). They were detected as active methylotrophs in several different studies (Radajewski et al., 2002; Lidstrom 2006), reinforcing that populations belonging to the genus of *Janthinobacterium* also utilized methanol in our study.

We also assessed changes in microbial diversity by analysing the phospholipid fatty acid (PLFA) composition of the enrichments. The major fatty acid in lipids (44 to 69 % abundance) in all samples was 18:1 ω 7c.

Table 3. OTU heat map from enrichment samples incubated at A= 4°C, B= 20°C,

C= 30°C at pH 5.5, and D= pH 4.5, E= pH 7 at 15°C. Warm colors indicate higher relative abundances.

#OTU ID	A	B	C	D	E	Order	Family	Genus
13						Rhizobiales	Beijerinckiaceae	
33						Rhodospirillales	Rhodospirillaceae	
102						Rhizobiales	Methylobacteriaceae	Methylobacterium
110						Sphingomonadales	Sphingomonadaceae	
123						Caulobacterales	Caulobacteraceae	
133						Rhizobiales	Xanthobacteraceae	Azorhizobium
136						Sphingomonadales	Sphingomonadaceae	
163						Rhizobiales	Methylobacteriaceae	Methylobacterium
170						Rhizobiales		
192						Caulobacterales	Caulobacteraceae	Caulobacter
239						Rhizobiales		
373						Rhizobiales	Methylobacteriaceae	Methylobacterium
376						Alphaproteobacteria		
450						Rhizobiales	Beijerinckiaceae	
489						Rhizobiales	Methylobacteriaceae	Methylobacterium
516						Rhizobiales		
596						Rhodospirillales	Rhodospirillaceae	
697						Alphaproteobacteria		
700						Rhizobiales	Methylobacteriaceae	Methylobacterium
774						Rhizobiales	Methylobacteriaceae	Methylobacterium
830						Rhizobiales	Methylobacteriaceae	Methylobacterium
862						Alphaproteobacteria		
905						Rhodospirillales	Acetobacteraceae	
864						Burkholderiales	Burkholderiaceae	Cupriavidus
878						Burkholderiales	Oxalobacteraceae	Janthinobacterium
814						Burkholderiales	Burkholderiaceae	Burkholderia
615						Burkholderiales	Oxalobacteraceae	Herbaspirillum
159						Burkholderiales	Oxalobacteraceae	
321						Pseudomonadales	Pseudomonadaceae	Pseudomonas
753						Pseudomonadales	Pseudomonadaceae	Pseudomonas
780						Pseudomonadales	Pseudomonadaceae	Pseudomonas
897						Acidobacteria GpI	GpI	
829						Acidobacteria GpI	GpI	
31						Sphingobacteriales	Sphingobacteriaceae	Pedobacter



An investigation of the effects of temperature on the biomass and analyzing the microbial community of soils, taken from cold, moderate and hot natural sites indicated that total PLFA concentration decreased with increasing incubation temperature, especially at 35°C (Wu et al., 2010). It was observed that monounsaturated PLFAs including 16:1 ω 9c, 16:1 ω 7c, 16:1 ω 5c, 17:1 ω 8c, 18:1 ω 9c, 18:1 ω 7c, 19:1 ω 11c decreased at increased temperature in all three soils and biomass decreased at 35°C (65-96%) (Wu et al., 2010).

In our study, we detected the highest 18:1 ω 7c amount at 20°C, pH 5.5, whereas the lowest amount was observed in the enrichment incubated at 15°C and pH 4.5. Our results corroborated with previous studies that found that the microbial PLFA composition was affected by the soil pH (Rousk et al 2010). Mono-unsaturated PFLAs including 16:1 ω 5c, 16:1 ω 7c and 18:1 ω 7c were less abundant at low pH, whereas the relative abundance of cy19:0 was high at low pH (Petersen and Klug, 1994; Rousk et al., 2010). Similarly, Pettersson and Bååth (2003) examined the effect of temperature changes on the bacterial community in both limed and unlimed soils and they found that increasing the temperature strongly affected the PLFA profiles. Besides the lipid fatty acids we also detected diplopterol in all samples (Table 2). The highest amount of diplopterol was observed at pH 4.5 and 15°C, whereas the proportion of diplopterol was stable in the other samples as compared to the initial enrichment. Hopanoids such as diplopterol are widespread in a broad range of different environments, and diplopterol is produced by a variety of bacteria including nitrate reducers, cyanobacteria and it is a very important membrane component in aerobic methylotrophs (Van Dorsselaer et al., 1977; Rohmer et al., 1984).

We detected branched GDGT production at 15°C and pH 5.5 (Table 2). Unfortunately, potential candidate(s) that produce branched GDGT were not enriched in further transfers to fresh medium, indicating that the branched GDGT-producing bacteria were outcompeted by bacteria that do not produce such lipids. We detected one single sequence that belongs to the phylum of *Acidobacteria* at 15°C and pH 5.5. This sequence was most similar to the 16S rRNA gene of *Bryocella elongata*, which was isolated from acidic sphagnum peat (Dedysh et al., 2012). Currently, it is not known if *B. elongata* is able to produce branched GDGT membrane lipids. Since we detected that about 20% of the sequences belonged to the genus of *Pedobacter* in this specific sample incubated at pH 5.5 and 15°C (Table 3), we investigated three taxonomically described representatives of this genus, namely *Pedobacter heparinus* (DSM2366), *Pedobacter cryoconitis* (DSM14825), *Pedobacter saltans* (DSM12145). Unfortunately, none of them was able to produce branched GDGT membrane lipids (data not shown).

In conclusion, microbial groups previously not associated with a methylotrophic lifestyle including *Pedobacter* and *Acidobacteria* subdivision 1 were enriched with methanol. This study furthermore showed that certain groups of known methylotro-

phic bacteria are strongly affected by pH, temperature or both. Members of *Methylobacteriaceae* and *Acidobacteria* were mainly enriched at 30°C at pH 5.5. It was also shown that members of *Methylobacteriaceae* grow at a broad pH range. Members of *Oxalobacteraceae* were mainly enriched at high pH, but their abundance was negatively correlated with high temperature, whereas enrichment of members of *Burkholderiaceae* was negatively correlated with pH and positively correlated with temperature. *Pedobacter* was mainly enriched at low pH and lower temperature. In general, *Betaproteobacteria* were mainly enriched at 4°C, whereas the opposite trend was observed for *Alphaproteobacteria*.

Future studies should focus on the isolation of novel representative species from the genus of *Pedobacter* and *Acidobacteria* subdivision 1 with methanol as substrate. Physiological studies with isolated species will result in more knowledge on their potential to grow methylotrophically. New trials with methanol at low temperature and low pH need to be done to get information whether these conditions will result in the selective enrichments of branched GDGT-producing bacteria, as was initially observed in this study.

Acknowledgement

We thank the Darwin Center for Biogeosciences (142.16.2051) for financial support, and T. van den Bogert for providing help with technical aspects related to the analysis of pyrosequencing data.

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CHAPTER 5

Supplemental Materials

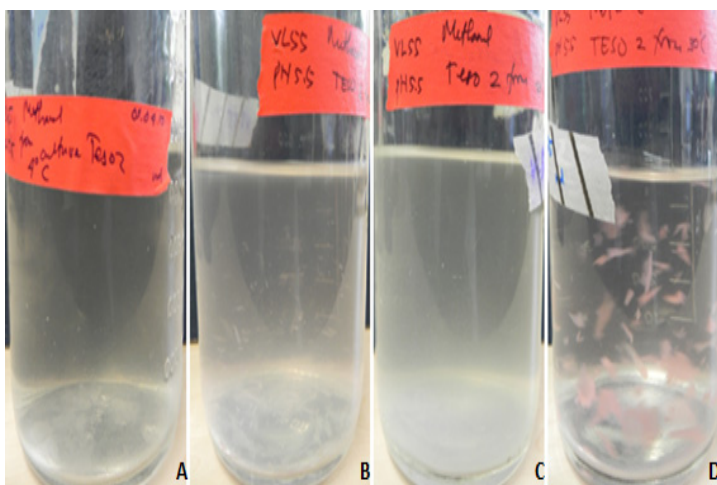


Figure S1. Cultivation experiment-temperature effect: A= 4°C; B= 15°C; C= 20°C; D= 30°C

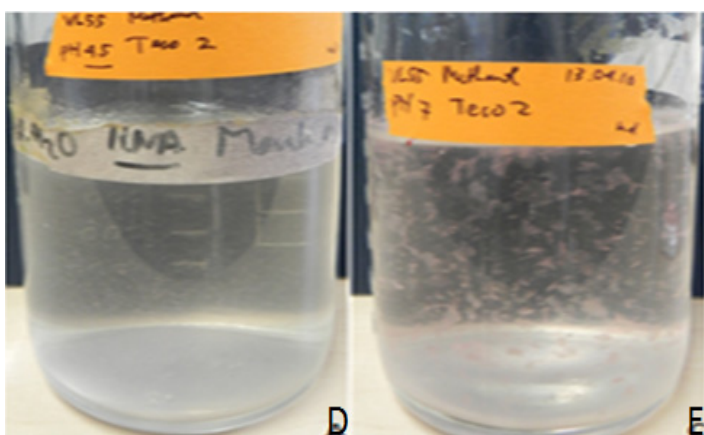


Figure S2. Cultivation experiment-temperature effect: D= pH 4.5; E= pH 7

CHAPTER 6

Isolation and Characterization of a Methanol Degrading *Azospirillum* Strain From Soil

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Manuscript in preparation

Abstract

An aerobic, white-pigmented, methanol-utilizing bacterium, strain SSRF, was isolated from an acidic soil, Têt watershed (France). Strain SSRF is Gram-negative, spiral-shaped, motile and non-spore forming. It grows optimally at 25°C and pH 6. Strain SSRF is able to grow with methanol, ethanol, glycine, pyruvate, lactate, succinate, malate, glutamate, α -ketoglutarate, citrate, fructose, glucose, mannitol, maltose, and mannose. The G+C content of the DNA is 64.7 mol%. The major fatty acids in lipids are C18:1 ω 7 and α OH-C18:1 ω 7. The intact polar lipids are phosphatidylethanolamine, monomethylphosphatidylethanolamine, phosphatidylglycerol, ornithine lipid, dimethylphosphatidyl-ethanolamine, 2-hydroxy ornithine lipid and phosphatidylcholine. The 16S rRNA gene sequence of strain SSRF was most closely related to that of *Azospirillum amazonense* (95%), *Azospirillum irakense* (91%) and *Azospirillum brasilense* (88%). A direct phenotypic comparison with these two type strains indicated that strain SSRF represents a novel species, for which the name *Azospirillum methanolicus* sp. nov. is proposed.

Introduction

The genus of *Azospirillum*, within the class of *Alphaproteobacteria*, consists of species that are highly versatile with respect to carbon and nitrogen metabolism. They are well-known plant growth promoting bacteria, with the ability to fix atmospheric nitrogen. *Azospirillum* spp. are known to survive harsh conditions. They form cysts and flocs to survive drought, synthesize reserve materials including poly- β -hydroxybutyrate and polysaccharides, and synthesize melanin conferring enhanced protection against UV light (Bashan, 1999; Baldani et al., 2006). Furthermore, *Azospirillum* spp. play a key role in reducing abiotic stress effects on plants, and they stimulate and facilitate plant growth (Bashan & Holguin, 1997; Tsavkelova et al., 2006). The ability of autotrophic growth with hydrogen as energy substrate has been described for *Azospirillum* strains (Malik and Schlegel, 1981). Some *Azospirillum* species are able grow with methanol or other C1 compounds, though this ability is rarely tested for newly isolated strains (Sampaio et al., 1982). Beijerinck (1925) isolated the first representative of the genus *Azospirillum*, initially described as *Spirillum lipoferum*, from a Dutch soil. This bacterium was later renamed as *Azospirillum lipoferum* by Tarrand et al. (1978). Members of the genus of *Azospirillum* have been isolated from a broad range of environments, including grasses, cereals, tropical soils, temperate regions, oil contaminated areas (Döbereiner et al., 1976; Bally et al., 1983; Ladha et al., 1987; Kirchhof et al., 1997; Gunarto et al., 1999; Young et al., 2008). Currently, the taxonomy of *Azospirillum* species is based on a polyphasic approach that includes 16S rRNA gene sequencing, DNA-DNA hybridization, fatty acid composition, DNA G+C content and phenotypic characterization (Eckert et al., 2001; Sly & Stackebrandt, 1999; Xie & Yokota, 2005). *Azospirillum* consists of 15 taxonomically described species: *A. lipoferum* (Tarrand et al., 1978), *A. brasilense* (Helsel et al., 2006), *A. amazonense* (Falk et al., 1985), *A. canadense* (Mehnaz et al., 2007a), *A. doebereineriae* (Eckert et al., 2001), *A. formosense* (Lin et al., 2012), *A. halopraeferens* (Reinhold et al., 1987), *A. irakense* (Khammas et al., 1989), *A. largimobile* (Ben Dekhil et al., 1997), *A. melinis* (Peng et al., 2006), *A. oryzae* (Xie & Yokota, 2005), *A. picis* (Lin et al., 2009), *A. rugosum* (Young et al., 2008), *A. thiophilum* (Lavrinenko et al., 2010) and *A. zeae* (Mehnaz et al., 2007).

In our study on the fate of methanol in soil samples, we isolated an aerobic methanol-degrading *Azospirillum* strain. The phylogenetic and physiological properties of the strain are described here.

Materials and Methods

Source of inoculum and culture medium

The sample was obtained from a slightly acidic (pH 4.9) soil near the Têt River in the south east of France. The sample was taken from the top 7cm of the soil. The soil temperature at the time of sampling was about 11°C. Half a gram of soil sample was homogenized in 48 ml of VL55 mineral medium (Janssen et al., 1997) without growth substrate or vitamins by stirring for 24 hours. From that sample further enrichments were made in VL55 medium supplemented with methanol as sole carbon and energy source. Medium VL55 contained 1.95 g 2-[*N*-morpholino]ethanesulfonic acid, 0.2 mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM (NH₄)₂HPO₄, 1 ml of trace element solution SL-10 (Widdel et al., 1983) per litre and 1 ml of selenite/tungstate solution (Tschech and Pfennig, 1984). The pH was adjusted to 5.5 with a mixture of 200 mM NaOH plus 100 mM KOH. 120-ml serum vials were used with rubber stoppers and aluminium crimp seals. Serum vials that contained 48 ml VL55 medium were autoclaved at 121°C for 20 minutes. Concentrated stock solutions of substrates, sterilized by filtration or autoclaving, were added to the medium to a final concentration 20mM, and 0.15 ml of vitamin solution was added per vial.

Unless indicated otherwise, the incubation temperature was 15°C and the headspace was filled with air prior to inoculation. Cultures were incubated in the dark without shaking. For routine cultivation the inoculum size was 2% (v/v).

Enrichment and isolation

A bacterial enrichment culture was obtained with 20 mM methanol as substrate. The enrichment was plated on solidified (1.5%, w/v, Agar Noble) VL55 medium amended with 20 mM methanol. After 3 weeks of incubation, white colonies were visible, and from the highest dilution colonies were picked, and then serially diluted

and spread on agar plates. This was repeated 3 times, after which a single colony was transferred to liquid medium. The culture was serially diluted 3 times to obtain a pure culture. Cell morphology and purity of the culture were checked by phase-contrast microscopy. Gram-staining was carried out according to the standard protocol (Doetsch, 1981). The pure culture was designated strain SSRF, and was used for further characterization. Its properties were compared with properties of *A. brasilense* (DSM 1690) and *A. amazonense* (DSM 2787) which were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig) and grown in the same medium as strains SSRF.

Substrate utilization test

The ability of strain SSRF to metabolize substrates was tested in VL55 medium, pH 6.15°C. Substrates were added from sterile, anoxic concentrated stock solutions to final concentrations of 20 mM, unless otherwise indicated. The pH range for growth was tested at pH 4.0, 6.0, and 7.0, in VL55 medium at 15°C, and the temperature range for growth was tested at 10, 20, 25, 28, 30°C at pH 6.

16S rRNA gene sequence analysis

For phylogenetic characterization of strain SSRF, DNA was extracted using the FastDNA®SPIN kit for soil, according to manufacturer's instructions (MP Biomedicals, Solon, OH, USA). PCR targeting the bacterial 16S rRNA gene was performed with bacterial primers 27F and 1492R (Park et al., 2006) and using the GoTaq DNA Polymerase Kit (Promega, Leiden, The Netherlands). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sequencing of the almost complete 16S rRNA gene was performed at Baseclear (<http://www.baseclear.com/>), yielding a sequence of 1363 nucleotides. The sequence was manually checked for misreading with alignment of Bioedit (www.mbio.ncsu.edu/bioedit/bioedit.html) closely related 16S rRNA gene sequences were identified using the BLASTN algorithm (<http://ncbi.nlm.nih.gov/blast>). Phylogenetic analysis was performed using MEGA 5 (Tamura et al., 2007). Phylogenetic distances of sequences were determined according to Kimura (1980). Phylogenetic trees were inferred by using parsimony (Fitch, 1971),

neighbour joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981). Jukes & Cantor (1969) correction was used for distance matrix calculation. The topology of the neighbour-joining tree was evaluated by bootstrap analysis with 1000 resamplings (Felsenstein, 1985).

G+C content

The G+ C content determination was performed at the DSMZ (Braunschweig, Germany). For analysis of DNA G+C content, DNA was isolated and degraded enzymatically into nucleosides according to the protocol from Mesbah et al. (1989), and the mixture of nucleosides was separated by HPLC.

Lipid analysis

Lyophilized cells were hydrolyzed with 5% HCl in methanol (MeOH) by reflux for 3 h. The hydrolysate was adjusted to pH 4 with 2N KOH/MeOH (1:1, v/v) and, after addition of water to a final ratio of 1:1 of the H₂O/MeOH, extracted three times with dichloromethane. The obtained extract was methylated with diazomethane and silylated with *N,O*-bis(trimethylsilyl)- trifluoroacetamide in pyridine at 60°C for 20 min and analyzed by gas chromatography (GC) and GC-mass spectrometry (MS). Double-bond positions of the monounsaturated fatty acids were determined on the basis of the mass spectra of their dimethyl disulfide derivatives as described by Nichols et al. (1986).

The intact polar lipids were extracted from the lyophilized cells using a modified Bligh and Dyer method as described by Pitcher et al. (2009). The extract was dissolved in hexane/2-propanol/water (72:27:1) at a concentration of 2 mg/ml and filtered through a 0.45-μm regenerated cellulose filter and analyzed as described elsewhere (Sinninghe Damsté et al., 2011). Briefly, an Agilent 1200 series LC, equipped with a T-controlled auto-injector and column oven was used, coupled to a Thermo LTQ XL linear ion trap with Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA).

Accession number

The 16S rRNA gene sequence of strain SSRF has been deposited in GenBank under

accession number JF340293.

Results

Morphological properties

Strain SSRF, isolated from acid soil with methanol as energy and carbon substrate is a spiral shaped, non-spore forming Gram-negative bacterium. On agar plates it forms white colonies with a rough appearance.

16S rRNA analysis and G+C content

Based on the analysis of the 16S rRNA gene sequence (1363bp), strain SSRF is a member of the genus *Azospirillum* within the family of *Rhodospirillaceae* of the order of *Rhodospirillales* of the class *Alphaproteobacteria* of the phylum of *Proteobacteria* (Figure 1). The rRNA gene sequence of strain SSRF has 95, 91 and 88% sequence identity with that of *A. amazonense*, *A. irakense*, and *A. brasiliense*, respectively. The strain is more distantly related to other *Azospirillum* species. The G+C content of the DNA of strain SSRF is 67.4 mol%.

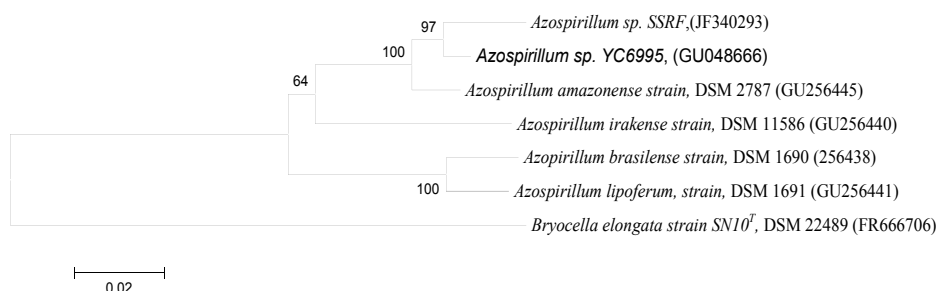


Figure 1. Neighbour-joining tree based on 16S rRNA sequences, showing the position of strain SSRF in the genus of *Azospirillum*. The reference bar indicates 0.02 substitutions per nucleotide position. Bootstrap values > 50% based on 1000 resamplings are given at respective nodes in the tree. GenBank accession numbers of all sequences are given in parentheses.

Growth and substrate utilization

Strain SSRF grows between 10°C to 25°C. Optimum growth was observed at 25°C and pH 6. Strain SSRF is able to utilize methanol, ethanol, glycine, pyruvate, lactate, succinate, malate, glutamate, α -ketoglutarate, citrate, fructose, glucose, mannitol, maltose, mannose and sucrose. Growth with α -ketoglutarate, glutamate, glucose, and peptone was much faster than with methanol. The strain is not able to grow with formate, propionate, 2-propanol, and butyrate. The growth spectrum of strain SSRF was compared with the growth spectrum of *A. amazonense*, *A. brasiliense* and *A. irakense*. *A. amazonense* and *A. brasiliense* were ordered from DSMZ (Braunschweig, Germany) and they were cultivated in VL55 medium. Data for *A. irakense* was taken from Khammas et al. (1989) (Table 1). Strain SSRF, *A. amazonense* and *A. brasiliense* can grow with ethanol, pyruvate, lactate, malate, succinate, α -ketoglutarate, glutamate, peptone, xylose and yeast extract (Table 1). They cannot grow with methanol. Formate is used by *A. amazonense*, but not by strain SSRF and *A. brasiliense*. Strain SSRF and *A. brasiliense* also use alanine, but *A. amazonense* did not. Strain SSRF, *A. amazonense* and *A. brasiliense* were not able to utilize benzoate. All 4 isolates are mesophilic, and strain SSRF and *A. amazonense* have a lower pH range of growth than *A. brasiliense* and *A. irakense*.

Lipid analysis

The fatty acid profile of strain SSRF was quite similar to that of *A. amazonense*, but not to that of *A. brasiliense* (Table 2). Although the relative contribution of major fatty acids C18:1 ω 7 and α OH-C18:1 ω 7 was similar among the tested species, there were some differences with respect to the proportion of fatty acids (Table 2). The abundances of C18:1 ω 7 and β OH-C16 in strain SSRF were higher than of *A. amazonense* and *A. brasiliense* (Table 2). C16:1 ω 5 is only present in strain SSRF and *A. amazonense*, whereas α OH-C14 is only present in *A. amazonense*.

The relative abundance of intact polar lipids showed minor differences between strain SSRF and the two reference strains. In total nine different IPLs were detected including phosphatidylethanolamine, monomethylphosphatidylethanolamine, phosphatidylglycerol, ornithine lipid, dimethylphosphatidylethanolamine, 2-hydroxy ornithine lipid, 2-hydroxy ornithine like lipid with a different amino acid

and phosphatidylcholine (Table 3). Strain SSRF and *A. amazonense* showed similar IPL composition with the exception of dimethylphosphatidylethanolamine, which was not found for the latter strain.

Table 1. Comparison of characteristic properties of strain SSRF and closely related representatives of the genus *Azospirillum* *1= Strain SSRF, 2= *A. amazonense* DSM2787, 3= *A. brasilense* DSM1690, 4= *A. irakense* DSM11586. Data for carbon source utilization by *A. irakense* was taken from Khammas et al. (1989); data for strain SSRF, *A. amazonense* and *A. brasilense* were from this study. Symbols: + = positive for strain and replicates; v = variable and replicates; - = Negative for strain and replicates; NA= not available.

ISOLATES*				
Characteristics	1	2	3	4
Optimum temp. (°C)	25	30	37	33
Cell size (µm)	1.0-1.2	0.9-1.0	1.0-1.2	NA
G+C content (mol%)	67.4	67-68	69-71	64-67
pH range	4.5-7	5.5-6	6-7.5	5.5-8.5
Habitat	Soil	Soil	Wheat roots	Rice roots
Formate	-	+	-	NA
Methanol	+	-	-	NA
Ethanol	+	+	+	NA
2-propanol	-	+	-	NA
Glycine	+	-	-	NA
Pyruvate	+	+	+	NA
Propionate	-	-	+	NA
DL-lactate	+	+	+	v
Succinate	+	+	+	NA
Malate	+	+	+	NA
Glutamate	+	+	+	NA
α-ketoglutarate	+	+	+	v
Citrate	+	-	-	v
Fructose	+	+	+	-
Glucose	+	+	-	v
Mannitol	+	-	-	-
Maltose	+	+	-	+
Mannose	+	+	-	+
Sucrose	+	+	-	+
Peptone	+	+	+	NA
Yeast extract	+	+	+	NA
Xylose	+	+	+	NA
Alanine	+	-	+	-
Lysteine	+	-	-	NA
Butyrate	-	-	-	NA
Benzoate	-	-	-	NA

Table 2. Fatty acid composition (%) of strain SSRF and 2 previously described representatives of the genus *Azospirillum* after acid hydrolysis. All strains were grown in VL55 medium. Major fatty acids are shown in bold.

Fatty acid	1*	2*	3*
C16:1 ω9	0.5	0.1	0.3
C16:1 ω7	5.9	3.8	13.8
C16:1 ω5	6.2	11.9	
C16:0	4.9	7.4	7.8
βOH-C14	1.9	1.4	8.0
αOH-C14		3.8	
C18:1 ω7	56.3	43.2	61.1
C18:0	0.5	0.8	0.9
βOH-C16	5.5	7.9	3.2
αOH-C18:1 ω7	18.0	19.2	4.0
αOH-C19:1 (cyclopropyl)	0.4	0.5	0.9

1*=Strain SSRF; 2*= *Azospirillum amazonense*; 3*= *Azospirillum brasilense*

Table 3. Relative abundances and fatty acid composition of intact polar lipids (IPLs) of strain SSRF and two described representatives of the *Azospirillum*

IPL	1*	2*	3*
PE	++	++	+
	(36:2, 34:2)	(36:2, 34:2)	(36:2, 34:2)
MMPE	++	+	-
	(34:2, 36:2)	(34:2, 36:2)	
PG	+	+	+
	(36:2, 34:2)	(36:2, 34:1)	(36:2, 34:2)
OL (115)	+	+	+
	(34:1)	(34:1, 35:1)	(34:1)
OL (129)	+	+	-
	(34:1, 35:1)	(34:1)	
DMPE	+	-	-
	(36:2, 34:2)		
2-OH-OL (115)	+++	++	+
	(34:1)	(34:1)	(34:1)
2-OH-OL (131)	++	+++	-
	(34:1)	(34:1)	
PC	-	-	+++
			(36:2, 34:2)

1*= Strain SSRF, 2*= *Azospirillum amazonense*, 3*= *Azospirillum brasilense*.

Abundance is given relative to the major peak in the LC/MS base peak chromatogram: +++ = base peak, ++ = 50-100% of base peak, + = 10-50% of base peak, - = less than 10% of base peak or not detected. Note that mass spectral response factors for different IPL groups can be quite different.

IPLs are listed in order of elution:

PE= Phosphatidylethanolamine, MMPE= Monomethylphosphatidylethanolamine, PG= Phosphatidylglycerol, OL (115) = Ornithine lipid, OL (131) = Ornithine like lipid with an amino acid 14 masses higher than ornithine, DMPE= Dimethylphosphatidylethanolamine, 2-OH-OL (115) = 2-hydroxy ornithine like lipid with a different amino acid, PC= Phosphatidylcholine. The predominant fatty acid composition is reported as the total number of carbon atoms of the acyl moieties and the number of double bonds.

Discussion

An *Azospirillum* strain was isolated that grows with methanol as the sole source of energy and carbon. The strain was phylogenetically closest related to the nonclassified *Azospirillum* sp. YC6995 and to the type strain of *A. amazonense* (97 and 95 % sequence identity, respectively) , and more distantly related to *A. irakense* and *A. brasilense*, with 91, and 88 % sequence identity of the rRNA gene, respectively. Physiological comparison of strain SSRF with *A. amazonense*, *A. brasilense* and *A. irakense* as the three most closely related validly described species within the genus *Azospirillum* revealed that only *A. amazonense* is able to use formate, whereas only strain SSRF was able to use methanol.

Members of the genus *Azospirillum* are able to fix nitrogen and use polymeric substrates such as hemicellulose for growth (Halsall et al., 1985a, 1985b; Ladha et al., 1986). Sucrose is not used by *Azospirillum brasilense*, whereas *A. amazonense* and strain SSRF use sucrose as a sole energy and carbon source. The ability of autotrophic growth is known for some members of this genus. *Azospirillum* spp. and *Azospirillum halopraeferens* are able to grow with C1 compounds i.e. methanol (Sampaio et al., 1982). In the description of novel *Azospirillum* species the ability of methylotrophic growth is rarely tested, which is remarkable as C1 compounds may represent important substrates derived from plant polymers, such as pectine and hemicellulose. The pathway of carbon assimilation by strain SSRF is not known. Methylotrophic bacteria may employ the ribulose monophosphate pathway or the serine pathway for carbon fixation (Lidstrom et al., 2006). Strain SSRF is able to grow with glycine, which is an intermediate of the serine pathway, whereas the other 2 isolates tested here, *A. amazonense* and *A. brasilense* are not able to utilize glycine. This finding suggests that the serine pathway might be employed by strain SSRF for growth with methanol. Further investigation is needed to get insight into the carbon assimilation pathways of strain SSRF.

Comparing the properties, strain SSRF is closer related, both phylogenetically and chemotaxonomically, to *A. amazonense* than to *A. brasilense*. Besides the fatty acids measurements, intact polar lipids were also determined in our study. The intact polar lipid composition of strain SSRF and *A. amazonense* is similar.

Dimethylphosphatidylethanolamine was mainly found in plant associated bacteria (Chapman, 2000). Since, it is represented only in few strains, including *A. formosense* (Lin et al., 2012) and strain SSRF, it might be used as a taxonomic marker for *Azospirillum* species. Based on the chemotaxonomic and phylogenetic differences with known *Azospirillum* species, strain SSRF represents a novel species within the genus of *Azospirillum* for which we propose the name *Azospirillum methanolicus*.

Description of *Azospirillum methanolicus* sp. nov.

Azospirillum methanolicus [me.tha'noli.cus. M. L. n. methanolicum, methanol; M. L. masc. adj. methanolicus, relating to methanol].

The cells are spiral shaped, motile, Gram-negative and the size is 1.0-1.2 μm . Growth occurs between 10 to 26°C at a pH ranging from 4.5 to 7.0. Optimum growth was observed at 25°C and pH 6.0. Strain SSRF was isolated from acid soil with methanol as substrate. After 3 weeks of incubation it forms whitish colonies with a diameter of 1 mm on VL55 agar supplemented with methanol. Growth was possible with methanol, glycine, various disaccharides, hexoses and pentoses. Formate, 2-propanol, propionate, butyrate and benzoate did not support growth. The major fatty acids were C18:1 ω 7, α OH-C18:1 ω 7. Intact polar lipids are phosphatidylethanolamine, monomethylphosphatidylethanolamine, phosphatidylglycerol, ornithine lipid, dimethyl phosphatidylethanolamine, 2-hydroxy ornithine lipid, and phosphatidylcholine. The DNA G+C content is 67.4 mol%.

16S rRNA gene sequence analysis indicated that strain SSRF belongs to the genus *Azospirillum* and is most closely related to *A. amazonense* (95%), *A. irakense* (91%), *A. brasilense* (88%) and *Azospirillum* sp. YC6995 (97%).

Acknowledgements

We would like to thank Ton van Gelder for providing help with technical aspects. We thank the Darwin Center for Biogeosciences (142.16.2051) for financial support.

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CHAPTER 7

General Discussion and Future Perspectives

GENERAL DISCUSSION

The main objective of this study was to identify, isolate and characterize the bacteria that produce branched GDGT membrane lipids, which are used as paleoenvironmental proxy for the estimation of past ambient temperatures. The bacterial community composition of soil samples with high and low GDGT content was determined to link GDGT content to specific phylogenetic bacterial groups. In addition, attempts were made to isolate potential candidates that produce these membrane lipids by using cultivation techniques in specific media. Based on previous data, members of the phylum of the *Acidobacteria* had been suggested as the prime candidates. The bacterial communities of the soil samples that were analysed turned out to be very diverse, and the majority of the detected bacteria remain to be uncultured. The abundances of different phylogenetic bacterial groups were significantly affected by environmental parameters, such as pH, temperature and substrate. By using methanol as growth substrate and applying high-throughput molecular techniques, well-known methylotrophs as well as possible novel methylotrophs were detected. Methylotrophs play an important role in the global carbon cycle, and initial enrichments indicated that branched GDGT producers can be enriched at low pH and low temperature with methanol as substrate. Even though further enrichment was not successful, new leads for future enrichment of branched GDGT membrane lipids producing bacteria were obtained.

The hunt for bacterial producers of branched glycerol dialkyl glycerol tetraether lipids in the domain of *Acidobacteria*

Branched glycerol dialkyl glycerol tetraether (GDGT) lipids are ubiquitously present in a broad range of different environments including peat bogs (Schouten et al., 2000; Sinninghe Damsté et al., 2000; Weijers et al., 2006), soils (Weijers et al., 2006, 2007a; Peterse et al., 2009), lakes (Blaga et al., 2009; Tierney et al., 2009), and coastal and marine environments (Hopmans et al., 2004; Kim et al., 2007). Two indices were developed based on these observations, namely the CBT (the Cyclisation of Branched Tetraethers) and the MBT (Methylation of Branched Tetraethers) index. The MBT-CBT proxy can be used to reconstruct the temperature of the living environment of branched GDGT producers. However, this promising

proxy, the relationship of branched GDGTs with MAT and pH, has so far only been tested in case studies, e.g. hot springs (Peterse et al., 2009) and soils sampled along a pH transect (Peterse et al., 2010), and thus still needs to be biologically validated and evaluated before it can be confidently applied. Previous studies suggested that members of the phylum *Acidobacteria* are the most likely candidates (Weijers et al., 2007, Peterse et al., 2010). This could recently be confirmed by Sinninghe Damsté et al., (2011), who showed that two isolates from *Acidobacteria* subdivisions 1 and 3 are able to produce branched GDGT-Ia membrane lipids, albeit at lower concentrations than what has been observed in environmental samples. Therefore, and based on the complex patterns of environmental distribution of these membrane lipids, it cannot be excluded that members of other subdivisions of *Acidobacteria* or yet other phyla are also able to produce branched GDGTs.

In chapter 3, 4 and 5 new leads towards the identity of potential additional producers of branched GDGT lipids were generated, by using both culture dependent and independent approaches. To this end, soil samples collected near two hot springs in California (Chapter 3) and near the Têt River in France (Chapter 4) were analysed. Samples from each of the different locations differed in the amount of branched GDGT lipids. By using 16S rRNA gene-targeted high-throughput screening methods, including cloning and sequencing, and pyrosequencing, the microbial community structure of samples containing high and low amounts of branched GDGT's was analysed and compared (Figure 1). Indications for the involvement of *Acidobacteria* in branched GDGT production was obtained, but was not unequivocally shown their involvement due to the failure to obtain branched GDGT producing bacterial isolates. Notably, the abundance of some other microbial groups, including taxa within the *Alphaproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes* coincided with the abundance of branched GDGTs (Chapters 3, 4 and 5). To this end, it is interesting to note that, even though there are considerable differences in the pH and temperature, soils that were taken from the different locations showed similar trends regarding the fact that high content branched GDGT soils were dominated by the phylum of *Proteobacteria*, mainly *Delta*- and *Alphaproteobacteria*, and *Bacteroidetes*.

A recent study showed that root associated microorganisms might be involved in

the production of branched GDGT membrane lipids (Huguet et al., 2012). Based on the natural ^{13}C abundance in the lipids, it was suggested that branched GDGT-producing bacteria should have a heterotrophic life style (Oppermann et al., 2010; Weijers et al., 2010; Huguet et al., 2012). Data obtained from this study are in line with those of previous studies. We detected a broad bacterial diversity in the soil samples, belonging to mainly heterotrophic phyla within the *Alpha*-, *Delta*-, *Betaproteobacteria*, *Bacteroidetes* and *Acidobacteria*.

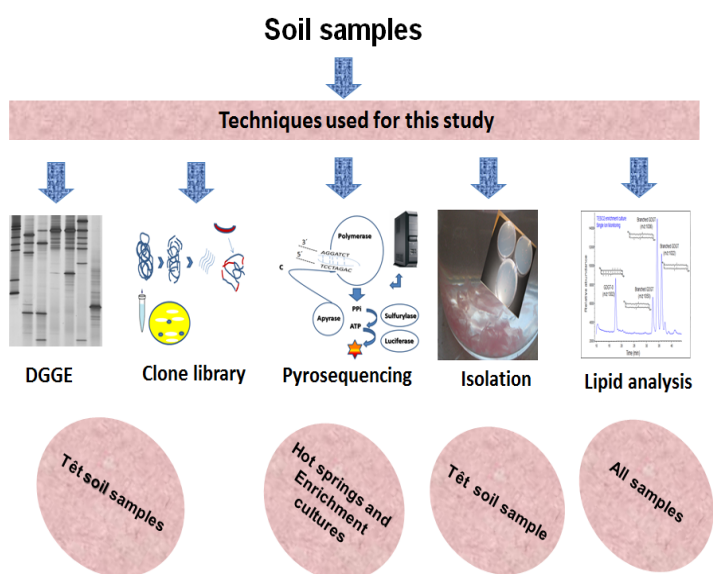


Figure 1. Integrated culture independent and culture dependent approaches used in this thesis.

In chapter 2 the current knowledge of the taxonomy, physiology and ecology of members of the phylum of *Acidobacteria* is presented. Innovative techniques for cultivating uncultured acidobacterial strains were addressed. Traditional cultivation of microorganisms is laborious, time consuming and the majority of microorganisms often do not form visible colonies on solidified media. Nevertheless, 16S rRNA surveys and metagenomics studies alone are not sufficient to get a complete picture of the physiology of members within the phylum of *Acidobacteria*. Possible strategies

to isolate novel representatives of this mysterious phylum are extended incubation time, using refinement of medium and cultivation conditions, i.e. incubating at lower temperature and using acidic medium, using polymeric substrates (Dedysh, 2011) and using high-throughput culture dependent techniques such as the micro-petri dish (Ingham et al., 2007). Taken together, a combination of cultivation dependent and cultivation independent techniques is needed to get more insight of the phylum of *Acidobacteria*.

In the course of the studies described in this thesis indications were obtained that specific bacterial interactions, i.e. syntrophy among members of different bacterial phyla might play an important role in the synthesis of branched GDGTs (Chapters 3, 4 and 5). Syntrophy is a type of symbiosis between two organisms that require each other for growth and it is well known for anaerobic methanogenic communities (Stams et al., 2009). Since we detected different anaerobic genera belonging to different phyla, it is tempting to speculate about a complex relationship between the different bacteria i.e. between *Deltaproteobacteria* and *Acidobacteria* that were always detected in high numbers in the high GDGT content samples. These interactions might also contribute to the difficulties to identify and isolate the target bacteria. In Chapter 5 microcosm incubations were described using a sample with high branched GDGT content as starting material. Production of branched GDGTs was observed in initial enrichment cultures incubated at 15°C and pH 5.5 in the presence of methanol as sole source of carbon and energy, suggesting that methylotrophic bacteria are able to produce branched GDGT lipids. Unfortunately, during subsequent enrichments of the branched GDGT producers were lost. This microcosm study furthermore led to the isolation of a novel *Azospirillum* strain that was proposed to represent a novel species, *Azospirillum methanolicus*. Up till now there is not much information available on the utilization of one carbon compounds by members of the genus of *Azospirillum*, even though this has been rarely tested in detail. This suggests that previous isolates from this genus should be re-evaluated for their ability to grow with reduced one-carbon substrates.

Influence of environmental factors on the bacterial community composition

Soil is a heterogeneous environment, containing numerous types of organisms. Notably, innovative techniques hold great promises to get more insight into the unexplored diversity and the drivers of geochemical cycles. The introduction of high-throughput techniques (Giovannoni et al., 1988; Torsvik et al., 1998) which allowed studying microbes without cultivation remarkably changed our perception of the diversity of microbes. Nevertheless, the vast majority of microorganisms are still uncultured, and hence our knowledge of the physiology and ecology of microbial communities in soil remains scarce. Therefore, it is important to get more insight into the microbial communities, especially how structure and function are influenced by biotic and abiotic factors.

More specifically, in the studies described in this thesis, the effect of temperature and pH on the bacterial community composition in different soil environments was studied (Chapter 3, 4 and 5). Soil samples were collected from 2 different areas, namely from different locations within the Têt watershed in the south east of France, and different hot springs in the US. A comparison of 2 high and 2 low pH samples revealed that certain bacterial groups including *Acidobacteria*, *Alpha*- and *Deltaproteobacteria* were more dominantly represented at lower pH (Chapter 4). To this end, we observed that members of *Acidobacteria* subdivisions 1 and 2 were enriched in acidic soils, whereas *Acidobacteria* subdivisions 4 and 6 were more abundant in neutral or slightly alkaline soils, which is in line with previous studies showed that some acidobacterial subdivisions, including subdivision 1, are only enriched at lower pH (Sait et al., 2006; Jones et al., 2009; Rousk et al., 2010). Furthermore, the families of *Beijerinckiaceae* and *Sphingobacteriaceae* were mainly detected at low temperature (**Chapter 4-5**).

The findings of this PhD study are:

- 1- More insight on the mysterious phylum of *Acidobacteria* was obtained, especially about the ecophysiology of *Acidobacteria* and possible strategies for successful isolation of novel strains.
- 2- High-throughput techniques allowed getting insight into the bacterial community composition in different soils and in methylotrophic enrichments.

Environmental parameters strongly influence the bacterial community composition.

- 3- Using culture dependent methods and high-throughput molecular techniques, possible branched GDGTs producers were identified. Besides members of the phylum of *Acidobacteria*, populations within the phylum of *Proteobacteria*, mainly *Delta*- and *Alphaproteobacteria* and the phylum of *Bacteroidetes* might also be involved in the synthesis of branched GDGTs.
- 1- Members of the *Acidobacteria* subdivision 1 and *Pedobacter* likely are methylotrophic. A methylotrophic *Azospirillum* was isolated, which is proposed to represent a novel species of the genus of *Azospirillum*.

Future perspective

In this thesis the microbial community structure of different soil samples was analysed by using high-throughput molecular techniques, but thus far, it was not possible to unequivocally identify the bacterial producers of branched GDGT lipids. Some enrichment attempts initially seemed promising, but in the end were not successful as the obtained isolates and enrichment cultures did no longer produce branched GDGT lipids under the tested conditions. By using innovative molecular approaches, the number of recognized bacterial phyla has increased considerably (Hugenholtz et al., 1998; Janssen et al., 2006). Nevertheless, still to date most of the phyla do not have cultured representatives. Classical and novel culture methods i.e. simple plating methods and innovative microscale cultivation technology (Joseph et al., 2003; Ingham et al., 2007) may be employed to obtain novel key players in culture. With such novel isolates, hypotheses developed based on molecular inventories can be tested regarding their potential role in microbial ecosystems. Since the discovery of simple plating techniques, microbiologists are trying to cultivate novel microorganisms. Yet, less than 1 % of the soil microorganisms have been cultured. By using low nutrient media, increasing the incubation time, changing the nutrient level and the oxygen concentration, novel bacteria can be obtained (Stevenson et al.,

2004; Janssen et al., 2006). Isolation of novel representative species from the genus of *Pedobacter* and *Acidobacteria* subdivision 1 will unveil their importance in the conversion of one-carbon substrates. Additionally, in order to get more insight into one-carbon transformations in soil, methylotrophic bacteria need to be isolated and their genomes analyzed. Additionally, ^{13}C stable isotope probing experiments may provide more insight of possible novel methylotrophic bacteria.

Since we do not know if besides pH and temperature, any other environmental parameters can influence branched GDGT production, well designed microcosm experiments are needed. We conducted an experiment with ^{13}C -labelled substrates to see whether the candidate organisms in microcosms incorporate labelled carbon from glucose in branched GDGTs. Unfortunately, these attempts were not yet successful. The main reasons might be that branched GDGT-producing bacteria are growing slowly or do not use glucose as substrate (unpublished data). Since we had some branched GDGT production in our microcosm study with methanol as substrate, labelled C1 compounds might be better candidate substrates for such studies.

An alternative approach might be to screen a large number of randomly isolated bacteria as well as representative strains currently available from culture collections for the ability to produce branched GDGT lipids. They need to be grown at different conditions with respect to pH, temperature and substrate and their lipids analysed. This approach is laborious, but at the end might allow linking branched GDGT production to bacterial groups and environmental conditions.

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Nederlandse Samenvatting

In grond en veen zijn bacteriën aanwezig die vertakte glycerol dialkyl glycerol tetraether membraan lipiden (GDGTs) kunnen maken. Vertakte GDGTs worden gebruikt als biologische indicatoren voor de analyse van fossiele monsters om de bodemtemperatuur en pH op geologische tijdschaal te schatten. Op basis van de samenstelling en hoeveelheid van vertakte GDGTs werden twee indices gedefinieerd; de mate van cyclisatie van vertakte tetraethers (CBT) is gerelateerd aan de pH en de mate van methylering van de vertakte tetraethers (MBT) is gerelateerd aan zowel de pH van de bodem als de over het hele jaar gemiddelde luchttemperatuur (MAT). Aangenomen wordt dat bacteriën vertakte GDGTs maken als een reactie op veranderingen in de pH en temperatuur. Op basis van hun voorkomen in veengebieden die een hoog gehalte aan vertakte GDGTs hebben is voorgesteld dat bacteriën van het geslacht *Acidobacteria* verantwoordelijk zijn voor de productie van vertakte GDGTs, en onlangs werd aangetoond dat twee vertegenwoordigers van de *Acidobacteria*, *Edaphobacter aggregans* Wbg-1T en *Acidobacteriaceae* stam A2-4 c, inderdaad deze lipiden bezitten. Echter, vertakte GDGTs komen naast in veengebieden ook nog in tal van andere bodems voor en daardoor is het aannemelijk te veronderstellen dat nog andere acidobacteriën en andere bacteriën vertakte GDGTs kunnen produceren.

Acidobacteria is een divers en veelvoorkomend geslacht van bacteriën. Ze spelen een belangrijke rol in met name terrestrische milieus. Informatie over de fylogenie en taxonomie van Acidobacterien alsook hun rol in het milieu, genoomeigenschappen en isolatiemethoden werd verzameld en vastgelegd in een van de hoofdstukken van dit proefschrift.

De bodem is een zeer dynamisch en complex ecosysteem. Bodemorganismen bepalen in sterke mate de eigenschappen van de bodem. Bacteriën zijn van cruciaal belang voor de bodemstructuur en geochemische omzettingen. De biologische activiteit in de bodem vindt voornamelijk in de bovengrond plaats, waar zich de meeste micro-organismen en plantenwortels bevinden.

Bodem micro-organismen, zowel bacteriën als archaea en schimmels, zijn verantwoordelijk voor de kringloop van nutriënten en de afbraak van organische verbindingen. De microbiële activiteit wordt sterk beïnvloed door een scala van abiotische en biotische factoren, en de interactie tussen verschillende micro-organismen speelt hierbij eveneens een belangrijke rol. Om inzicht te krijgen hoe milieuparameters de bacteriële diversiteit en productie van vertakte GDGTs beïnvloeden, werden bodemmonsters van verschillende locaties die verschillende hoeveelheden vertakte GDGTs bevatten geanalyseerd. Het betrof bodemmonsters dicht bij twee verschillende heetwaterbronnen (Surprise Valley, Californië, VS) en uit het stroomgebied van de rivier de Têt (Frankrijk). De bacteriesamenstelling werd bepaald door gebruik te maken van kweek-onafhankelijke high-throughput technieken. Tevens werd getracht middels selectieve ophoping inzicht te krijgen van vertakte GDGT-producenten. Het bleek dat de pH en de temperatuur een grote invloed hebben op de microbiële samenstelling. De resultaten in dit proefschrift laten zien dat er naast acidobacteriën ook andere bacteriën verantwoordelijk zijn voor de waargenomen productie van vertakte GDGTs. Het vóórkomen en de relatief hoge aantallen *Alfa*- en *Deltaproteobacteria* en *Bacteroidetes* zijn in verband te brengen met de hoeveelheid vertakte GDGTs in de bodemmonsters. Bovendien, incubaties bij verschillende pH en temperatuur en met verschillende substraten leidde tot de veronderstelling dat methanol een goede koolstofbron is om bacteriën op te hopen die vertakte GDGTs kunnen maken. Helaas, waren de pogingen om bacteriën die vertakte GDGTs kunnen produceren te isoleren niet succesvol. Echter, er werd wel een nieuwe methylootrofe *Azospirillum* soort geïsoleerd, welke we *Azospirillum methanolicus* genoemd hebben. Tevens toonden ophopingsstudies aan dat bacteriën behorende tot *Acidobacteria* subdivisie 1 en *Pedobacter* met methanol kunnen groeien.

Uit onderzoek dat in dit proefschrift beschreven is blijkt dat de temperatuur en

pH een sterke invloed hebben op de bacteriële samenstelling van alle bodems die bestudeerd zijn. Hoewel de bacteriën die vertakte GDGTs produceren niet geïsoleerd konden worden, kan het hier beschreven onderzoek helpen om nieuwe strategieën te ontwikkelen die kunnen leiden tot het isoleren en identificeren van deze groep van bacteriën.

Trefwoorden: Vertakte GDGTs, proxie, pH, temperatuur, *Acidobacteria*, methylootrofie, high-throughput technieken

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Rozelin Aydin was born in Turkey on the 5th of January 1982. From 1999 until 2003, she studied at the Faculty of Fisheries at Çukurova University in Adana, where she graduated completing her MSc program. She was awarded Socrates-Erasmus grant for 9 months at the groups of Cell Biology and Immunology, and Aquaculture and Fisheries. Her MSc thesis entitled “Alteration of the distal intestinal permeability and the endocytosis process in Atlantic salmon (*Salmo salar* L.) fed a soybean meal-based diet” was defended in 2006. From March 2007 onwards, she continued practicing science as a PhD student in the Laboratory of Microbiology at Wageningen University under the supervision of Prof dr . ir. A.J.M Stams and Prof. dr. Hauke Smidt. Since July 2011, she is working as a researcher in the same laboratory, working on the bioremediation of polluted soil and groundwater systems.

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Acknowledgements

The completion of this thesis is never an individual work. Being surrounded with great people kept me finishes my thesis. Therefore, I would like to take this opportunity to express my deepest gratitude to many people who involved to this PhD project.

First and foremost among them is one of my promoter, Fons Stams, who gave me an enormous amount of support, encouragement and great guidance. Sometimes words are simply not enough to express my appreciation and now, I am really having difficulties to find the best words to thank you. I always feel deeply grateful for your supervision. Next, I owe my deepest gratitude to my other promoter, Hauke Smidt. Dear Hauke, thank you so much for your invaluable support, encouragement, endless help. Also, your availability 24/7 is astonished.

Dear Willem, thank you very much for giving me this wonderful opportunity.

Beste Francien, Irene, Johan, Stefan and Jaap, bedankt voor alles! Bedankt voor de goede samenwerking. Dear Svetlana, it was a great pleasure to work with you. Many thanks for your great contribution to my thesis (Chapter 2).

Melikecigim, iyilik melegim, nerden baslasam , ne kadar tesekkur etsem az kalir, yetmez. 5 yil suresince her zaman sevecen bir dost, bir hoca olarak arkamda durdugun icin cok ama cok tesekkur ederim. Sonsuz destegin icin cok ama cok tesekkurler. Derya deniz bilgin varken bir o kadar da mutevazi olman inan bana cok sey ogretti.Sen bana bu sekilde sadece mikrobiyal fizyolojiyi ogretmedin, hayati da ogretmis oldun. Iyi ki seni tanima firsatina erisebildim. Mikrobiyoloj Labi'nin diger eski ve yeni Turk tayfalari ; Neslihancik hersey icin cok sagolasin. Dostlugun ve sonsuz destegin icin cok ama cok tesekkur ederim. Seattle ve San Francisco bir sey cagristirmistir sanirim, degil mi Oylum? Guzel ciceklerin esliginde balkonun da ictigimiz o enfes kahve sefalarini ve ustune bir de erkekler ve hayat uzerine bitmek bilmeyen sohbetleri unutmak ne mumkun. Sevgili Derya ve Sameto; mini mini birler, yuvarlak ikiler diyesim geldi birden. Sizi cok seviyorum, iyi ki varsiniz, hersey icin cok ama cok tesekkur ederim.

Beste Ton, it's been a great pleasure working with you. I wish to thank you so much for your friendship, invaluable support and help. Dear past and present MicFys's; Caroline, Ton, Gosse, Wim, Ana, Marjet, Vicente, Peer, Micheal, Susakul, Nam, Martin, Joana, Derya, Samet, Sidnei, Lara, Irene, Teun, Aura, Jueeli, Urania, Alona, Farrakh, Melike, Elsemik, Flavia, Christian, Marcel, Jeanine, Petra, and MolEco's; Detmer, Farai, Mauricio, Hans, Erwin, Coline, Sebestian, Susana, Basak, Teresita, Carmen, Tian, Jing, Floor, Clara, Noora, Janneke, Gerben, Naim, Kyle, Milkha, Tom,

Vincent, Dennis, Toygun, Gianina, Mark, Javier, Leo, Oylum, Wilma, Philippe, Ineke, Odette, Petia, Sahar, Neslihan, Rocio, Mirjana, Muriel, Carien, Diana, Eline, thank you all. Thank you very much for your great company and invaluable support. Also, I wish to thank for the group of Bacgen and Fungen; Fab, Mathijs, Mark, Woulter, Bram, Miguel, Ruud and so on. Thanks for the nice chatting. A special thanks to Wim, Anja, Carolein, Roelfina, and Petra for their enormous help.

I also thank to PyroTeam; Detmer, Jose, Mauricio, Tom, Hans, Naim and Gianina. Thanks a million for all your support and invaluable comments.

To all my close friends, Ana, Marjet, Burcu, Ton, Daniel, Esra, Martin, Teun, Joos, Farai, Jose, Irene, Rie, Maria, Robin, Yesim, Yusuf, Ali, Eylem, Nil, Fatma, Emeka, Petra, Katrin, Naiara, Juanan, Jeanine, Ellen, Abhishek, Merel, Ayhan, Serap, Zehra, Onur, Farrakh, Flavia, Marta, Nicolas, Pierpaolo, Selma, Yuliya, Pilar, Sonia, Coline, thanks a million times! I LOVE YOU ALL and I feel so lucky that I met you. My dear Ana, thank you very much for always being there for me. I wish to thank you again for your invaluable support. I will never ever forget our adventures in the hospital during my walking disaster period. Beste Marjet, bedankt voor alles! Thank you for your friendship. I am truly grateful.

Fatmacim guzel insan, can insan, sartsiz kosulsuz her zaman yanimda olan ender insanlardan birisin ve be seni tanidigim icin cok sansliyim. Hersey icin cok ama cok tesekkur ederim. Bucum yazmaya baslasam sayfalar yetmez yasadiklarimiza. Hastalikta, saglikta, iyi ve kotu gunde..kendimi nikah memuru gibi hissettim simdi. Daha da katlanarak cogalsin dostlugumuz. Daha ismini sayamadigim nice dostlarim, hayatima guzellik katan ve hayatimi anlamlı kilan canlar, zor gunlerimde benden destegini hic esirgemeyen tum guzel dostlarim, hepinizi cok seviyorum. İyi ki sizleri tanimisim ve ne sansliyim ki desteginiz her daim benimle.

Annecigim, yanimda oldugun icin, hayata tekrar dondugun, bizi sensizlikle bas basa birakmadigin ve sonsuz destegin icin ne kadar tesekkur etsem azdir. Kendimi kotu hissettigim anlar da bile sesinizi duymak bana fazlasiyla yetiyordu. Babacim, annecim ve Firat Can sizleri cok seviyorum, iyi ki varsiniz.



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- o *Identity and ecophysiology of anaerobic soil bacteria producing branched GDGT membrane lipids.* PhD Study Trip, 15 - 16 April 2009, Woods Hole Oceanographic Institute & Kolter Lab, Harvard University, MA & Boston, USA.
- o *Can anaerobic soil bacteria produce branched GDGT membrane lipids?* Konstanz Symposium, 7 March 2009, Wageningen, the Netherlands

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Cover : Ayhan Algur

Layout: Rozelin Aydın

Printed by : GVO Printers & designers B.V Ponsen & Looijen

