Assessment of soil bacterial communities with emphasis on the phylum *Acidobacteria*

DISSERTATION

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CONTRIBUTIONS BY THE COAUTHORS

Chapter 3

Dr. F. Gich has supervised the diploma-thesis from H. Hoffelner who has contributed the alpine soil sample, the data for the ATP activity in soil slurries, the data of the preliminary tests for detachment of soil bacteria from soil particles, and has produced the MicroDrop cultures.

Chapter 4

Dr. F. Gich has supplied the unidentified acidobacterial enrichment culture of the alpine soil sample. Dr. P. F. Dunfield has contributed the acidobacterial pure culture of strain Wbg-1 of the forest soil sample and its complete 16S rRNA gene sequence.

Chapter 5

Dr. F. Gich has supervised the diploma-thesis from H. Hoffelner who has contributed the alpine soil sample.

I hereby confirm the above statements.

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TABLE OF CONTENTS

I ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHL	N-(butyryl)-DL-homoserine lactone
C	cytosine
cAMP	cyclic adenosine monophosphate
CON	control without inducer molecules
DAPI	4',6-diamidino-2-phenylindol
ddH_2O	double-distilled water
DGGE	denaturing gradient gel electrophoresis
DNA	desoxyribonucleic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EPS	extracellular polymeric substances
G	guanine
HA	humic analogs
HD-medium	yeast extract-dextrose-medium
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HSL	homoserine lactone
IND	inducer molecules
LB	Luria-Bertani medium
MES	2-(N-morpholino)ethanesulfonic acid
MLSA	multilocus sequence analysis
NRCS	Natural Resources Conservation Service
OHHL	*N*-(oxohexanoyl)-DL-homoserine lactone
PCR	polymerase chain reaction
PHB	poly-β-hydroxybutyrate
POL	polymer mixture
PP/Tween	pyrophosphate and Tween 80
RT-PCR	real-time polymerase chain reaction
RO	artificial root exudates
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SSU	small subunit
v/v	volume per volume
VBNC	viable but non culturable
w/v	weight per volume

Chapter 1

Summary

1 SUMMARY

The seasonal culturability (February, April, August) of bacterial cells from a microbial community of an alpine calcareous soil was assessed employing the MicroDrop technique using different laboratory media with humic acid analogs (HA), a mixture of polymers (POL), artificial root exudates (RO), nutrient broth, or soil extract as carbon and energy sources. Thereby, the summer August sample showed the highest culturability value in media supplemented with soil extract (13.5%). Since only 81 wells of a total number of 1008 individual growth tests were overgrown with the February soil sample, the cultivation success was the lowest for the winter environment (0.16%). The major aim of the present study, however, was to assess the cultivation success for cells even exposed to extreme environmental conditions by using defined media. Therefore, subsequent analysis focused on the cultures obtained from the February sample and in media supplemented with RO. It was shown that the monomeric organic carbon of RO proved to be superior to POL and HA for the optimization of the cultivation success (i.e., 71 of the total number of 81 cultures).

The quantitative PCR approach confirmed the high coverage of the present analysis since the target groups (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*) constituted 73.6% of all eubacteria in the sample whereas the major part was composed of *Alphaproteobacteria* (49.2%) and *Acidobacteria* (20.1%). A total of 251 bacteria were analyzed representing 53 distinct phylotypes of which 73% are previously unknown. The majority of the cultured fraction was closely related to the *Alphaproteobacteria* with the largest number of different phylotypes and the highest evenness value. Although this phylum dominated the cultivated fraction, its cultivation success was hundredfold lower than its abundance in the natural community (0.4% of total cell numbers). Also the *Bacteroidetes* were most frequently cultured but were dominated by one phylotype (*Sphingoterrabacterium pocheensis*). The relative culturability of the *Bacteroidetes* was the highest of all groups and reached 25% of the numbers detected by real-time PCR. The lowest culturability was assessed for the *Acidobacteria* with only one single cultivated phylotype using media with POL supplemented with signal compounds. However, this phylotype represents a novel, previously unknown acidobacterium, strain Jbg-1.

The phylum *Acidobacteria* mostly consists of environmental 16S rRNA gene sequences and so far comprises only the four validly described species *Holophaga foetida*, *Geothrix fermentans*, *Acidobacterium capsulatum* and *Terriglobus roseus*. In the present thesis two different novel strains of acidobacteria were isolated. Strain Jbg-1 and the second strain Wbg-1, which was recovered from a coculture with a methanotrophic bacterium established from

calcareous forest soil. Both strains represent members of subdivision 1 of the phylum *Acidobacteria* and are closely related to each other (98.0 % 16S rRNA gene sequence similarity). At a sequence similarity of 93.8-94.7%, strains Jbg-1 and Wbg-1 are only distantly related to the closest described relative, *Terriglobus roseus*, and accordingly are described as members of the novel genus *Edaphobacter* gen. nov. Based on the DNA-DNA-similarity between strains Jbg-1 and Wbg-1 of 11.5-13.6% and their chemotaxonomic and phenotypic characteristics, the two strains are assigned to two separate species, *Edaphobacter modestus* sp. nov. with strain Jbg-1^T (= ATCC BAA-1329^T = DSM 18101^T) as the type strain, and *E. aggregans* sp. nov. with strain Wbg-1^T (= ATCC BAA-1497^T = DSM 19364^T) as the type strain. The two novel species are adapted to low carbon concentrations and to neutral to slightly acidic conditions. It was shown that strain Jbg-1 was also well adapted to long-term survival and to higher carbon concentrations after subcultivation.

Unexpectedly, a high percentage of interspecific interaction was obtained for the cultivation approach of the February alpine soil (75% cocultures), which represented the major reason for the low cultivation success. Only 16 out of 71 cultures with RO consisted of single cultivated strains. Due to the frequent occurrence of different bacteria in the same cultures, the actual cultivation success was 4.9 fold higher than the value calculated from the abundance of positive cultures. For subsequent analysis, the effect of different treatments during the cultivation approach on the number and composition of bacteria cultured was investigated. In order to differentiate between free-living and attached cells, bacteria were detached from soil particles and used to set up parallel incubations. The detachment from soil particles prior to inoculation had no effect on the total cultivation success and on co-cultivation. Furthermore, signal compounds (cyclic AMP and *N*-butyryl homoserine lactone), however, increased the cultivation success and co-culturability. Addition of signal compounds yielded different types of activated bacteria and enhanced the total number of phylotypes per co-culture towards 4, 5, 6, and 7 different bacteria. The major part of the single cultivated strains represented a single phylotype, which was related to *Sphingoterrabacterium pocheensis*. In contrast, most co-cultures contained members of the *Alpha*- and *Betaproteobacteria* whereas relatives of *Phyllobacterium brassicacearum*, *Rhodospirillum rubrum*, *Inqulinus ginsengisoli*, *Delftia tsuruhatensis*, and *Rhodocyclus tenuis* were the most abundant ones.

In conclusion, it is supposed that cell-to-cell interaction routinely occurs between different species of microorganisms, although the way, how these aerobic microorganisms beneficially interact remained to be shown. The elucidation of such interactions seems to be the most successful approach to enhance the culturability of interesting soil bacteria to promote their growth in pure or defined co-cultures.

Chapter 2

Introduction

2 INTRODUCTION

2.1 BACTERIAL BIODIVERSITY

Prokaryotes are the most diverse and abundant type of organisms (Whitman *et al*., 1998; Torsvik *et al*., 2002), which catalyze many of the environmental processes that sustain life on Earth. Therefore, their diversity is of important concern for bioremediation and bioprospecting applications. However, the factors determining the microbial diversity are poorly understood (Ward *et al*., 1998; Horner-Devine *et al*., 2003). Not until the understanding of bacterial biodiversity become more profound, the microbial potential can be used to its full extension.

2.1.1 Recent advances in bacterial biodiversity research

Biodiversity describes the number of prokaryotic species and their relative abundance in a community (Torsvik *et al*., 2002). For a long period of time, the identification of microbial species was limited to pure cultures or defined co-cultures (Amann *et al.*, 1995; Pace, 1997). The advent of culture-independent molecular techniques allowed a deeper insight into microbial diversity. These molecular techniques mainly focus on the small-subunit (SSU or 16S) ribosomal RNA (rRNA) (Woese, 1987). The 16S rRNA gene has several characteristics that explain why is so widely used to study bacterial diversity: ubiquitous distribution among prokaryotes, relatively slow evolution rate, and the coexistence of highly variable and conserved regions. The variable regions enable a comparison between very divergent bacteria, while the highly conserved domains serve as templates for designing specific PCR amplification primers or specific nucleotide probes. Thereby, the diversity of a bacterial community in a natural environment can be investigated without any culture, solely based on molecular phylogeny (Giovannoni *et al*., 1990; Santos and Ochman, 2004). Potentially, most of the sequences present in the environment can be detected by PCR. Consequently, there is a tremendous difference in the estimation of bacteria diversity based on culture-independent and culture-dependent approaches since cultivation has inherent selection towards certain bacteria.

Initially, Carl Woese (1987) primarily classified the domain *Bacteria* into 12 related phyla, which were tripled in the following decade up to 36 divisions (Hugenholtz *et al*., 1998; Santos and Ochman, 2004), based on 8,000 bacterial 16S rRNA gene sequences at that time available as obtained by cloning environmental DNA directly, or after amplification by PCR. A high proportion of the bacterial divisions, however, was predominantly represented by uncultured organisms. Even 13 divisions were entirely characterized by environmental sequences and hence

named as 'candidate divisions'. In the following years, the number of bacterial divisions increased to 52 (Fig. 1; Rappé and Giovannoni, 2003; Handelsman, 2004), from which 25 divisions were still represented by only uncultured organisms. At present, almost 80 phyla including 54 'candidate-divisions' are recognized (Fox *et al*., 2005; Kamagata and Tamaki, 2005). Due to the progress in the molecular techniques, the number of phyla doubled in the last few years (from 36 to 80), whereas the number of phyla with cultivated representatives failed to increase significantly (from 23 to 26). Taxonomic lineages, which predominantly comprise cloned sequences are, e.g. *Verrucomicrobia*, *Chloroflexi* and *Acidobacteria*.

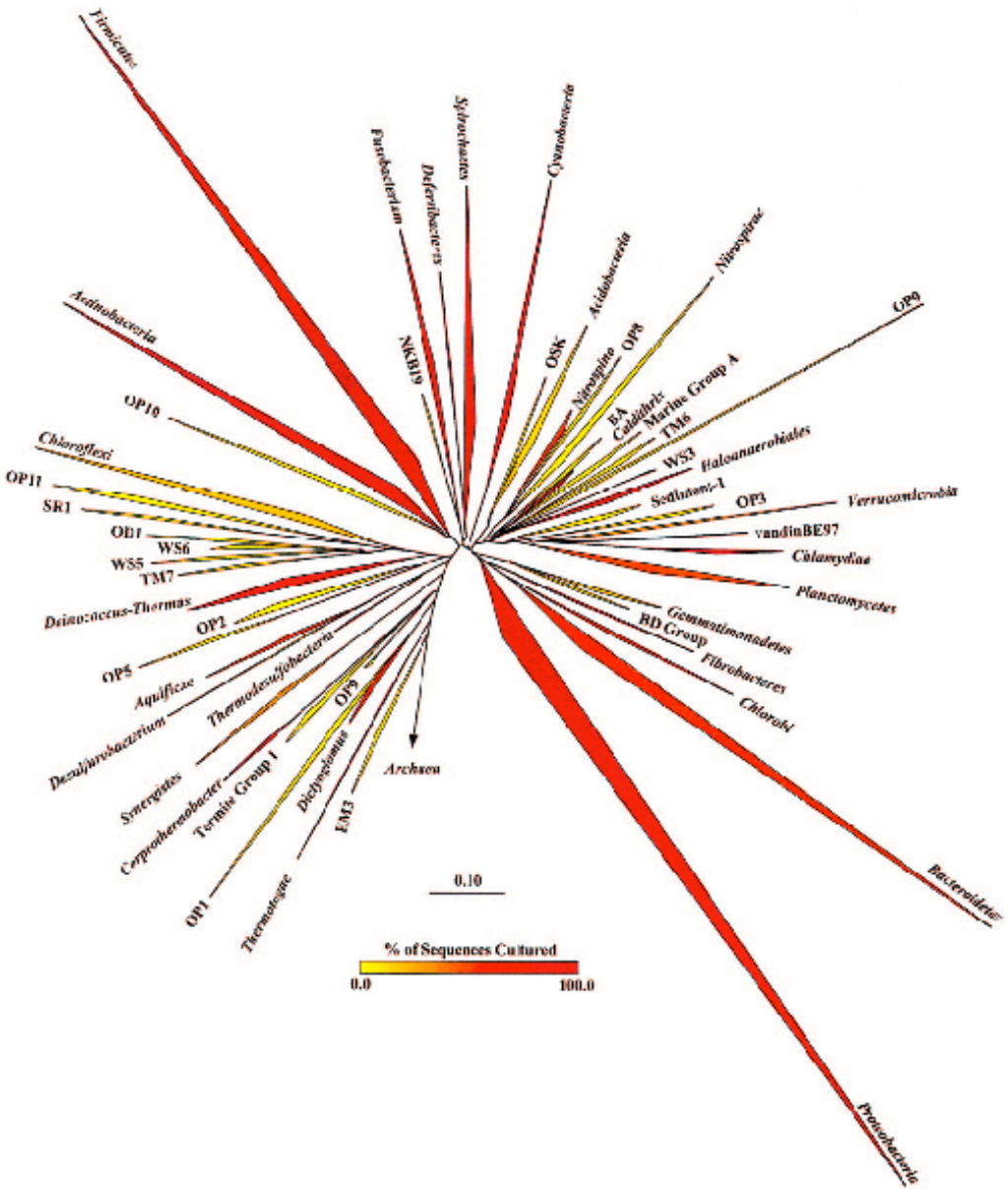

Figure 1. Phylogenetic tree of *Bacteria* (Handelsmann, 2004) showing established phyla (Latin names in italics) and candidate phyla. The vertex angle of each wedge indicates the relative abundance of sequences in each phylum; the length of each side of the wedge indicates the range of branching depth found in that phylum; the redness of each wedge corresponds to the proportion of sequences in that phylum obtained from cultured representatives. Candidate phyla do not contain any cultured members.

2.1.2 Quantifying biodiversity

In biodiversity research the species is the basic unit for its classification and quantification. However, quantification is hindered since it is difficult to define 'species' in a manner that applies to all naturally occurring organisms ('species problem'). Ernst Mayr (1996) developed the widely used biological species definition according to which 'species are groups of interbreeding natural populations that are reproductively isolated from other such groups.' Since it is based on sexual reproduction, this definition cannot be applied to organisms that reproduce asexually (for example some plants, the *Eubacteria* and *Archaea*). Therefore, an alternative species definition was established, which was initially arbitrary and artificial (Staley, 1997) but also pragmatic, operational and universally applicable: 'A species is a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions' (Rosselló-Mora and Amann, 2001). At present, molecular typing methods and whole genome sequences of different strains indicate that the species definition is not as artificial as one would have thought some years ago (Mougel *et al*., 2002). Accordingly, 1,257 bacterial genera are described to date, (Nomenclature up to date, Deutsche Sammlung von Mikroorganismen und Zellkulturen, http://www.dsmz.de/microorganisms/bacteria_catalogue.php, 21/11/2007), while current estimates amount to several millions (Torsvik *et al*., 2002), even over thousand millions of different bacterial species (Bach *et al*., 2002).

Presently, different 'concepts' (Istock *et al*., 1996) defining a prokaryotic species exist. Firstly, the polyphasic or phylophenetic approach incorporates phenotypic (for example, results of biochemical tests, fatty acid composition), genotypic and phylogenetic information (Stackebrandt *et al.*, 2002; Torsvik *et al.*, 2002; Gevers *et al*., 2005). Hereby, a prokaryotic species is considered as a group of strains (including the type strain) that are characterized by a certain degree of phenotypic consistency and showing 70% of DNA-DNA binding and ≥97% of 16S rRNA gene sequence identity. Yet, strains that have ≥97% identity might or might not meet the 70% DNA-DNA binding criterion (Fox *et al*., 1992; Stackebrandt and Goebel, 1994). Therefore, other methods have to be applied to determine whether isolates are sufficiently similar to be assigned to the same species.

The second step may represent the 'multilocus sequence analysis' (MLSA) (Maynard Smith *et al*., 1993; Spratt, 1999; Santos and Ochmann, 2004) that defines species by loci evolving more rapidly than rRNA genes. A cluster of strains would merit species status when they are subject to a higher frequency of lateral gene transfer among orthologous genes than with strains of

another cluster. Hereby, prokaryotic species are demarcated based on their inter- and intraspecific level, using allelic mismatches of a small number of housekeeping genes (usually 7) (Maiden *et al*., 1998). Accordingly, this universal set of genes allows the hierarchical classification of all prokaryotes (Santos and Ochmann, 2004).

In the former concepts, however, ecology is not yet incorporated into the species definition. This is especially important for those groups whose ecological distinctness namely diverse metabolic capabilities (Feldgarden *et al*., 2003) are obviously important. The 'ecotype-model', then, suggests a rational basis for demarcating such bacterial taxa (Cohan, 2001, 2002). Protein-coding genes are to be used as molecular markers for ecologically distinct populations (Palys *et al*., 2000). 'Ecological populations' that are genetically cohesive and ecologically distinct (Gevers *et al.*, 2005) would hence be induced by adaptive mutations. They drive to extinction from the same, but not from other ecotypes.

Finally, a combination of all approaches should be used to identify the demarcation of prokaryotic lineages. Thereupon, bacterial species can be demarcated in practice by the same sequence-cluster criteria used in eukaryotic systematics. But one has to consider that bacterial species are not necessarily natural entities, whatever method is applied (Gevers *et al.*, 2005).

2.2 THE SOIL ECOSYSTEM

2.2.1 Functions of soil

At present there is particular interest in the relation between microbial biodiversity and function in soil ecosystems since even 80 to 90% of the processes in soil are reactions mediated by microbes (Nannipieri and Badalucco, 2003). To date, many studies have been conducted in soils, lakes and oceans (Bruns *et al*., 2002, Connon and Giovannoni, 2002; Bruns *et al*., 2003a,b; Gich *et al*., 2005) and succeeded in recovering their biodiversity. The fact that prokaryote diversity is extraordinary in soil (Torsvik *et al*., 1990) and that the microorganisms which can be isolated and studied from soil represent only a small fraction of the microbial groups present *in situ* (Hugenholtz *et al*., 1998), has limited a comprehensive functional understanding of soil biodiversity. Yet, the assessment of the basic soil quality plays an important role at various levels of scale corresponding to the functions of soil, as proposed by Karlen *et al*. (1997):

1. sustaining biological activity, diversity, and productivity;
2. regulating and portioning water and solute flow;
3. filtering, buffering, degrading, immobilizing, and detoxifying organic and inorganic materials, including industrial and municipal by-products and atmospheric deposition;

4. storing and cycling nutrients and other elements within the earth's biosphere; and
5. providing support of socioeconomic structures and protection for archeological treasures associated with human habitation.

2.2.2 Composition and types of soil

Soil is defined as an unconsolidated mineral or organic substance on the earth's surface that primarily provides a natural medium for growth of land plants. Different soil types grouped by field and laboratory properties, among which soil texture, moisture, pH, temperature regimes and soil horizons reflect soil development (Tate, 2000). Most soils are mineral soils, which are primarily composed of mineral matter. They contain less than 1% to approximately 20% colloidal soil organic matter. Organic soils, however, generally contain more than 20% organic matter and are characterized by peats and mucks (Tate, 2000).

Currently, the U.S. soil classification system (Natural Resources Conservation Service (NRCS) of United States Department of Agriculture) divides world soils into 12 borders (Miller and Gardiner, 1998; Brady and Weil, 2002). Examples of soil orders are aridisols (dry soils), andisols (volcanic soils), gelisols (permafrost-impacted soils), histosols (organic soils), inceptisols (embryonic soils), mollisols (typical of grassland systems), and spodosols (typical of forest systems). At its lowest level of organization the U.S. system recognizes approximately 15,000 different soil series.

Soil horizons drive to disposition of the soil types. Typical soil horizons are O, A, and B horizons, which represent layers of soil parallel to the land surface and differing among one another physically, chemically and biologically, or in characteristics such as color, structure, texture, consistency, biotic populations, and pH. O horizons contain predominantly organic material, whereas A horizons represent mineral layers, formed on the soil surface, or below the O horizon. B horizons, however, are commonly referred to as the subsoil, which formed below an A, or O horizon. They represent a zone of accumulation where rainwater percolating through the soil has leached material from above (usually iron but also humus, clay, carbonates, etc.) which was deposited below (Tate, 2000). Microbial activity is generally highest in A, or O horizons (Tate, 2000).

2.2.3 Factors determining bacterial diversity in soil

The number of bacterial cells per gram of soil range from 10^8 in forest soil to 10^{10} in manure (Stackebrandt, 2003) while DNA reassociation experiments revealed between 4,000 to 10,000,000 genome equivalents per 10 g or 30 g of soil (Torsvik *et al*., 1990; Torsvik *et al*., 1996; Dykhuizen, 1998; Øvreås and Torsvik, 1998; Øvreås *et al*., 1998; Gans *et al*., 2005). Schloss and

Handelsman (2006) developed statistical models applied to molecular data, which predict that 0.5 g of soil samples from Alaska and Minnesota had a richness of 5,000 and 2,000 species, respectively, and approximately 20% of the bacteria were endemic to both soils. The diversity in soil is extraordinary enormous for the following distinctive properties, which refer to geographic, geologic, hydrologic, climatic, and anthropogenic factors, as well as vegetation and fauna (Liesack and Dunfield, 2002). Due to the fact that many factors are responsible for the composition of soil bacterial community, soil approaches always lead to various results. Minor differences in these factors may cause major differences in the distribution of their species.

Genetic factors: Large populations of organisms in soil microbial communities support its high genetic diversity. Accordingly, the capacity to accumulate large number of mutations is given. This is due to the fact that a prokaryotic population represents a mixture of genetically diverging clonal cell lines, which are exposed to natural selection. Furthermore, in high-density populations molecular mechanisms like the horizontal gene transfer are facilitated (Torsvik *et al*., 1996; Torsvik *et al*., 2002).

Spatial factors: Another reason for the enormous diversity is the spatial isolation of microbial populations in soil caused by its structural complexity. Hence, ecological niches are created in which the heterogeneity of carbon resources is very high. Thereby, prokaryotes specialize and divide into distinct ecological species. The structural complexity can be alleviated by the addition of water, e.g. rainfall. The rapid drainage of surface soils determines the major difference to subsurface soils. Saturation of soil with water in the subsurface result in a predominance of one or a few species whereas the species diversity and evenness would remain high in the dryer conditions in the surface (Torsvik *et al*., 2002; Nannipieri *et al*., 2003; Treves *et al*., 2003).

Temporal factors: Soil is also exposed to a strong temporal heterogeneity; this means disturbances such as starvation, desiccation, freezing/thawing, or human activity, which cause the desintegration of the microhabitats and disruption of the boundaries between populations (Torsvik *et al*., 1996; Torsvik *et al*., 2002). Therefore, the microbial diversity is affected by the changing availability of limiting resources and the ability of populations to utilize these resources.

Biological factors: The bacterial diversity is also controlled by biological factors such as plants (Torsvik *et al*., 1996), e.g. released root exudates of plants can stimulate, or inhibit the growth of soil organisms (Watt *et al*., 2006). Furthermore, mucilage and sloughed-off root cells provide an additional nutritional source for microbial cell multiplications (Chow *et al*., 2002). It was observed that even specific bacterial divisions, e.g. *Acidobacteria*, were affected by the plant

rhizosphere because the plant roots of *Trifolium repens* and *Lolium perenne* had a selective effect towards *Gammaproteobacteria*, *Actinobacteria* and *Acidobacteria* (Marilley and Aragno, 1999).

2.3 COMPOSITION OF SOIL BACTERIAL COMMUNITIES

2.3.1 Diversity estimates based on clone libraries and culture collections

Clone libraries often yield only incomplete pictures of natural bacterial communities. Different factors lead to biases in the clone library composition, especially in soil. For example, small libraries such as those with less than 400 clones do not present the full genetic diversity in soil (Janssen, 2006). Furthermore, different efficiencies in the DNA extraction corresponding to specific phyla may cause shifts in the bacterial contribution (Amann *et al*., 1995; Dunbar *et al*., 1999; Martin-Laurent *et al*., 2001; Janssen, 2006;). Thicker cell walls or formation of spores can limit the efficiency of DNA-extraction, leading to under-representation of some phyla such as Gram-positive bacteria. Moreover, chimeric sequences may be formed, which lead to false diversity estimates because microbial species are discovered that do not really exist (Gonzalez *et al*., 2005). Additionally, the number of 16S rRNA operons per bacterial genome varies from one to as many as 15 (Rainey *et al*., 1996) as an adaptation to fluctuating growth conditions (Klappenbach *et al*., 2000; Sharma *et al*., 2007). Finally, PCR approaches do not necessarily amplify rRNA genes belonging to all members of each targeted group (Fierer et al., 2005) and 16S rRNA genes of specific phyla are preferentially amplified.

By all means, clone libraries still describe bacterial community compositions of soil more precisely than culture collections, since less than 0.5% of the cells in soil are able to multiply in laboratory media (Torsvik *et al*., 1990) whereby soil bacteria account only 2.7 to 3.7% in culture collections (Janssen, 2006). As opposed to culture collections, however, clone libraries do not provide information on physiological and ecological function of soil bacteria (Felske *et al*., 2000). In order to analyze a large number of isolates generated in culture collections, efficient, rapid and sensitive screening techniques are required. Fingerprinting of phylogenetic group-specific 16S rRNA gene fragments by denaturing gradient gel electrophoresis (DGGE) has been proven to be an efficient approach for the rapid comparison of environmental isolates (Bruns *et al*., 2002, 2003a,b; Jaspers and Overmann, 2004; Gich *et al*., 2005). Most species of environmental samples occur at a very low abundance and hence would escape detection by conventional eubacterial 16S rRNA gene fingerprinting.

2.3.2 Abundant bacterial phyla in soil

To gain an understanding of the general composition of soil bacterial communities, Janssen (2006), compared 21 clone libraries derived from soil with a total of 2,920 clones, which he treated as one global set. He reviewed and subsumed the abundance of the different phyla in soil and the corresponding phylum level diversity (Fig. 2).

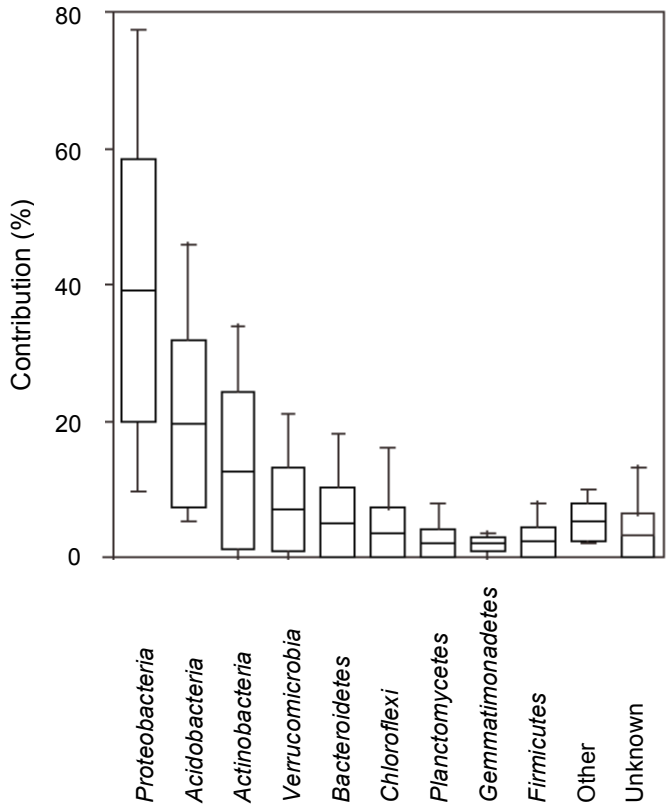

Figure 2. Contributions of 16S rRNA and 16S rRNA genes from members of different phyla in libraries prepared in soil bacterial communities (2,920 clones 21 libraries). The horizontal line in the middle of each block indicates the mean, the block represents 1 standard deviation on either side of the mean, and the vertical lines extending above and below indicate the minimum and maximum distribution of each phylum (Janssen, 2006).

Nine different bacterial phyla dominate in soil (Dunbar *et al*., 1999; Chow *et al*., 2002; Zhou *et al*., 2003; Lipson and Schmidt, 2004; Janssen, 2006) and members of the phylum *Proteobacteria* and *Acidobacteria* represent the most dominant groups with 39% (10 to 77%) and 20% (5 to 46%), respectively (Janssen, 2006). The third most abundant group is the phylum *Actinobacteria*. Although this phylum is the most commonly related one to soil, its relative frequency is only 13% (0 to 34%). In addition, 7% (0 to 21%) of soil bacterial communities are members of the phylum *Verrucomicrobia*, whereas the phyla *Bacteroidetes* and *Chloroflexi* receive 5% (0 to 18%) and 3% (0 to 16%) on average, respectively. *Planctomycetes*, *Gemmatimonadetes* and *Firmicutes* contribute up to 2% while a low percentage is particularly

remarkable for the phylum *Firmicutes*. The Gram-positive genera *Clostridium* and *Bacillus* have been considered to be frequent in soils. Possibly lower efficiencies of DNA extraction may lead to an under-representation of the corresponding 16S rRNA gene sequences in clone libraries.

Some phyla such as *Acidobacteria* are of particular interest. They are often very abundant in soil (Dunbar *et al*., 1999; Chan *et al*., 2006), yet the overlap between culture-collections and 16S rRNA gene clone libraries is particularly little. Consequently, research has recently focused on the cultivation of representatives of the predominantly not-yet-cultured groups, such as *Acidobacteria*.

2.4 THE TAXON *ACIDOBACTERIA*

Based on analyses of 16S rRNA gene clone libraries, members of the phylum *Acidobacteria* represent a fraction of about 20% of typical soil bacterial communities (Janssen, 2006) but in some cases can even contribute up to 51% (Dunbar *et al*., 1999), or even 80% (Chan *et al.,* 2006). *Acidobacteria* have been detected in almost every clone library of soil but also in many other habitats including a peat bog, acid mine drainage, a contaminated aquifer, a hot spring, a freshwater lake, and a sample of the Atlantic ocean from a depth of 1,000 m (Hugenholtz *et al*., 1998). Since they occur in many different environmental conditions, a high physiological diversity is predicted (Hugenholtz *et al*., 1998; Barns *et al*., 1999). It is assumed that their phylogenetic diversity is nearly as great as in the phylum *Proteobacteria* (Ludwig *et al*., 1997; Hugenholtz *et al*., 1998). Recently, the number of subdivisions was extended to 26 (Barns *et al*., 2007).

In pronounced contrast to the high overall ecological and phylogenetic diversity, the cultivation success of *Acidobacteria* is usually low. Only four species have been validly described to date: *Terriglobus roseus* DSM 18391^T, *Acidobacterium capsulatum* DSM 11244^T (strain 161^T) (Kishimoto *et al*., 1991), *Holophaga foetida* DSM 6591^T (Liesack *et al*., 1994), and *Geothrix fermentans* DSM 14018^T (Coates *et al*., 1999). But even these four isolates indicate a high diversity within this phylum. *Geothrix fermentans* and *Holophaga foetida* are representatives of subdivision 8. *G. fermentans* is a strictly anaerobic bacterium that oxidizes acetate and other simple organic acids with Fe(III) as sole electron acceptor (Coates *et al*., 1999). On the contrary, *H. foetida* is a strictly anaerobic demethylating homoacetogen that degrades aromatic compounds to acetate and is capable of transferring methyl groups from phenylmethylesters to sulfide, thus forming methanthiol and dimethyl sulfide (Bak *et al*., 1992). Although additional aerobic chemoorganotrophic strains have been isolated, *Acidobacterium capsulatum* and *Terriglobus roseus* are the sole validly described representatives of subdivision

1 (Sait *et al*. 2002; Joseph *et al*., 2003; Stevenson *et al*., 2004; Eichhorst *et al.*, 2007). Since *A. capsulatum* is an acidophile (pH 3.0 to 6.0), its physiology is not representative of many other *Acidobacteria*. Yet, *Acidobacteria* of subdivision 1 are still more abundant in clone libraries from acidic soils. Recently, it has been demonstrated that members of this subdivision can be cultivated selectively on solid laboratory media at a low pH (Sait *et al*., 2006).

In general, in soils subdivisions 1, 4 and 6 are dominant but to date no isolate was found in the subdivisions 6 and 7 so far (Janssen, 2006). Based on the high diversity of soil bacterial communities, one has to conclude that an enormous hidden number of novel species exist for the phylum *Acidobacteria*.

2.5 LOW CULTIVATION SUCCESS

To increase the culturability in the laboratory, the causes for the low cultivation success have to be determined. Generally, it is presumed that only those bacterial cells have low cultivation successes, which fail to adapt themselves to shifts in environmental parameters (McDougald *et al*., 1998; Roszak and Colwell, 1987). These adaptive capabilities may be even used to respond to culture conditions in the laboratory, which usually radically differ from their natural environment. Examples for adaptive responses are: appropriate modification of enzyme synthesis to take up growth-limiting nutrient, modulation of uptake rates for nutrients available in excess, rerouting of metabolic pathways to avoid possible blockages due to specific nutrient limitation, and coordination of synthetic rates to maintain balanced growth (Roszak and Colwell, 1987).

2.5.1 Hypotheses for low cultivation success

There are several reasons for the failure of the adaptive capabilities, which have to be addressed during cultivation approaches of soil bacteria. The most prominent but most discussed hypothesis reveals that altered environmental conditions may mediate the formation of inactive cells such as dormant, or ‘viable but non culturable cells’ (VBNC) in non-sporeforming cells. These cells were shown to be analog to spores, the stress response of differentiating bacteria (Xu *et al*., 1982; Roszak and Colwell, 1987; Kell *et al*., 1998; McDougald *et al*., 1998; Oliver, 2005). But it is still controversially discussed whether the VBNC-state is a survival strategy such as spores, or a moribund condition where the cells become debilitated until cell death occurs (Kell *et al*., 1998; Barer and Harwood, 1999; Bogosian and Bourneuf, 2001; Oliver, 2005). One theory of the survival strategy was illustrated as an active process including the ability to reverse and resuscitate to become viable. Triggers for the resuscitation are shifts of temperature (*Vibrio vulnificus*) (Oliver, 2000), addition of nutrients (Kell *et al*., 1998; Barer and Harwood, 1999), or

addition of culturable cells (*Micrococcus luteus*) (Votyakova *et al*., 1994; Mukamolova *et al*., 1998, 2006). Yet, the remaining question is whether the re-growing cells are a true result of resuscitation, or growth of a few culturable cells, which were not detected in the otherwise totally VBNC population (Oliver, 2005).

Furthermore, adaptation to shifts in the environment may be impeded due to 'substrate-accelerated death'. Under these conditions, growth under high concentrations of a substrate is inhibited, which was the limiting substrate in a previous growth phase (Calcott and Postgate, 1972; Postgate and Hunter, 1963), such as under natural conditions. Consequently, one of the widely method used to increase the cultivation success is the reduction of organic carbon concentration in the media for cultivation, simulating the oligotrophy typical of natural environments (Chin *et al*., 1999; Aagot *et al*., 2001; Breznak, 2002; Bruns *et al*., 2003a; Schoenborn *et al*., 2004; Stevenson *et al*., 2004; Davis *et al*., 2005; Fierer *et al*., 2007). Thereby, starved cells are protected from the 'substrate accelerated death' (Postgate and Hunter, 1963; Calcott and Postgate, 1972).

An additional possible reason for the low cultivation success of environmental bacteria represents the lack of cell-to-cell communication in laboratory media due to the absence of signal molecules (Barer and Harwood, 1999; Bruns *et al*., 2002, 2003a). They have been shown to be involved in the gene expression under starvation and resuscitation and the addition to cultivation media already increased the cultivation efficiency of planktonic bacteria (Bruns *et al*., 2002, 2003a). For instance, cyclic AMP (cAMP) prevents 'substrate-accelerated death' in starved laboratory cultures (Schultz *et al*., 1988), or *N*-acyl homoserine lactones trigger resuscitation (Batchelor *et al*., 1997). However, interaction between natural bacteria can also be stimulated in diffusion chambers, which already allowed the cultivation of not-yet-cultured bacteria in a previous study (Kaeberlein *et al*., 2005).

2.5.2 Further methods to increase the cultivation success

Extending the incubation period of the cultures in the laboratory was shown to be another possibility to get more not-yet-cultured bacteria growing (Janssen *et al*., 2002; Stevenson *et al*., 2004; Davis *et al*., 2005). Especially the growth of oligotrophic bacteria is induced (Whang and Hattori, 1988).

Furthermore, liquid serial dilutions such as the 'most probable number' (MPN) technique is an appropriate method to isolate bacterial strains, which belong to abundant 16S rRNA gene sequences. Most rapidly growing bacteria are eliminated by the dilution (Eilers *et al*., 2000; Bruns *et al*., 2002; Schoenborn *et al*., 2004). Automated methods (e.g., MicroDrop technique) can significantly accelerate and standardize this so-called 'extinction culturing' (Bruns *et al*.,

2003b); wells of microtiter plates (96 wells) are inoculated within less than 1 min with a constant number of bacteria. Due to the high number of replicates at a high dilution, highly enriched or even pure cultures are obtained at a much higher frequency than in conventional MPN dilutions.

Additionally, cultivation of soil bacteria may be increased due to dispersion of soil samples because 80 to 90% of the microorganisms inhabiting soil occur on solid surfaces (Nannipieri *et al*., 2003). Soil bacteria produce extracellular polymeric substances (EPS), which promote the adhesion. EPS may be dissolved with chemical substances such as pyrophosphate (Böckelmann *et al*., 2003) and Tween 80 (Kuczynska and Shelton, 1999). Moreover, the chemical treatment can be displaced with a physical one such as sonication, which increased the number of viable bacteria in a previous study (Janssen *et al*., 2002).

Another method to increase the cultivation efficiency especially of soil bacteria is to add more recalcitrant substances (Chin *et al*., 1999) like polymeric substances in the cultivation media as organic carbon source. Furthermore, roots are the largest fraction of the biological material in most arable soils and the excreted root exudates feed the organisms in the rhizosphere (Watt *et al*., 2006). Therefore, artificial root exudates can also be used as carbon source under laboratory conditions.

2.6 SCOPE OF THE PRESENT THESIS

The scope of the present thesis was the coverage and analysis of the natural bacterial community of an alpine soil with a combination of modern culture-dependent and 16S rRNA based methods. Since systematic tests of the effect of media composition on the cultivation success have been conducted only for a very limited range of lowland soils so far (Davis *et al*., 2005), the microbial diversity of alpine soils was understudied at the beginning of this thesis. Hence, the seasonal cultivation success of alpine soil bacteria (February, April, and August Jochberg soil sample) was assessed in different laboratory media with humic acid analogs (HA), a mixture of polymers (POL), artificial root exudates (RO), nutrient broth, or soil extract as carbon and energy sources employing the high-throughput MicroDrop approach (Bruns *et al*., 2003b). In order to identify and characterize novel, not-yet-cultured typical representatives of an extreme soil bacterial community (i.e., February soil sample), the analysis of the cultured fraction focused on six (sub)phyla *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Acidobacteria* (Chapter 3), which were known to be abundant in 16S rRNA gene clone libraries of soil samples (Janssen, 2006). For subsequent analysis of the coverage of the present cultivation approach, the abundance of these target groups in the cultured fraction was compared with their abundance in the natural soil community.

The assessment of the alpine soil bacterial community from February was emphasized on the phylum *Acidobacteria*, which mostly consists of environmental 16S rRNA gene sequences and so far comprises only four valid described strains. Therefore, the following section of this thesis (Chapter 4) is dedicated to the description of the novel, previously unknown acidobacterium strain Jbg-1, which was isolated in the present cultivation approach. Chapter 4 also revealed the description of a second novel acidobacterium strain Wbg-1, recovered from another soil sample by another working group.

The third section of this thesis represents the analysis of the low total cultivation success of the alpine soil bacteria from the February Jochberg soil sample (Chapter 5). Since the first section already proposed the unexpected high percentage of interspecific interactions (i.e., co-cultures) as the major reason, the present chapter elucidates the significance of these interactions for the cultivation success. For subsequent analysis, chapter 5 also identified the composition of these co-cultures and the effect of different incubation conditions (i.e., free-living versus attached bacteria, addition of inducer molecules) on their development.

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Chapter 3

Inverse correlation of bacterial culturability and abundance in an alpine soil

3 INVERSE CORRELATION OF BACTERIAL CULTURABILITY AND ABUNDANCE IN AN ALPINE SOIL

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3.1 SUMMARY

The seasonal culturability of bacterial cells from a microbial community of an alpine soil was assessed in different laboratory media containing humic acid analogs (HA), a mixture of polymers (POL), artificial root exudates (RO), nutrient broth, or soil extract as carbon and energy sources. Thereby, the summer sample showed the highest culturability value in media supplemented with soil extract. The analysis of correlation of bacterial culturability and phylogenetic group-specific abundance, however, was focused on cultures obtained from the extreme winter environment in defined media containing RO and POL whereas the monomeric organic carbon of RO proved to be superior to POL for optimization of the cultivation success. The real time (RT)-PCR approach confirmed the high coverage of our analysis since our target groups (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*) constituted 73.6% of all eubacteria in the sample. A total of 251 bacteria were analyzed which represented 53 distinct, containing 73% previously unknown phylotypes. The majority was closely related to the *Alphaproteobacteria* with the largest number of different phylotypes and the highest evenness value. Also the *Bacteroidetes* were most frequently cultured but were dominated by one phylotype. The lowest culturability was assessed for the *Acidobacteria* with only one single cultivated phylotype (*Edaphobacter modestus* strain Jbg-1), which was well adapted to long-term survival and to higher carbon concentrations after subcultivation. Unexpectedly, a high percentage of interspecific interaction was obtained (i.e., 75% co-cultures), which represented the major reason for the low cultivation success of the winter sample.

3.2 INTRODUCTION

Based on culture-independent analysis of the 16S rRNA gene sequence inventory, the majority of bacteria thriving in the soil environment represent not-yet-cultured *Bacteria* and *Archaea* with unknown genetic and physiological properties. Using culture-independent metagenomic approaches and functional gene analyses, particular functions of certain not-yet-cultured soil bacteria have been detected (Quaiser *et al.*, 2003; Leininger *et al.*, 2006). Given the large complexity of soil bacterial communities, assembling entire genomes of not-yet-cultured soil bacteria at present appears impractical (Tringe *et al.*, 2005). Hence, the study of bacterial genetics and physiology using laboratory cultures often remains a prerequisite for the functional understanding of such bacteria in the soil environment. Due to the large diversity of soil bacteria, such culture attempts need to be focused on dominant phylotypes or phylotypes, which are known to participate in key transformations.

Where tested, collections of cultured phylotypes and 16S rRNA gene sequence clone libraries were not congruent (Felske *et al.*, 1999; Hengstmann *et al.*, 1999; McCaig *et al.*, 2001; Sait *et al.*, 2002; Lipson and Schmidt, 2004) and environmental phylotypes have only rarely been recovered by cultivation from the same sample (Zul *et al.*, 2007). From the very early studies of soil microbial communities on, the maximum values for the cultivation success of soil bacteria has been in the range of 4-19 % (Conn, 1918; Janssen *et al.*, 2002; Sait *et al.*, 2002; Stevenson *et al.*, 2004). Only occasionally, higher values were reported (Shrestha *et al.*, 2007). However, growth of bacteria is widespread among bacterial genera and not confined to a few active species (Harris and Paul, 1994) and activity staining revealed that over 90% of the cells are potentially viable (Janssen *et al.*, 2002). These observations support the view that a large fraction of bacteria should be capable of growing if their requirements are met in laboratory media.

Whereas a plethora of different media formulations has been used in these recent attempts to isolate previously uncultured types of soil bacteria (e.g., Balestra and Misaghi, 1997; Lipson and Schmidt, 2004), representatives of dominant soil bacterial groups such as the *Acidobacteria* were often missed in the cited studies. Systematic tests of the effect of media composition on the cultivation success are still rare (Davis *et al.*, 2005) and so far have been conducted for a very limited range of lowland soils. The microbial diversity of alpine soils is understudied and previous work has been largely limited to a high alpine soil of a dry meadow site (Lipson and Schmidt, 2004). This analysis of bacterial clone libraries indicated that *Acidobacteria* are particularly prevalent and constitutes up to 43% of all sequences (Lipson and Schmidt 2004).

Nevertheless, it is unknown whether these *Acidobacteria* resemble the known isolates from lowland soils.

The aim of the present study was to assess the cultivation success in different defined laboratory media for cells from a winter microbial community of an alpine soil, employing the high throughput microdrop approach (Bruns *et al.*, 2003). The six (sub)phyla *Acidobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* together account for an average of 68% in 16S rRNA gene clone libraries (Janssen, 2006) and specific PCR-techniques are available for these groups which are suitable for a rapid and high throughput phylogenetic fingerprinting of natural communities, cultivated members and quantification of natural communities by real time-PCR (Fierer *et al.*, 2005; Gich *et al.*, 2005; Zul *et al.*, 2007). Therefore these six groups were chosen as target groups for analysis of the cultured fraction of soil bacteria and comparison with their abundance in the natural soil community, in order to identify and characterize novel, not-yet-cultured typical representatives of soil bacterial communities.

3.3 MATERIAL AND METHODS

3.3.1 Sampling site and soil characteristics

The sampling site was an alpine rendzina (mollisols: rendolls) located on Jochberg (close to Kochel in southern Germany) on a north-facing cliff at an altitude of 1,400 m. Soil cores were collected on February 8, April 4 and August 26 in the year 2002. On February 8, the sampling site was covered by 20 cm of snow. During each sampling, three independent soil samples were aseptically recovered at a distance of 1 m employing autoclaved stainless steel cores (length 20 cm, width 3 cm). The upper 3 to 5 cm of each core consisted mainly of grass and litter and hence was discarded. Afterwards, soil cores were pooled, roots and pebbles were removed with sterile forceps and the soil homogenized with a motar and pestle. For dry weight determination, 2 g aliquots of soil homogenate were dried at 110°C for 72 hours and weighed. The pH was determined in double-distilled water and in 0.1 M KCl using a pH meter 763 (Knick, Berlin, Germany). For determination of total bacterial numbers, subsamples were suspended in sterile filtered tap water (0.1 μm-pore-size polycarbonate filters; Millipore, Eschborn, Germany) and fixed in 2% (vol/vol) glutaraldehyde. After fixation, total cell numbers were determined by epifluorescence microscopy after staining with SYBR-Green-II as described previously (Zul *et al.*, 2007).

3.3.2 ATP measurements

15 g of soil were suspended in 60 ml of soil solution equivalent (SSE; Angle *et al.*, 1991) buffered with 10 mM HEPES (pH 6.7). 5 ml-aliquots of the slurries were distributed in 50 ml Erlenmeyer flasks containing two glass beads each in order to maintain the soil particles in suspension. For stimulation experiments, slurries were amended with two types of substrate mixtures, a combination of polymers and artificial root exudates (see next paragraph). Slurries without substrates served as controls. The samples were incubated on a rotary shaker at 15°C in the dark (the incubation temperature of the cultivation experiments) and 200 µl-subsamples were withdrawn after 0, 6, 24, 70 and 240 h using autoclaved, disposable pipet tips with their ends cut open. ATP was extracted employing the phosphoric acid method (Ciardi and Nannipieri, 1990) by adding 1.8 ml of extraction solution [0.67 mM H_3PO_4, 2 M urea, 20% (v/v) DMSO, 0.02% (w/v) adenosine] to each aliquot, followed by ultrasonic treatment (1 min, Branson Sonifier Cell disruptor B15). After centrifugation, the supernatants were pipetted into 5 ml of measuring buffer (40 mM Tris/HCl, pH 8.2, 1 $mg \cdot ml^{-1}$ BSA, 10 mM Mg-acetate, 2 µM Na-pyrophosphate, 0.1 mM dithiothreitol) and ATP concentrations were determined after addition of 80 µg luciferin and 10 µg luciferase. In order to attain higher sensitivity, a Tricarb 2199 TR (Canberra Packard, Dreieich, Germany) scintillation counter was used for these measurements (Rieger, 1997). A calibration curve was obtained for amounts of 5.5 pg to 550 µg using a freshly prepared ATP stock solution. In addition, each sample was internally calibrated by addition of 0.55 ng of ATP.

3.3.3 Cultivation of soil bacteria

Cultivation experiments were conducted using samples collected on the three dates in 2002. SSE was used as the general base for the liquid cultivation media and buffered with 10 mM HEPES at a pH value of 6.7 according to pH values determined in soil samples (compare Table 1). The following supplements were added as carbon and energy sources of bacterial growth: (i) a mixture of humic acid analogs (HA) consisting of abietin, 9,10-anthraquinone-2,6-disulfonic acid, benzoic acid, coumestrol, 3,5-dimethoxy-4-hydroxycinnamic acid, quercetin, sodium salicilate and solanidine (500 µM each) (Coates *et al.*, 2002), (ii) a mixture of the polymers containing cellulose, chitin, curdlan, pectin, soluble starch, xylan (0.1% [wt/vol] each) (Chin *et al.*, 1999), (iii) artificial root exudates comprising 21 different sugars, alcohols, organic acids and amino acids at different concentrations (Kozdrój and van Elsas, 2000), (iv) nutrient broth (5 $g \cdot l^{-1}$ peptone, 3 $g \cdot l^{-1}$ meat extract), and (v) soil extract. Soil extract was prepared by autoclaving 40 g of air-dried soil with 100 ml of distilled water for 1 hour, filtering the supernatant through filter

paper and autoclaving the extract again. Nutrient broth and soil extract were only tested with samples obtained in April and August 2002.

Basal medium was supplemented with the respective carbon substrates and 200 µl-aliquots were distributed into sterile 96-well polysterene microtiter plates (Greiner, Frickenhausen, Germany). High throughput cultivation was performed employing the MicroDrop technique for inoculation of the microtiter wells (Bruns *et al.*, 2003; Gich *et al.*, 2005). In order to prevent clogging of the microdispenser pipette, soil suspensions (see above) were prefiltered through 12 µm-pore-size nitrocellulose filters (Sartorius, Göttingen, Germany) prior to inoculation. The total cell number in the filtrate was determined by epifluorescence counting and each well was inoculated with 50 bacterial cells using the MicroDrop AutoDrop microdispenser system version 5.50 (Microdrop GmbH, Norderstedt, Germany). On each microtiter plate, 12 wells were left without inoculation and served as controls for contamination. A total of 20 microtiter plates were inoculated for samples obtained in April and August, representing 1680 individual growth tests and a total of 12 microtiter plates for the sample obtained in February, representing 1008 individual growth tests. After 6 weeks of incubation at 15°C in the dark, growth was monitored by visual inspection of turbidity.

Culturability was calculated based on the fraction of positive wells p (wells positive for growth /total inoculated wells). The number of culturable cells per well x and the corresponding 95% confidence interval $CI_{95\%}$ were calculated from p and the total number of inoculated wells n based on a binomial distribution (Button *et al.*, 1993)

$$x = - \ln(1 - p)$$

$$CI_{95\%} = \pm 1.96 \cdot \sqrt{\frac{p}{n(1-p)}}$$

For growth experiments of the isolated acidobacterium strain Jbg-1, HD medium containing 0.5% (w/v) casein peptone, 0.1% glucose, 0.25% yeast extract was employed. The medium was buffered at a pH of 5.5 using 2-(N-morpholino)ethanesulfonic acid (MES; 0.2% w/v). Previous to inoculation, cells from exponentially growing cultures were washed twice with MES-buffered (0.2% w/v, pH 5.5) TS salt medium (Kishimoto *et al.*, 1991), which does not contain any organic carbon source. Cells were resuspended in the original volume of TS medium. Growth experiments were inoculated with 5.0% of the washed cell suspensions and growth was followed by measurement of the optical density at 580 nm. Different dilutions of HD medium were tested. In parallel, growth experiments were conducted in SSE medium (pH 6.3) supplemented with 0.0025% yeast-extract, 0.1% glucose and trace element solution SL10 ($1 ml \cdot l^{-1}$; Widdel *et al.*, 1983).

3.3.4 High-resolution phylogenetic fingerprinting, sequencing and phylogenetic analyses

Cells in microtiter plate wells exhibiting turbidity were harvested by centrifugation at 15,000 × g for 20 min at a temperature of 4°C. Cell pellets were lysed by 6 freeze-thaw cycles (each cycle consisting of 3 min at 100°C and 3 min at −20°C). Aliquots of 0.2 µl of the cell extracts were directly used for PCR amplifications of 16S rRNA gene fragments. Chromosomal DNA of the soil samples was extracted using the UltraClean™ Mega Prep Soil DNA Kit (MoBio Laboratories, Solana Beach, CA). DNA was recovered by additional standard ethanol precipitation, then resuspended in 500 µl of 10 mM Tris-HCl, pH 8.0 and purified using the Wizard DNA-Clean-up kit (Promega, Madison, WI). The DNA extract was transferred to a Centricon dialysis filtration unit (Millipore) and washed 2 times with Tris-HCl (2 mM, pH 8.0). The extracted DNA was concentrated to a volume of 50 µl and the DNA quantified by fluorescent dye binding (PicoGreen; Molecular Probes, Eugene, OR).

For initial identification, MicroDrop cultures were screened by PCR using group-specific primer sets targeting 16S rRNA genes of six taxonomic groups of prokaryotes including Gram-positive bacteria with low G+C content (*Firmicutes*), Gram-positive bacteria with high G+C content (*Actinobacteria*), *Bacteroidetes*, *Alpha-* and *Betaproteobacteria*, and *Acidobacteria* and employing the PCR conditions reported previously (Zul *et al.*, 2007). Subsequently, the PCR products were separated with denaturing gradient gel electrophoresis (DGGE). Denaturing gradient gel electrophoresis was carried out in an Ingeny phorU system (Ingeny International BV, Goes, The Netherlands) employing 6% (w/v) polyacrylamide gels in 1xTAE (pH 8.0). Denaturing gradients ranged from 30 to 70% where 100% denaturant is defined as 7 M urea and 40% (v/v) formamide (Muyzer *et al.*, 1995). Gels were stained for 45 min with SYBRGold (1:10,000 dilution; MoBiTec, Göttingen, Germany), visualized on a UV transilluminator (LTF Labortechnik, Wasserburg) and photographed (Visitron Systems GmBH, Puchheim).

For sequencing, DNA bands were excised with a sterile scalpel, transferred to a 1.5 ml eppendorf tube containing 25 µl of 10 mM Tris-HCl buffer (pH 8.0) and the DNA was eluted for 2 hours at 65°C. Depending on the band intensity on the DGGE gel, between 1 and 3 µl of the eluate was reamplified using the corresponding primers without a GC clamp. 16S rRNA gene fragments of *Firmicutes* and *Bacteroidetes* were reamplified with a nested amplification method employing primers 517f (Lane, 1991) and 907r (Muyzer *et al.*, 1995) in a step down PCR protocol (10 cycles annealing at 61°C for 0.75 min, 20 cycles at 56°C for 0.75 min). For reamplification of *Betaproteobacteria* sequences primers Beta680f and 907r were combined (10 cycles annealing at 55°C for 1 min, 25 cycles at 50°C for 1 min). PCR products were separated

from free PCR primers with a QIAquick Spin Kit (Qiagen) and sequenced as described earlier (Zul *et al.*, 2007). Some DGGE bands contained multiple 16S rRNA gene sequences, which were first separated by cloning using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Recombinants were plated on selective LB agar plates, picked randomly and the plasmids were extracted from an overnight culture in liquid LB media with a QIAprep Spin Miniprep kit (Qiagen). After differentiation by enzymatic digestion with Eco R1 (Fermentas GmbH, ST. Leon-Rot, Germany) clones with different inserts were sequenced. All 16S rRNA gene sequences were checked for the presence of chimeras by use of the CHIMERA-CHECK online analysis program of the RDP-II database (Maidak *et al.*, 2001).

For the construction of phylogenetic trees, the closest relatives of all 16S rRNA gene sequences were retrieved from the GenBank database employing BLAST 2.0.4 (Altschul *et al.*, 1997) and the sequences were added to the program package ARB (Ludwig *et al.*, 2004). Phylogenetic trees were constructed from sequences longer than 1,300 bp, employing the neighbor joining and maximum likelihood algorithms as described previously (Gich *et al.*, 2005). Tree topologies were evaluated by 100 bootstrap resamplings. Shorter sequences obtained from DGGE bands were then added without changing the overall tree topology using the parsimony tool and the *Escherichia coli* filter.

3.3.5 Diversity analyses of the cultivated fraction

Based on the results of the phylogenetic analysis of the cultivated fraction obtained from Jochberg soil, phylotype richness was calculated for each (sub)phylum separately, employing the EstimateS software package (Version8.0.0) (Colwell, 2005). The nonparametric Chao1 estimator was chosen for quantification (Chao, 1984) a bias correction was applied. In addition, the evenness of each (sub)phylum was determined. The relative abundance p_i of each individual cultivated phylotype i (number of isolates n_i per total number of isolates N within the group) was used to calculate the Shannon-Weaver index of diversity H' according to

$$H' = -\Sigma p_i \ln p_i$$

(Shannon and Weaver, 1963). The evenness index was then calculated from H′ and the total number of different phylotypes S for the respective group:

$$E = \frac{H'}{\ln S} \quad .$$

3.3.6 Quantitative PCR

The abundance of *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria, Betaproteobacteria*, *Bacteroidetes, Firmicutes*, and *Verrucomicrobia* was quantified by quantitative PCR using the same group-specific primer sets as in conventional PCR. In addition, the total fraction of eubacterial DNA was quantified with a combination of primers EUB338-I/EUB338-II/EUB338-II and 907r (Daims *et al.*, 1999). The real time (RT)-PCR reactions were performed in an iCyclerQTM Multi-Color Real Time Detection System (Bio-Rad, München, Germany) using the iQ SYBR Green Supermix (Bio-Rad). 11 ng of extracted soil DNA were used in a reaction volume of 25 µl reaction and each determination was run in triplicate.

For calibration of the RT-PCR measurement, genomic DNA of the following representatives of the six phylogenetic target groups served as positive controls: *Acidobacterium capsulatum* DSM 11244^T (*Acidobacteria*), *Mycobacterium phlei* DSM 43239^T (*Actinobacteria*), *Rhodospirillum rubrum* DSM 467^T (*Alphaproteobacteria*), *Flavobacterium aquatile* DSM 1132 (*Bacteroidetes*), *Rhodocyclus tenuis* DSM 109^T (*Betaproteobacteria*), *Bacillus subtilis* DSM 10 (*Firmicutes*), and *Verrucomicrobium spinosum* DSM 4136 (*Verrucomicrobia*). For each phylogenetic group, a dilution series ranging from 50 ng to 50 fg of genomic DNA of the respective positive control strain served as a standard. Finally, the values obtained were corrected for differences in *rrn* gene copy number between the control strains and their relatives in soil, using the ratio of copy number of the control strains as available in the Integrated Microbial Genomes database of the DOE Joint Genome Institute (http://img.jgi.doe.gov) and the average copy number reported for soil bacteria (Klappenbach *et al.*, 2000; Shrestha *et al.*, 2007).

3.3.7 Effect of starvation on culturability of strain Jbg-1

Cultures of strain Jbg-1 were grown to stationary phase in 1:10 diluted HD-medium, harvested by centrifugation at 8,000 x g for 20 min and washed twice with 10 ml MES-buffered TS-medium (pH 5.5; see above). Afterwards, cultures were adjusted to an OD_{580} of 0.1 by addition of the washing medium and incubated at 15°C in the dark. Changes in cell density were monitored measuring the optical density at 580 nm. Total cell counts were determined by epifluorescence microscopy (see above), and the number of culturable cells was quantified by plating onto agar-solidified MES-buffered (pH 5.5) TS-medium with 0.01% (w/v) tryptic soy broth and 0.1% (w/v) glucose. The agar plates were incubated for at least 6 weeks at 15°C and the visible colonies were counted.

3.3.8 Nucleotide accession numbers

The 16S rRNA gene sequences obtained in the present study have been deposited in the GenBank database under accession numbers AM932441-AM932483, AM932376-AM932424, AM932486-AM932487.

3.4 RESULTS

3.4.1 Soil characteristics

An alpine rendzina (mollisols: rendolls) was selected for the present study. Samples were obtained from the organic-rich A_h horizon, which extended over a depth of 22 cm. On the three sampling dates, *in situ* temperatures ranged between -3 and 12.3 °C (Table 1). The water content of the soil was between 57.3 to 68.5% and pH values were in the neutral range. Total bacterial numbers between $3.1 \cdot 10^{10}$ - $11.5 \cdot 10^{10}$ cells·(g dry weight)$^{-1}$ were determined between February and August 2002 (Table 1). In the samples obtained on February, 97.8% of the cells were found to be attached to soil particles >12 µm. These bacteria were inhomogeneously distributed, as reflected by the high standard deviations of total cell counts.

Table 1. Physicochemical characteristics and total cell numbers of the soil samples

Parameter	08.02.02	29.04.02	26.08.02
Temperature (°C)	– 3.0	8.0	12.3
Water content (%)	57.30±0.83	68.50±1.32	65.10±8.76
pH (H_2O)	7.80	7.40±0.48	7.55±0.58
pH (0.1 M KCl)	6.70	6.80±0.47	6.60±0.63
Total cell count [10^{10} (g dry weight)$^{-1}$]	3.12 ±1.53	5.45±3.92	11.50±9.13

3.4.2 Response of soil ATP content to substrate addition

An ATP content of 2.3 μg·(g dry weight)$^{-1}$ was determined for soil sampled in February 2002. This value corresponds to 0.074 fg ATP·cell^{-1} and an average intracellular concentration of 0.09 mM. The February sample was chosen to investigate the effects of substrate addition on the physiological state of the bacterial cells. Slurries were supplemented with artificial root exudates or the polymer mixture, and the ATP content was followed over 10 days (Fig. 1). Slurries without substrate amendments were included as controls. During the first 24 hours, a decrease in ATP content was detected in all slurries. This initial decrease was much more pronounced after the addition of artificial root exudates and in the controls than in slurries supplemented with the polymer mixture. After 3 days of incubation, the ATP content in slurries with artificial root exudates and the polymer mixture increased, and within ten days of incubation reached (1.4 ± 0.4) and (2.9 ± 0.3) μg ATP·(g dry weight)$^{-1}$, respectively. In contrast, the ATP content of control slurries did not recover. Over this incubation period, no changes in bacterial cell numbers could be detected in any of the slurries.

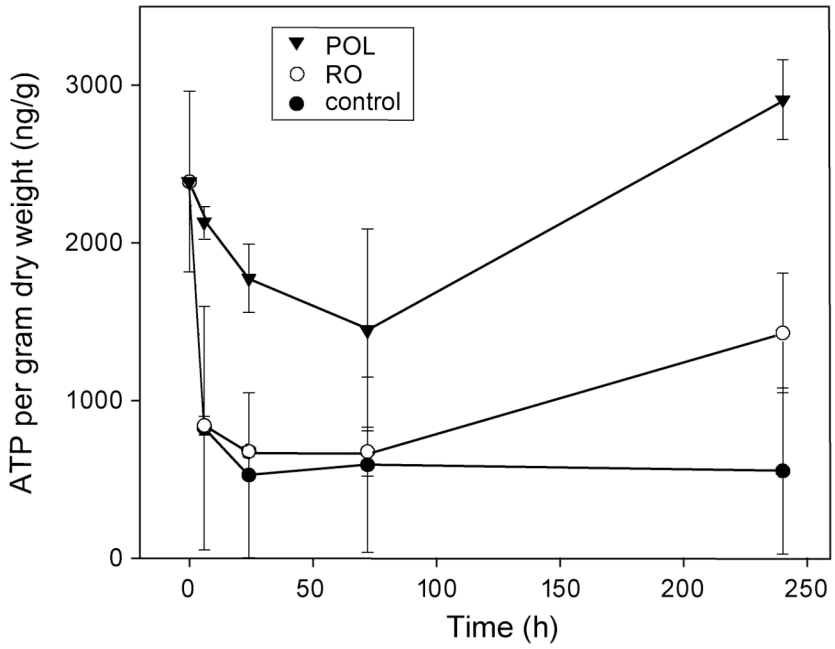

Figure 1. Time course of ATP concentrations in slurries prepared with samples collected in February 2002 and amended with artificial root exudates (○) or a polymer mixture (▼). Values in unamended controls (●) are shown for comparison. Vertical bars depict standard deviations.

3.4.3 Cultivation success, phylogenetic affiliation and diversity of the cultivated bacteria

The cultivation success of bacteria from Jochberg soil, as determined from the number of wells, which show turbidity, was observed to depend strongly on the type of carbon substrates added and varied significantly with season (Fig. 2). The lowest value of 0.012 % was obtained with soil sampled in February 2002 and in media containing humic acid analogs. In all cases, a significantly higher cultivation success was attained in the presence of artificial root exudates (RO) than with the polymer mixture (POL) or humic acid analogs (HA). This differential effect of substrate composition was most pronounced for the February sample, which yielded a tenfold higher culturability in the presence of RO than with POL. In the presence of the same substrate mixture, the culturability values for the August sample surpassed those for February by one to two orders of magnitude (Fig. 2). Overall, the values obtained with HA ranged from 0.012 to 1.8%, those with the POL from 0.045 to 2.3%, and those with RO from 0.48 to 5.3%. For comparative purposes, additional carbon substrates were tested in the cultivation assays in the months of April and August. Whereas the addition of nutrient broth resulted in a cultivation success, which was comparable to that for RO, the culturability values for the August samples reached 13.5 % in media supplemented with soil extract. The major aim of the present study was to assess the cultivation success for cells exposed to extreme environmental conditions by using defined media. Therefore, subsequent analysis focused on the cultures obtained from the February sample and in media supplemented with RO and POL.

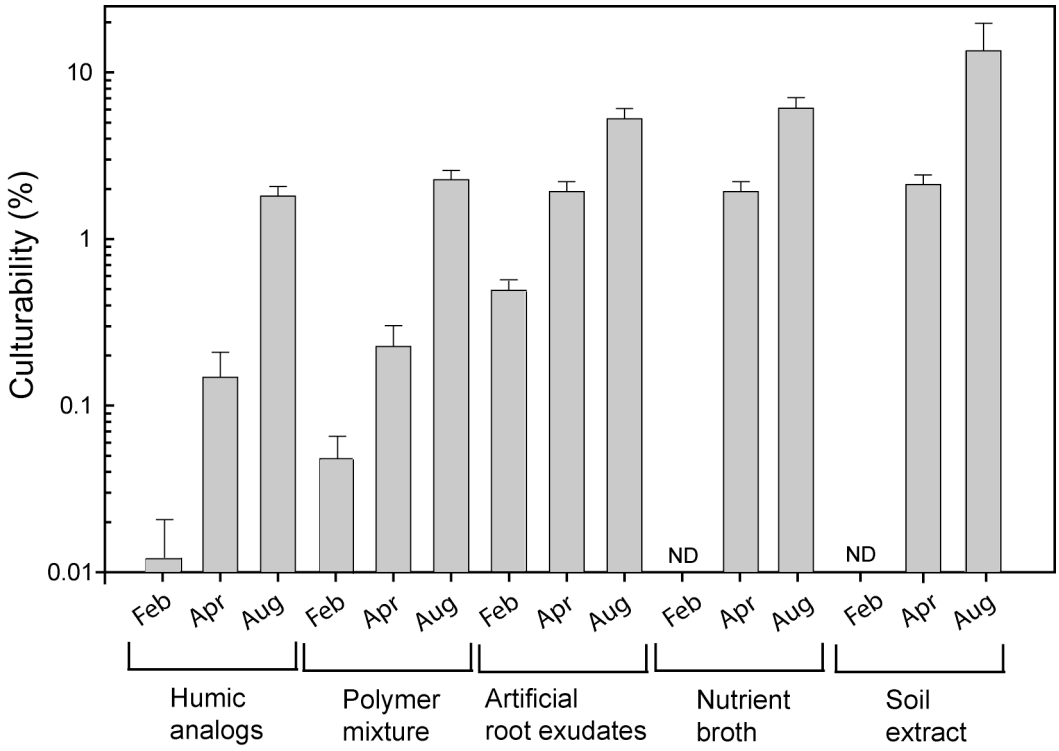

Figure 2. Culturability of bacteria in Jochberg soil based on the most probable numbers as determined by the MicroDrop technique. Data for the three sampling dates are shown. Vertical bars indicate 95% confidence interval. n.d., not determined.

For the subsequent comparative phylogenetic analysis of the 81 cultures that had been recovered from the February sample, we employed a group-specific PCR/DGGE approach. This approach, which separately targets the 16S rRNA genes of *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Alpha-* and *Betaproteobacteria*, and *Acidobacteria*, is especially well suited for the detection and analysis of mixed cultures since it permits the detection of the respective target bacteria even if they represent only a minor fraction of a culture (Overmann *et al.*, 1999; Gich *et al.*, 2005). The PCR products generated for the different cultures were separated side to side on denaturing gradient gel electrophoresis (DGGE) gels and the resulting DNA bands analyzed by sequencing.

Of the three carbon substrate mixtures tested, the artificial root exudates consistently yielded the largest number of different bacterial isolates for all taxonomic groups except the *Acidobacteria*, which were also detected in culture media supplemented with the polymer mixture. Unexpectedly, the majority (i.e., 75%) of the MicroDrop cultures yielded amplification products with several sets of group-specific primer pairs. These co-cultures of members of different bacterial phyla harbored up to 6 different phylogenetic groups. Thus, a total of 251 fingerprints could be generated from the 81 cultures. Since several 16S rRNA gene sequences were detected in multiple wells, the 251 fingerprints contained 53 distinct phylotypes (Table 2).

Table 2. Phylogenetic and diversity analyses of the MicroDrop cultures and the most frequently cultivated phylotypes obtained from the February 2002 soil sample

(Sub)phylum	Total no. of fingerprints	Frequency of individual phylotypes	Total no. of phylotypes	Total phylotype richness[1]	Evenness	Max.cultur-ability (%)[3]	Frequent isolates[2]	
							Closest relative	Max. cultur-ability (%)[3]
Firmicutes	23	15/3/1/1/1/1/1	7	17.0±10.1	0.63	0.10±0.04	*Staphylococcus schleiferi* CD22-1	0.07±0.02
Actinobacteria	17	4/3/2/2/2/1/1/1/1	9	10.5±2.2	0.94	0.09±0.04	*Rhodococcus erythropolis* EK5 *Agromyces cerinus* DSM8595	0.02±0.01 0.02±0.01
Bacteroidetes	54	37/12/2/1/1/1	6	7.5±2.6	0.52	0.32±0.08	*Sphingoterrabacterium pocheensis* Gsoil032 *Flavobacterium* sp. WB4.4-73	0.23±0.04 0.06±0.02
Alphaproteobacteria	69	17/13/11/8/4/3/3/2/ 2/1/1/1/1/1/1	15	20.0±5.5	0.82	0.40±0.08	*Phyllobacterium trifolii* PETP02 *Rhodospirillum rubrum* DSM107	0.09±0.02 0.07±0.02
Betaproteobacteria	60	23/21/9/4/1/1/1	7	10.0±4.4	0.72	0.32±0.08	*Delftia tsuruhatensis* 180282 *Rhodocyclus tenuis* DSM110	0.11±0.03 0.10±0.02
Gammaproteobacteria	24	14/3/2/1/1/1/1/1	8	13.0±6.0	0.69	0.14±0.05	*Pseudomonas* sp. HH.Sph.064	0.09±0.02
Acidobacteria	4	4	1	-	-	0.02±0.01	*Edaphobacter modestus* DSM18101^T	0.02±0.01
Sum	251	-	53	-	-	1.39	-	0.86

[1] Mean values ± standard deviation of the nonparametric Chao1 estimator
[2] closest relative in the database, compare Fig. 5 and Suppl. Figs. 1 through 6
[3] Mean ± 95% confidence interval. Maximum culturability values for all groups except the *Acidobacteria* are given only for medium supplemented with artificial root exudates as carbon source, since this medium yielded the highest values (compare Fig. 2). Values for cultivated *Acidobacteria* are for media containing the polymer mixture and artificial root exudates.
-, not applicable

In addition to the target groups, four primer sets (*Firmicutes*, *Bacteroidetes* and *Alpha*- and *Betaproteobacteria*) also recovered a few sequences of the *Gammaproteobacteria,* which were therefore included in the analysis. A detailed comparison of the cultured phylotypes revealed distinct differences with respect to abundance, richness, evenness and culturability between the seven individual (sub)phyla. Cultured *Alphaproteobacteria* represented the largest fraction and reached 0.4% of total bacterial numbers in media supplemented with artificial root exudates. This group also yielded the by far largest number of different phylotypes. In addition, the *Betaproteobacteria* and *Bacteroidetes* were most frequently cultured (Table 2) whereas significantly fewer cultures were found to contain *Firmicutes* and *Actinobacteria*. In pronounced contrast to all other bacterial (sub)phyla, only a single and rare phylotype of *Acidobacteria* was detected in four of the microtiter wells containing media supplemented with organic polymers (1 well) and artificial root exudates (3 wells; Table 2). In a few instances did bacteria of the same (sub)phylum co-occur in the same microtiter plate well (e.g. member s of the genus *Bacillus* in well 1, Supplementary Fig. 1). Phylogenetic distances between these co-occurring sequences were 9% or larger. Since the nucleotide divergence of multiple *rrn* operons of the same bacterial genome in almost all known cases is <2.2% (Acinas *et al.*, 2004) our analyses suggest that the phylotypes cultivated in the present study represent different types of bacteria.

The cultivated fractions of the *Firmicutes*, *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteria* were strongly dominated by one or two bacterial phylotypes and, accordingly, exhibited an uneven composition with low evenness values of 0.52 - 0.72 (Table 2). The dominance of individual phylotypes was less pronounced for the *Alphaproteobacteria*, and the collection of cultivated *Actinobacteria* showed the highest eveness value of 0.94. More than half of the cultivated *Firmicutes* were related to *Staphylococcus schleiferi* at a 16S rRNA gene sequence similarity of 98.9% (Supplementary Fig. 1). The two most frequent phylotypes of the *Actinobacteria* were identified as *Rhodococcus erythropolis* and close relatives of *Agromyes cerinus* (98.8%; Supplementary Fig. 2). Among all (sub)phyla, the cultivated fraction of the *Bacteroidetes* exhibited by far the lowest eveness value of 0.52. One of the 7 phylotypes of the *Bacteroidetes* strongly dominated the collection of cultured phylotypes and was detected in 70% of the *Bacteroidetes* cultures (Table 2); this bacterium showed a sequence similarity of 98.2% to *Sphingoterrabacterium pocheensis*. The culturability for this bacterium reached 0.23% (±0.04%) and thus significantly surpassed the culturability of all other isolates (Table 2). This frequent *Bacteroidetes* phylotype was found in distant wells on different microtiter plates (the numbering of wells in Supplementary Figures does not reflect their position on the microtiter plates), confirming that inoculation of these cultures represented independent events and does not represent progeny from one microcolony, which was disintegrated upon loading the MicroDrop

capillary. The second most frequent cultivated *Bacteroidetes* phylotype belonged to an already known *Flavobacterium* sp. (Supplementary Fig. 3). The *Alphaproteobacterium* most frequently recovered by cultivation was *Phyllobacterium brassicacearum* and the second most frequent isolate a distant (99.8% sequence similarity) relative of *Rhodospirillum rubrum* (Supplementary Fig. 4). Distant relatives of *Delftia tsuruhatensis* and *Rhodocyclus tenuis* dominated the cultivated fraction of the *Betaproteobacteria* (99.2% and 98.8% sequence similarity, respectively; Supplementary Fig. 5) (Table 2). Finally, a member of genus *Pseudomonas* was the most frequent *Gammaproteobacterium* detected in our culture collection (99.0% sequence similarity; Supplementary Fig. 6). Combining the results of our phylogenetic analyses (Suppplementary Figs. 1 - 6, Fig. 5), a total of 182 out of the 251 cultivated bacteria (i.e., 73%) represented novel phylogenetic lineages whereas only a limited number of known phylotypes was recovered. The fraction of known phylotypes was largest for the *Alphaproteobacteria*, reaching 46% of all cultured bacteria of this group.

The repeated recovery of identical phylotypes in all groups suggested that our culture collection covers a significant fraction of all the phylotypes, which are capable of growing in our media. Indeed, the values for the nonparametric Chao1 estimator as calculated from the frequencies of individual phylotypes of the *Actinobacteria*, *Bacteroidetes*, and *Betaproteobacteria* surpassed the actual number of cultivated phylotypes only slightly (Table 2). Thus, for these three groups, only few of the phylotypes capable of growing in our media were missed in our present study. The coverage of culturable phylotypes was still reasonable for the subphyla *Alphaproteobacteria* and *Gammaproteobacteria*. In contrast, the collection of cultivated *Firmicutes* comprised only 40% of the estimated phylotype richness of this phylum. The collector´s curve calculated for the combined data of all bacterial (sub)phyla reached saturation (Fig. 3), indicating that our culture collection comprises most of the phylotypes which can be retrieved by the three different cultivation media.

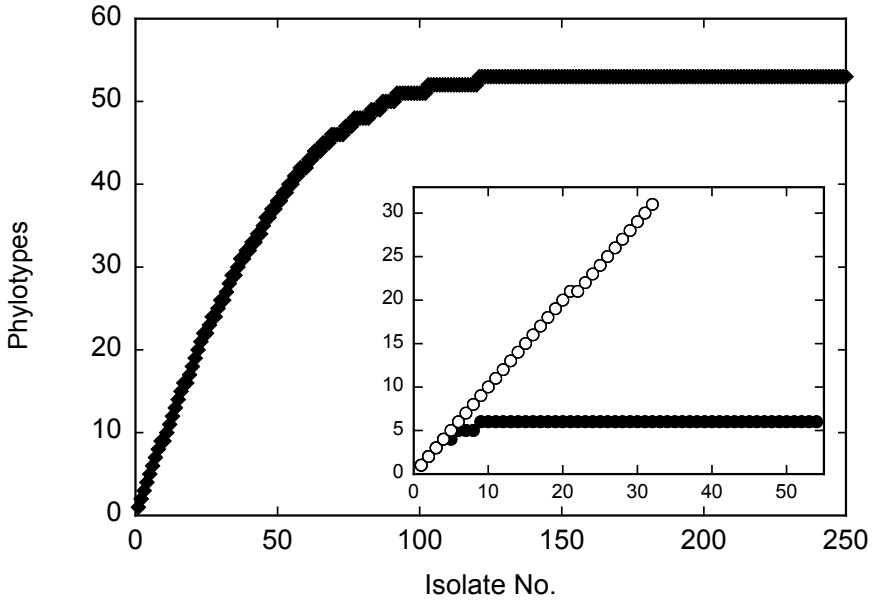

Figure 3. Collectors curve plotted for all 251 bacterial phylotypes cultured in the present study (♦). A total of 53 different phylotypes was detected. The insert shows the collectors curves for bacteria of the phylum *Bacteroidetes* including the phylotypes recovered by cultivation (●) and the phylotypes cloned from environmental DNA (○).

3.4.4 Culture-independent analysis of the bacterial community and detection of cultured phylotypes

In order to assess the selectivity of the different cultivation media, we determined for each (sub)phylum, (i) which fraction of the natural bacterial community had been recovered and (ii) whether cultured phylotypes were also detectable by culture-independent methods.

Chromosomal DNA was isolated from the soil sample collected in February 2002 and 16S rRNA genes of each target group were quantified by RT-PCR employing the group-specific primers for *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Alpha- and Betaproteobacteria* and *Acidobacteria*. As described above, the primers targeting the *Alphaproteobacteria* also cover some *Gammaproteobacteria* in this analysis. In addition, members of the *Verrucomicrobia* were quantified. Based on the RT-PCR results, members of the target groups constituted between 1.2 and 49.2% of all eubacteria in the soil sample (Fig. 4). Similar to the results of our cultivation assays, the *Alphaproteobacteria* dominated the bacterial community and constituted 49.2% (± 3.5%) of all eubacteria. In pronounced contrast to the cultivated fraction, however, the *Acidobacteria* represented the second most abundant bacterial group in Jochberg soil, reaching a fraction of 20.1% (±0.2%). All other phylogenetic groups, including the *Bacteroidetes*, were present in low abundances, which ranged between 1.2 and 4.0% of the eubacteria. Together, the target groups constituted 78% of all eubacteria in the sample, confirming that the major bacterial groups were covered by the RT-PCR approach.

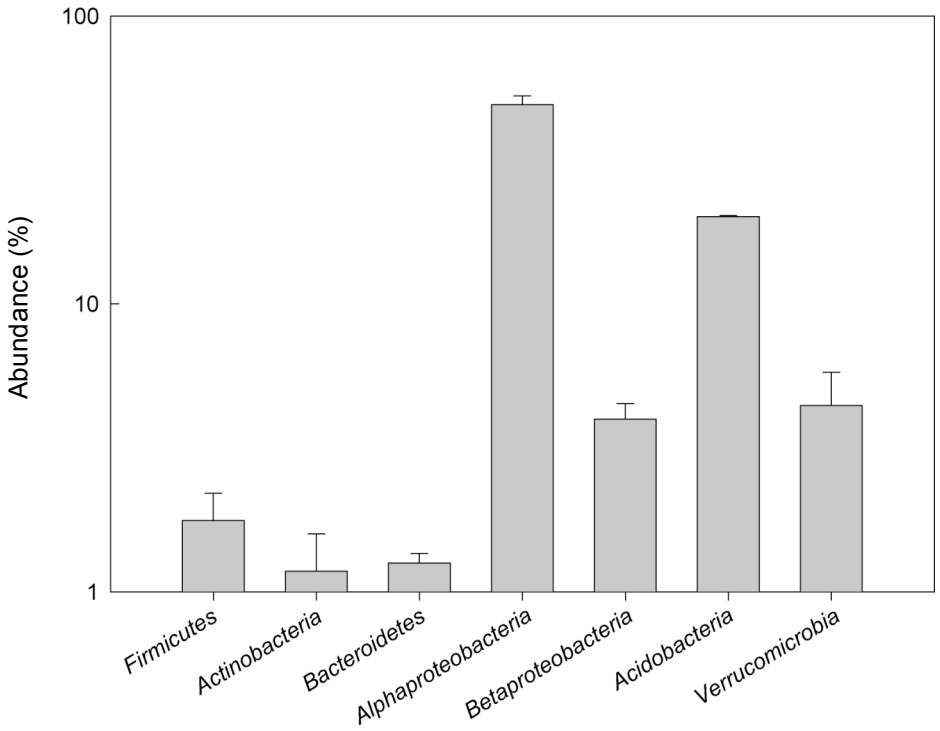

Figure 4. Abundance of bacteria of the six target groups in the natural community, determined by quantitative PCR and using Jochberg soil sampled in February 2002. Abundance values are given as percentage of the total amount of 16S rRNA genes of eubacteria.

The cultivated fraction of the *Bacteroidetes* obtained in our study showed a high coverage and accounted for 25% of the fraction determined by RT-PCR. Therefore this phylum represented the most promising candidate for a comparison of culture-independently detected and cultured phylotypes. Since DGGE-fingerprinting of the *Bacteroidetes* resulted in numerous but incompletely separated bands, the RT-PCR product for this group was cloned and sequenced. Of the 32 sequences obtained, only two were found to be identical (Fig. 4). 59% of the cloned 16S rRNA gene sequences (labeled with suffix "clone" in Supplementary Fig. 3) were closely related to previously uncultured bacteria but none was identical to the cultured phylotypes. Even the most frequently cultivated relative of *S. pocheensis* was not detected by the culture-independent approach, indicating a pronounced bias of the cultivation and/or the cloning approaches for this particular bacterial group.

The *Acidobacteria* represented the second most frequent bacterial group in Jochberg soil. Yet, only few members of this group have been isolated while the factors underlying the low cultivation success of *Acidobacteria* in defined laboratory media has remained largely unclear. Therefore, the *Acidobacteria* were selected as the second group for a comparative analysis of the natural community and the cultivated fraction. The phylotypes present in the natural community were amplified with group-specific primers and separated by DGGE (Fig. 5A). For comparison, the amplification products of strain Jbg-1 isolated from the acidobacterial cultures and of isolate

Wbg-1 recovered from a calcareous forest soil (Koch *et al.*, 2008) were analyzed in parallel. Although the cultivation success of phylotype Jbg-1 was only 0.02% of total bacterial cell numbers and thus exactly four orders of magnitude lower than the total abundance of *Acidobacteria* (20.1%), a melting type corresponding to isolate Jbg-1 could be detected in the natural community (melting type 2; Fig. 5A). The respective DNA band plus seven additional bands were excised, eluted from the gel, cloned and sequenced. Overall, the 8 melting types contained 19 different 16S rRNA gene sequences which were affiliated with subdivisions 1, 3, 4, 5, and 6 of the phylum *Acidobacteria* (Fig. 5B). A detailed phylogenetic analysis revealed that one of the sequences (clone 2a) corresponded exactly to that of strain Jbg-1 (Fig. 5B). The two cloned sequences 1a and 1b exhibited 98.9% and 99.3% similarity to the sequence of strain Wbg-1 and hence may represent members of the same species (based on an average value of 97% for delineating bacterial species; Stackebrandt and Goebel, 1994).

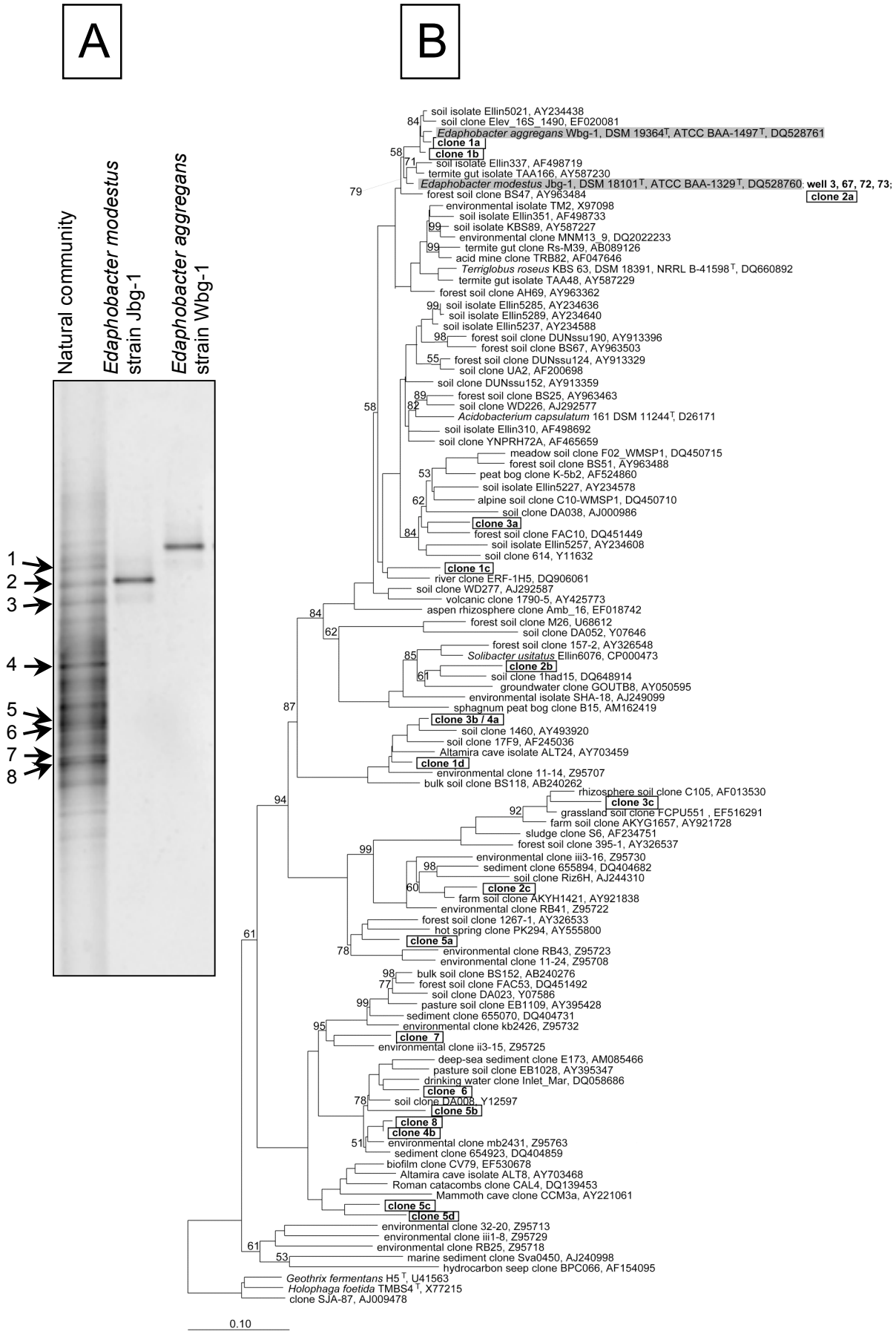
A
B
Natural community
Edaphobacter modestus strain Jbg-1
Edaphobacter aggregans strain Wbg-1
1
2
3
4
5
6
7
8
soil isolate Ellin5021, AY234438
soil clone Elev_16S_1490, EF020081
Edaphobacter aggregans Wbg-1, DSM 19364T, ATCC BAA-1497T, DQ528761
clone 1a
clone 1b
soil isolate Ellin337, AF498719
termite gut isolate TAA166, AY587230
Edaphobacter modestus Jbg-1, DSM 18101T, ATCC BAA-1329T, DQ528760; well 3, 67, 72, 73;
forest soil clone BS47, AY963484
clone 2a
environmental isolate TM2, X97098
soil isolate Ellin351, AF498733
soil isolate KBS89, AY587227
environmental clone MNM13_9, DQ2022233
termite gut clone Rs-M39, AB089126
acid mine clone TRB82, AF047646
Terriglobus roseus KBS 63, DSM 18391, NRRL B-41598T, DQ660892
termite gut isolate TAA48, AY587229
forest soil clone AH69, AY963362
soil isolate Ellin5285, AY234636
soil isolate Ellin5289, AY234640
soil isolate Ellin5237, AY234588
forest soil clone DUNssu190, AY913396
forest soil clone BS67, AY963503
forest soil clone DUNssu124, AY913329
soil clone UA2, AF200698
soil clone DUNssu152, AY913359
forest soil clone BS25, AY963463
soil clone WD226, AJ292577
Acidobacterium capsulatum 161 DSM 11244T, D26171
soil isolate Ellin310, AF498692
soil clone YNPRH72A, AF465659
meadow soil clone F02_WMSP1, DQ450715
forest soil clone BS51, AY963488
peat bog clone K-5b2, AF524860
soil isolate Ellin5227, AY234578
alpine soil clone C10-WMSP1, DQ450710
soil clone DA038, AJ000986
clone 3a
forest soil clone FAC10, DQ451449
soil isolate Ellin5257, AY234608
soil clone 614, Y11632
clone 1c
river clone ERF-1H5, DQ906061
soil clone WD277, AJ292587
volcanic clone 1790-5, AY425773
aspen rhizosphere clone Amb_16, EF018742
forest soil clone M26, U68612
soil clone DA052, Y07646
forest soil clone 157-2, AY326548
Solibacter usitatus Ellin6076, CP000473
clone 2b
soil clone 1had15, DQ648914
groundwater clone GOUTB8, AY050595
environmental isolate SHA-18, AJ249099
sphagnum peat bog clone B15, AM162419
clone 3b / 4a
soil clone 1460, AY493920
soil clone 17F9, AF245036
Altamira cave isolate ALT24, AY703459
clone 1d
environmental clone 11-14, Z95707
bulk soil clone BS118, AB240262
rhizosphere soil clone C105, AF013530
clone 3c
grassland soil clone FCPU551 , EF516291
farm soil clone AKYG1657, AY921728
sludge clone S6, AF234751
forest soil clone 395-1, AY326537
environmental clone iii3-16, Z95730
sediment clone 655894, DQ404682
soil clone Riz6H, AJ244310
clone 2c
farm soil clone AKYH1421, AY921838
environmental clone RB41, Z95722
forest soil clone 1267-1, AY326533
hot spring clone PK294, AY555800
clone 5a
environmental clone RB43, Z95723
environmental clone 11-24, Z95708
bulk soil clone BS152, AB240276
forest soil clone FAC53, DQ451492
soil clone DA023, Y07586
pasture soil clone EB1109, AY395428
sediment clone 655070, DQ404731
environmental clone kb2426, Z95732
clone 7
environmental clone ii3-15, Z95725
deep-sea sediment clone E173, AM085466
pasture soil clone EB1028, AY395347
drinking water clone Inlet_Mar, DQ058686
clone 6
soil clone DA008, Y12597
clone 5b
clone 8
clone 4b
environmental clone mb2431, Z95763
sediment clone 654923, DQ404859
biofilm clone CV79, EF530678
Altamira cave isolate ALT8, AY703468
Roman catacombs clone CAL4, DQ139453
Mammoth cave clone CCM3a, AY221061
clone 5c
clone 5d
environmental clone 32-20, Z95713
environmental clone iii1-8, Z95729
environmental clone RB25, Z95718
marine sediment clone Sva0450, AJ240998
hydrocarbon seep clone BPC066, AF154095
Geothrix fermentans H5T, U41563
Holophaga foetida TMBS4T, X77215
clone SJA-87, AJ009478
0.10
Subdivisions
1
2
3
5
4
6
7
8

Figure 5. A. DGGE fingerprinting of 16S rRNA gene fragments of *Acidobacteria* amplified from Jochberg soil sampled in February. A negative image of a SYBR-Gold-stained gel is shown. Numbers denote bands that were cloned and sequenced. Amplification products of the novel isolates *Edaphobacter modestus* Jbg-1 and *E. aggregans* Wbg-1 were run in parallel for comparison. **B.** Neighbour joining phylogenetic tree of the *Acidobacteria* recovered by cultivation of Jochberg soil samples collected in February 2002 and by cloning of environmental sequences from the DGGE bands obtained for the same sample. Cultured phylotypes are shaded in grey and denoted by the suffix 'well' followed by the serial culture number. Cloned environmental sequences are shown in boxes and denoted by the suffix `clone´. Percentages at nodes indicate bootstrap values out of 100 resamplings. Only values ≥50 are shown. Numbers on the right margin denote subdivision of *Acidobacteria* (Hugenholtz *et al*., 1998). Bar indicates 0.1 fixed point mutations per nucleotide base.

3.4.5 Growth characteristics and viability of strain Jbg-1

The physiology of strain Jbg-1 has recently been investigated in detail (Koch *et al.*, 2008). Based on the results of this previous study, the strain represents the novel species *Edaphobacter modestus* within subdivision 1 of the *Acidobacteria*. After inoculation of the three types of media with bacteria of the natural sample, strain Jbg-1 was found to grow in the medium supplemented with polymers or with artificial root exudates. As a basis for an improvement of the cultivation strategy for *Acidobacteria*, the growth requirements of strain Jbg-1 were tested using different types of media and a range of organic carbon concentrations. The lowest culturability values of bacteria in Jochberg soil were observed for the February sample, which suggested an effect of the limiting organic carbon supply on the cultivation success. Therefore, the effect of an extended starvation period on the capability of multiplying in defined laboratory media was studied.

In comparison to the SSE medium with polymeric carbon compounds which was used for isolation of strain Jbg-1, the growth rate was doubled in SSE supplemented with 0.0025% yeast-extract and 0.1% glucose (pH 6.3) or in 1:10 diluted HD medium (containing 0.05% casein peptone, 0.01% glucose, 0.025% yeast extract, pH 7.0) (Table 3). No growth of the freshly isolated strain Jbg-1 was observed in undiluted HD medium (0.5% casein peptone, 0.1% glucose, 0.25% yeast extract), in tenfold concentrated HD medium or in LB medium. After 36 months of subcultivation in diluted HD-medium, however, strain Jbg-1 was capable of growing at a maximum growth rate of 0.088 h^{-1} (doubling time, 7.9 h), although the cells displayed an irregular morphology under these conditions. The lowest concentration of HD medium, which supported growth, was a dilution of 1:100, corresponding to an organic carbon concentration of 37 $mgC \cdot l^{-1}$ (Table 3). At these concentrations, growth rates of 0.005 h^{-1} (doubling time, 150 h) were determined.

The effect of extended starvation on culturablity was tested with stationary phase, washed cells of strain Jbg-1 after growth in HD medium (Fig. 6). Over a time period of 200 d

subsamples were withdrawn and culturable cells quantified by plating onto HD medium; total cell numbers were determined by epifluorescence microscopy. During the first 20 days, no significant changes of total cell numbers and culturable cells could be detected. Afterwards, the titer of culturable cells declined, and reached 4.8% of the initial number after 200 days of incubation. This decline did not proceed in a negatively exponential fashion, however (Fig. 6).

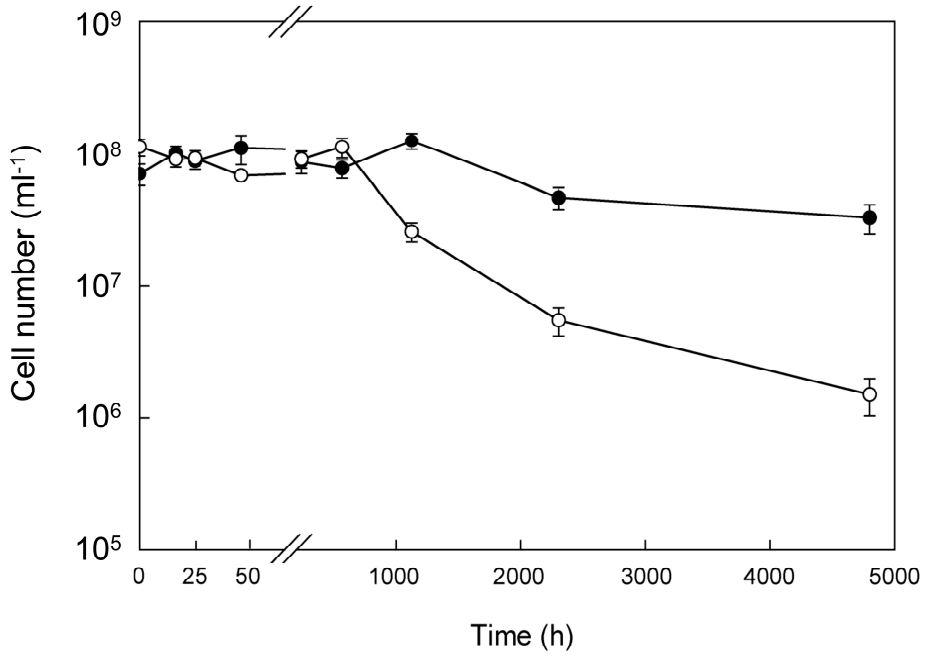

Figure 6. Time course of total (●) and live (○) cell counts during extended incubation of stationary cells of *Edaphobacter modestus*. The error bars indicate 1 standard deviation.

Table 3. Dependence of growth of strain Jbg-1 on organic carbon concentration and medium composition

Medium		OC[a] ($gC \cdot l^{-1}$)	Growth rate μ (h^{-1})	
			Without acclimatisation	After 36 months of subcultivation
HD	10fold	36.5	0	0
	Full strength	3.65	0	0.088±0.006
	1:10 dilution	0.365	0.014±0.003	0.026±0.003
	1:100 dilution	0.037	n.d.	0.005±0.001
	1:1000 dilution	0.004	n.d.	0
SSE + polymers cellulose, chitin, curdlan, pectin, soluble starch, xylan 0.1%		2.7	0.007±0.002	n.d.
SSE + yeast extract+glucose		0.411	0.015±0.004	n.d.
MES-buffered (pH 5.5) TS-medium 0.01% (w/v) tryptic soy broth 0.1% (w/v) glucose			0.008±0.004	n.d.
LB medium (Miller, DIFCO)		6.60	0	n.d.

[a] organic carbon
n.d., not determined

3.5 DISCUSSION

3.5.1 Composition of the alpine soil bacterial community and the cultivated fraction

Based on a recent comparison of 21 different 16S rRNA gene clone libraries, *Acidobacteria* and *Alphaproteobacteria* dominate soil bacterial communities worldwide and contribute 20 and 19 % of the clones, respectively. *Acidobacteria* in some cases can even contribute up to 51 or even 80 % (Dunbar *et al.*, 1999; Chan *et al.*, 2006). Next in abundance are sequences of *Actinobacteria* and *Betaproteobacteria* with 13 and 10 %, respectively, while *Bacteroidetes* constitute 5 and *Firmicutes* 1.8 % of cloned sequences (Janssen, 2006). The data from clone libraries in principle were confirmed by real-time PCR analysis of three different soil bacterial communities in which *Acidobacteria* and *Alphaproteobacteria* constituted 14-23 and 7-14 %, of *rrn* copy numbers, respectively (Fierer *et al.*, 2005).

By comparison with these lowland soils, samples from Jochberg showed a distinct composition of the bacterial community. The abundance of *Alphaproteobacteria* of 49.2% exceeds the upper limit of previous values (Janssen, 2006), whereas our values for *Betaproteobacteria* and *Actinobacteria* are at the lower limit reported for other soils. A similar high abundance of *Alphaproteobacteria*, based on PCR/DGGE fingerprinting, was recently reported for various other alpine soils (Labbé *et al.*, 2007) and may therefore be a common characteristic of high altitude soil ecosystems with large seasonal and diurnal temperature fluctuations and a high precipitation of up to 2000 mm per year. Whereas the abundance of *Acidobacteria* in Jochberg samples (20.1%) was similar to the average value in lowland soils, this group constituted up to 43% of all sequences in another alpine soils at a higher elevation of 3550 m (Lipson and Schmidt 2004).

The isolation of key species of the above bacterial groups provides an opportunity to study and understand their function in the soil environment. However, in clone libraries of 16S rRNA genes, the sequences of the 14 most frequently cultured soil bacteria occur at only low frequencies between 0 and 1.6% (Janssen, 2006). Yet, existing cultivation techniques in principle seem to be suitable to recover previously uncultured soil bacteria like subdivision 1 *Acidobacteria* or *Verrucomicrobia* (Janssen *et al.*, 2002). So far, however, the potential of different media to grow typical soil bacteria has not been assessed in an exhaustive manner. Employing our high throughput cultivation and screening approach, a total of 251 different cultivated bacteria were analyzed. This number significantly surpasses that of previous collections (McCaig *et al.*, 2001; Sait *et al.*, 2002; Lipson and Schmidt, 2004; Shrestha *et al.*, 2007) with the exception of very few studies (Joseph *et al.*, 2003; Davis *et al.*, 2005). In the

present study, a high coverage was attained for most of the target groups. Many of the isolates recovered were closely related to soil bacteria. Two cultivated *Firmicutes*, 3 cultures of *Actinobacteria* and 11 cultures of *Alphaproteobacteria* were identical to sequences of not-yet-cultured bacteria in other soils, demonstrating that more widely distributed soil bacteria can be grown in the available defined laboratory media. The high fraction (73%) of previously unknown phylotypes, which were obtained in the present study, clearly indicates that the potential of the cultivation approach so far has not been exploited to its full extent.

Although *Alphaproteobacteria* dominated the cultivated fraction, the cultivation success of these bacteria (0.4% of total cell numbers) was hundredfold lower than their abundance in the natural bacterial community (49.2%). In addition, about half of the cultivated *Alphaproteobacteria* represented previously isolated phylotypes. In 16S rRNA gene clone libraries, however, less than a third of the sequences can even be assigned to a known genus (Janssen, 2006). With respect to future cultivation attempts, *Alphaproteobacteria* obviously represent a target group for which novel approaches will me most rewarding.

The relative culturability of the *Bacteroidetes* was the highest of all groups and reached 25% of the numbers detected by RT-PCR. All cultivated members and all clones of the *Bacteroidetes* were affiliated with the two classes *Sphingobacteria* and *Flavobacteria*, which compares well with previous analyses of soil clone libraries (Janssen, 2006). In contrast to previous studies, however, our phylogenetic data revealed that most (21 out of 32) environmental sequences recovered from the alpine Jochberg soil microbial community did not fall into established genera. Similarly, 41 out of 54 *Bacteroidetes,* which were recovered by cultivation, represented previously unknown phylotypes. Studies of additional samples are required to test whether alpine soils typically harbor a particularly high diversity of previously unknown *Bacteroidetes*.

Unlike the recently frequent cultivation from lowland soils (Joseph *et al.*, 2003; Stevenson *et al.*, 2004; Eichorst *et al.*, 2007), *Acidobacteria* could not be recovered from a high alpine soil despite a pronounced numerical prevalence (Lipson and Schmidt, 2004). Similarly, the culturability for *Acidobacteria* from Jochberg soil was very low and only one single phylotype grew in our media. This may indicate that *Acidobacteria* in high altitude soil environments require specific cultivation conditions.

3.5.2 Effect of carbon substrates and co-cultivation on the physiological state and culturability of alpine soil bacteria

In order to evaluate the physiological effects of the different cultivation conditions, the ATP content was used as a sensitive parameter to follow the effects of substrate addition and of soil

homogenization on the physiological state of the soil bacteria. The initial ATP content determined in the present study was 4.5 nmol·(g dry weight)$^{-1}$ and thus comparable to values reported for other soils (1.2-11.1 nmol·(g dry weight)$^{-1}$; Tsai *et al.*, 1997; Contin *et al.*, 2002; Joergensen and Raubuch, 2002). Since bacterial cell numbers were rather high in Jochberg soil, the calculated intracellular ATP concentrations of 0.03 mM were significantly lower than the average of 2.4 mM determined for a range of different soils (calculated from these literature data based on a ratio of 0.22 pgC·µm^{-3}; Bratbak and Dundas, 1984) (Contin *et al.*, 2001). As another marked difference, substrate addition did not lead to an instantaneous rise but rather a significant drop in ATP levels over the first 72 hours in Jochberg soil. In other soils, the addition of easily degradable carbon substrates has been shown to induce transient increases in the intracellular ATP content which were followed by an increase in microbial biomass (Tsai *et al.*, 1997). Also, the microbial ATP content typically increases with incubation temperature and reaches optimum values between 30 and 35°C (Joergensen and Raubuch, 2003a). The pronounced initial drop in cellular ATP levels observed in the present study thus is unprecedent. It has been suggested that the use of polymers as substrate may eliminate sudden exposure of oligotrophic soil bacteria to high substrate concentrations and thereby alleviate the problem of substrate-accelerated death (Sait *et al.*, 2002). In Jochberg soil samples, the initial drop was also observed in the unamended controls and therefore not due to a substrate effect.

Continuous mixing has been shown to decrease the cellular ATP content (Joergensen and Raubuch, 2003b) and in the Jochberg soil may initially supersede the stimulating effect of substrate addition, possibly by destroying the microenvironments of the bacterial cells. The sensitivity of the Jochberg microbial community towards mixing is more pronounced than observed for lowland soils, but is commensurate with the very low culturability values observed. Interestingly, the initial drop in ATP concentrations was significantly dampened in the presence of polymeric carbon compounds, suggesting a protective effect of these compounds.

In our liquid media and for the bacterial community of alpine Jochberg soil, monomeric organic carbon compounds proved to be superior to a mixture of polymers for optimization of the cultivation success. These results contrast with those obtained on gellan-solidified media and for a temperate Australian lowland soil (Sait *et al.*, 2002; Davis *et al.*, 2005) and emphasize that results for a particular soil type cannot be generalized and that cultivation approaches need to be adapted on a case-by-case basis for the study of particular soil ecosystem.

An unexpected outcome of the present study was the high number of co-cultures, which was obtained in our growth media. Out of the 1008 cultivation assays set up for the February sampling date, only 81 wells displayed growth after 6 weeks of incubation. Based on this rare occurrence of positive cultures, it was expected that most of the cultures contained a single type

of bacterium because of the following reasoning. Assuming a Poisson distribution, if 92 % of the wells are empty, the expected number of cells per well is –ln 0.92 = 0.083. The probability of a well containing more than one cell is $1 - 0.92 - ((e^{-0.083}) \cdot (0.083^1))/1!) = 0.00361$. Consequently, at maximum, only 4.3% of all positive wells would be expected to contain more than one type of bacterium. Indeed, a dominance of pure cultures was generated with the MicroDrop technique in a previous study of freshwater bacterioplankton communities (Bruns *et al.*, 2003). In the case of Jochberg soil, however, the majority (i.e. 75 %) of the positive wells harbored co-cultures. This suggests that most bacteria grew only if accompanied by unrelated bacteria of other phyla and that this dependence on interspecific interaction was a major reason for the low cultivation success of February samples. Since co-cultures also dominated in the media containing monomeric carbon substrates, this bacterial interaction is probably not based on the hydrolysis by exoenzymes by one type providing the substrates for the accompanying bacteria.

3.5.3 Specific adaptations of the newly isolated acidobacterium strain Jbg-1

Although representatives of *Acidobacteria* subdivision 1 have repeatedly been isolated over the past years, only limited information on their specific adaptations is available. It has been shown that these bacteria grow best at a slightly acidic pH of 5.5 and increased partial pressures of CO_2 (Sait *et al.*, 2006; Eichorst *et al.*, 2007). In addition, previous cultivation trials suggested a superior effect of polymeric carbon substrates for the isolation of several members of subdivisions 1 and 3 (Sait *et al.*, 2002). Accordingly, strain Jbg-1 was also isolated in media containing polymers in the present study, whereas representatives of all other bacterial groups grew preferentially with artificial root exudates. Organic carbon has been found to represent the limiting substrate in high-pH, calcareous soils (Aldén *et al.*, 2001). In alpine soils, specific cellulase activity and microbial utilization of vanillic acid increased significantly during winter compared to the rest of the year (Lipson *et al.*, 2002). Polymers and derivatives of lignin or humic acids therefore may constitute important carbon sources during this time. Metagenomic analyses of *Acidobacteria* in soil have proven the presence of endo-1'- 4' beta xylanase and chitin deacetylase in the genome of members of this phylum (Quaiser *et al.*, 2003). In our concomitant study (Koch *et al.*, 2008) detailed characterization of strain Jbg-1, described as the novel genus and species *Edaphobacter modestus*, revealed that this strain expresses a whole series of hydrolytic exoenzymes. Besides the glycosidases α-and β-glucosidase, α-and β-galactosidase, β-glucuronidase and α-fucosidase, the strain tested positive for activities of acid and alkaline phosphatase, several esterases, as well as leucyl amidase, valyl amidase and α-chymotrypsin. Corresponding to the exoenzymes formed, strain Jbg-1 prefers sugars as growth

substrates and also grows on low concentrations of peptone and casamino acids (Koch *et al.*, 2008). These first results provide a foundation for future studies of polymer degrading exoenzymes in other representative soil *Acidobacteria*.

The increased cultivation success in media containing reduced concentrations of organic carbon (Davis *et al.*, 2005) theoretically would indicate that a major fraction of soil *Acidobacteria* is oligotrophic. Typical oligotrophic bacteria are capable of growing at substrate concentrations of ~1mg DOC · l^{-1}, but cannot grow at ~1g DOC · l^{-1} (Vancanneyt *et al.*, 2001; Cho and Giovannoni, 2004), with the growth of some obligate oligotrophs being inhibited by peptone at concentrations as low as 10 mg· l^{-1} (corresponding to 4.3 mg DOC · l^{-1}) (Rappé *et al.*, 2002). In contrast, facultative oligotrophic bacteria are also capable of growing at 10 g DOC · l^{-1} in complex media (Vancanneyt *et al.*, 2001). By comparison, strain Jbg-1 directly after its isolation exhibited only moderately oligotrophic characteristics, being able to grow up to 0.4 mg DOC · l^{-1}. After prolonged subculturing, the strain could be adapted to 10fold higher DOC concentrations. Strain Jbg-1 therefore can be classified a facultative oligotroph.

In starvation experiments conducted with *Pseudomonas* sp., the number of colony-forming cells dropped by three orders of magnitude within three weeks of incubation (Morita, 1993) and a drop by almost five orders of magnitude was observed during a 50-day incubation of *Micrococcus luteus* (Mukamolova *et al.*, 1995). The decline of culturability for five *Enterobacteriaceae* was even more pronounced and amounted to nine orders of magnitude in twelve days (Bogosian *et al.*, 1998); similar results have been reported for *Campylobacter jejuni* (Federighi *et al.*, 1998), *Xanthomonas campestris* (Ghezzi and Steck, 1999) and *Shewanella algae* (Gram *et al.*, 1999). Compared to the data available for other bacteria, strain Jbg-1 thus is extraordinarily well adapted to long-term survival in organic carbon-free media. The marked deviation from negative exponential decline of the curve shown in Fig. 6 indicates the survival of a rather large subpopulation of especially adapted cells, which maintain culturability even over a period of more than half a year.

Most probable number dilution series have repeatedly been found to be superior for the isolation of numerically dominant but slow-growing bacteria (Hengstmann *et al.*, 1999). Our results indicate, that the high throughput feature of the MicroDrop technique (or equivalent approaches) permit a cultivation also of those phylotypes of which only few cells are capable of multiplying in artificial laboratory media. In previous studies of soil bacterial communities, 16S rRNA gene sequences in clone libraries and cultivated phylotypes did not match (Felske *et al.*, 1999; Hengstmann *et al.*, 1999; Sait *et al.*, 2002; Lipson and Schmidt, 2004), which most likely has to be attributed to the vast phylotype richness of soil bacterial communities (50,000 up to millions; Sandaa *et al.*, 1990; Gans *et al.*, 2005). Although representatives of the phylum

Acidobacteria have been cultivated more frequently over the past years, *Edaphobacter modestus* strain Jbg-1 to our knowledge represents the first case of a cultivated acidobacterial phylotype, which corresponds to a sequence detected in the same sample by culture-independent methods. Strain Jbg-1 was found to be well adapted to extended periods of starvation and expresses a large variety of exoenzymes, thereby providing first indications of its possible functions in the nutrient cycling within soil environments.

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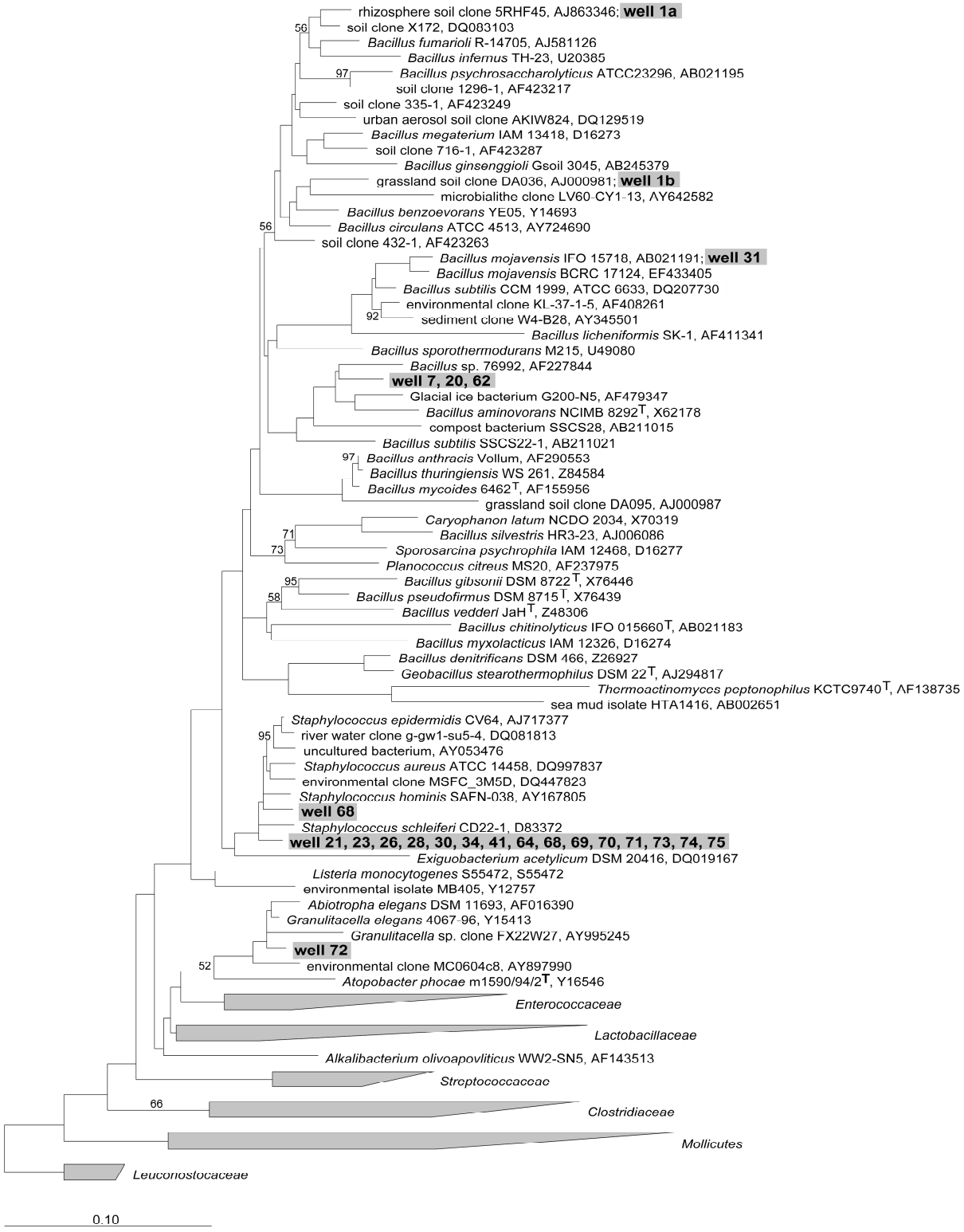
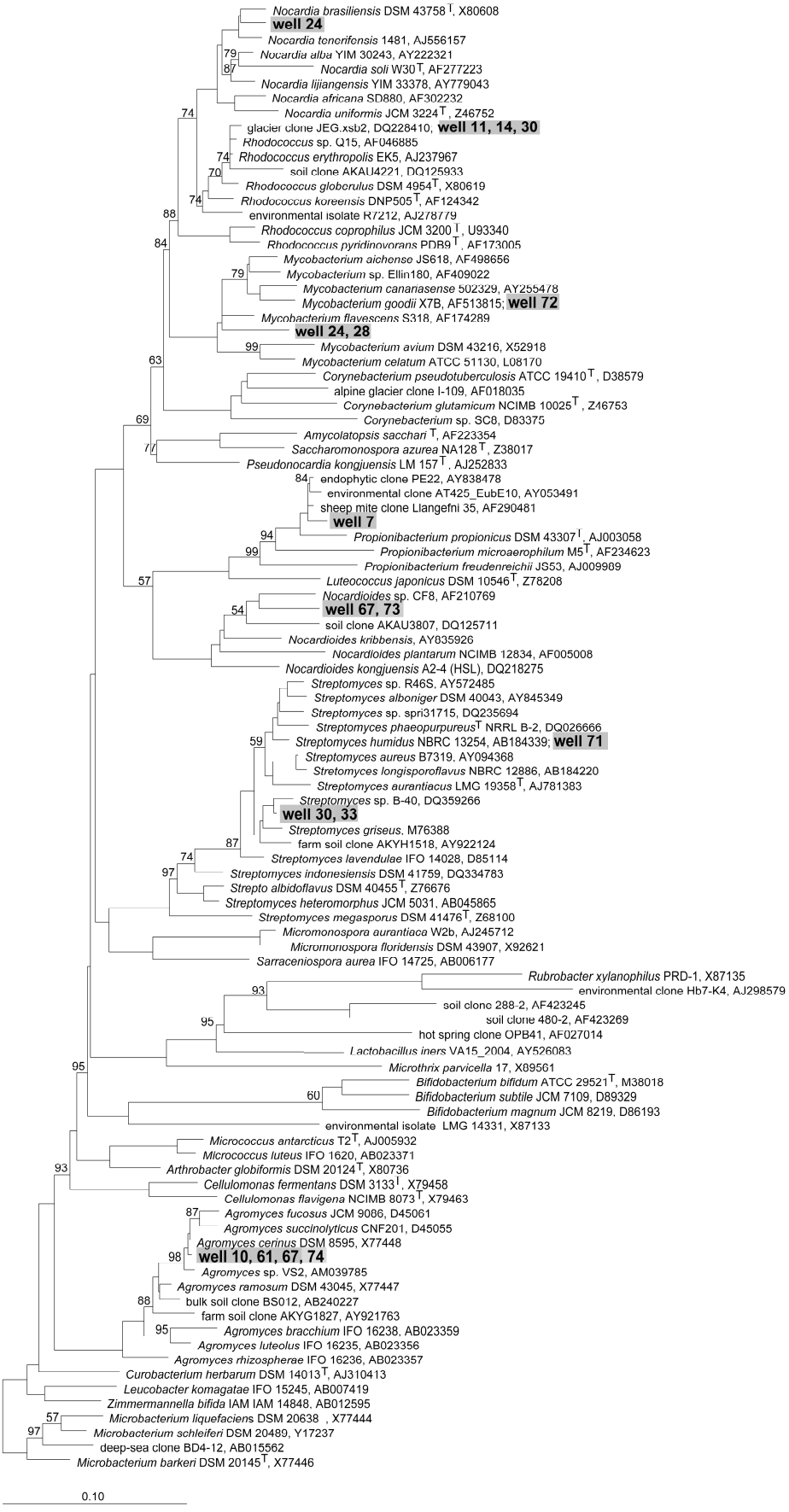
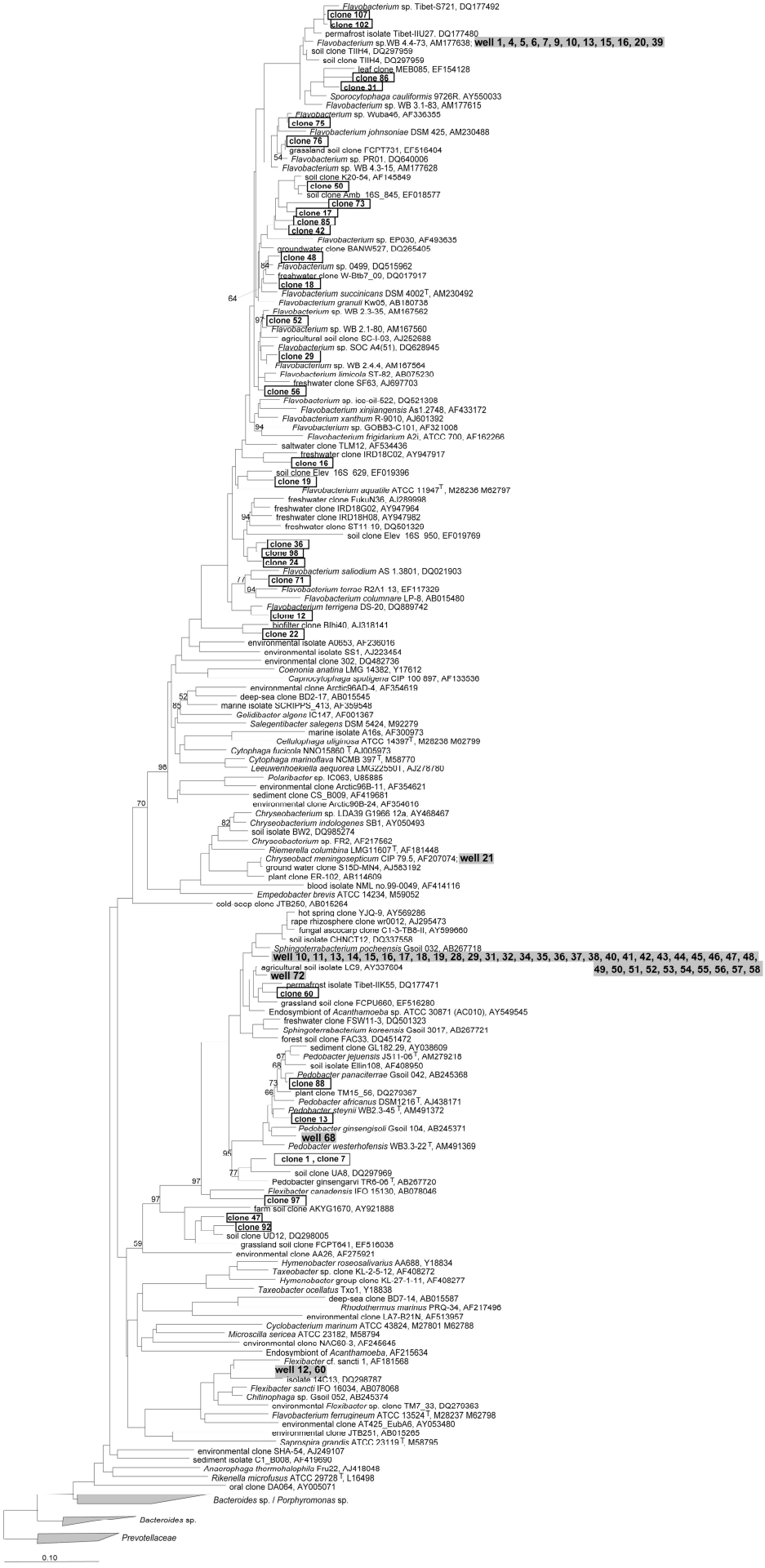
3.8 SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Phylogenetic affiliation of the *Firmicutes* recovered by cultivation of Jochberg soil samples collected in February 2002. A neighbour joining tree was calculated for the 16S rRNA gene sequences of the cultures and their closest relatives. Cultured phylotypes are shaded in grey and denoted by the suffix 'well' followed by the serial culture number. The designations "well1a" and "well 1b" refer to two phylotypes detected in the same well. Percentages at nodes indicate bootstrap values out of 100 resamplings. Only values ≥50 are shown. Bar indicates 0.1 fixed point mutations per nucleotide base.

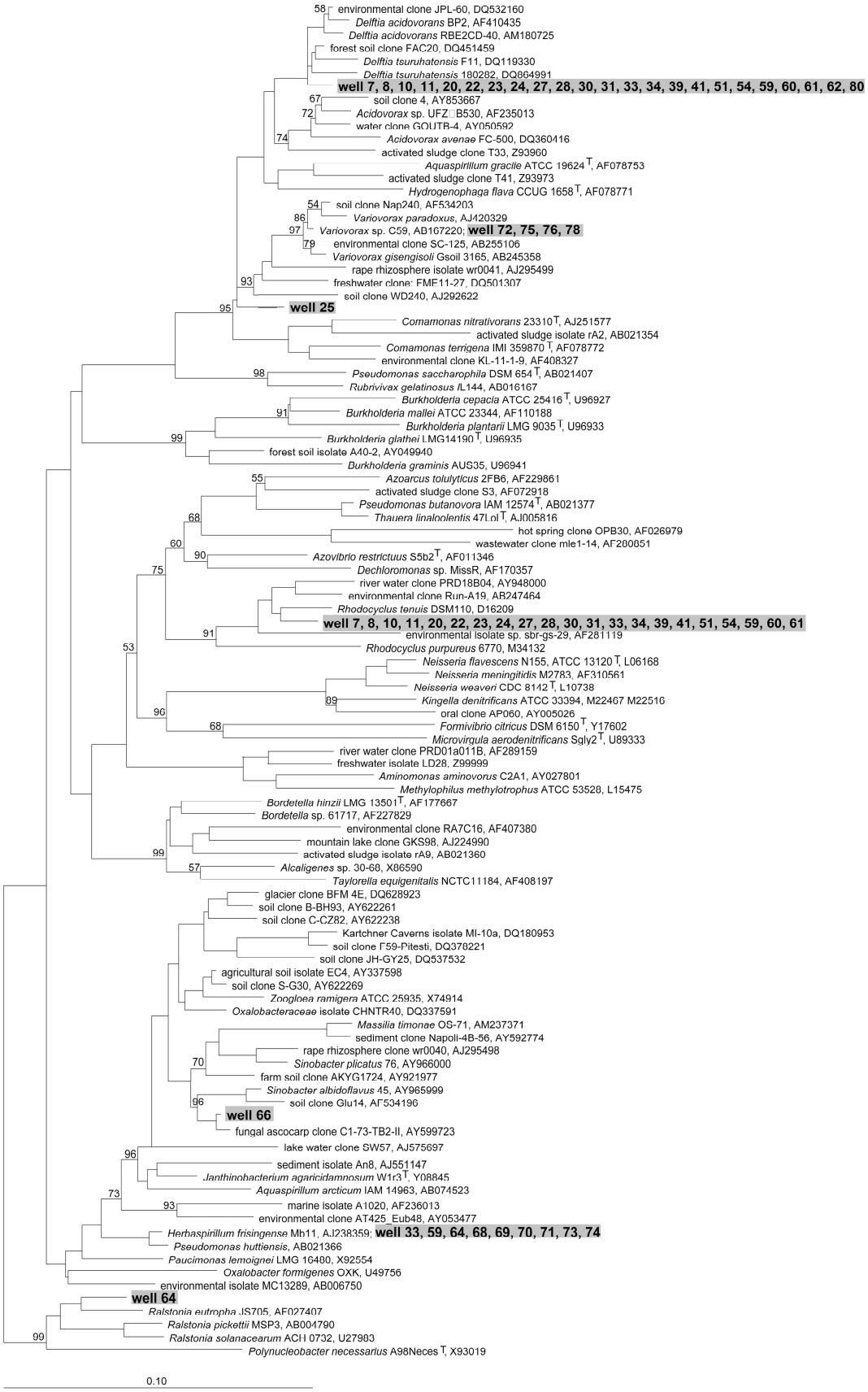


Supplementary Figure 2. Neighbour joining phylogenetic tree of the *Actinobacteria* recovered by cultivation of Jochberg soil samples collected in February 2002. See legend to Supplementary Fig. 1 for further explanations. Bar indicates 0.1 fixed point mutations per nucleotide base.

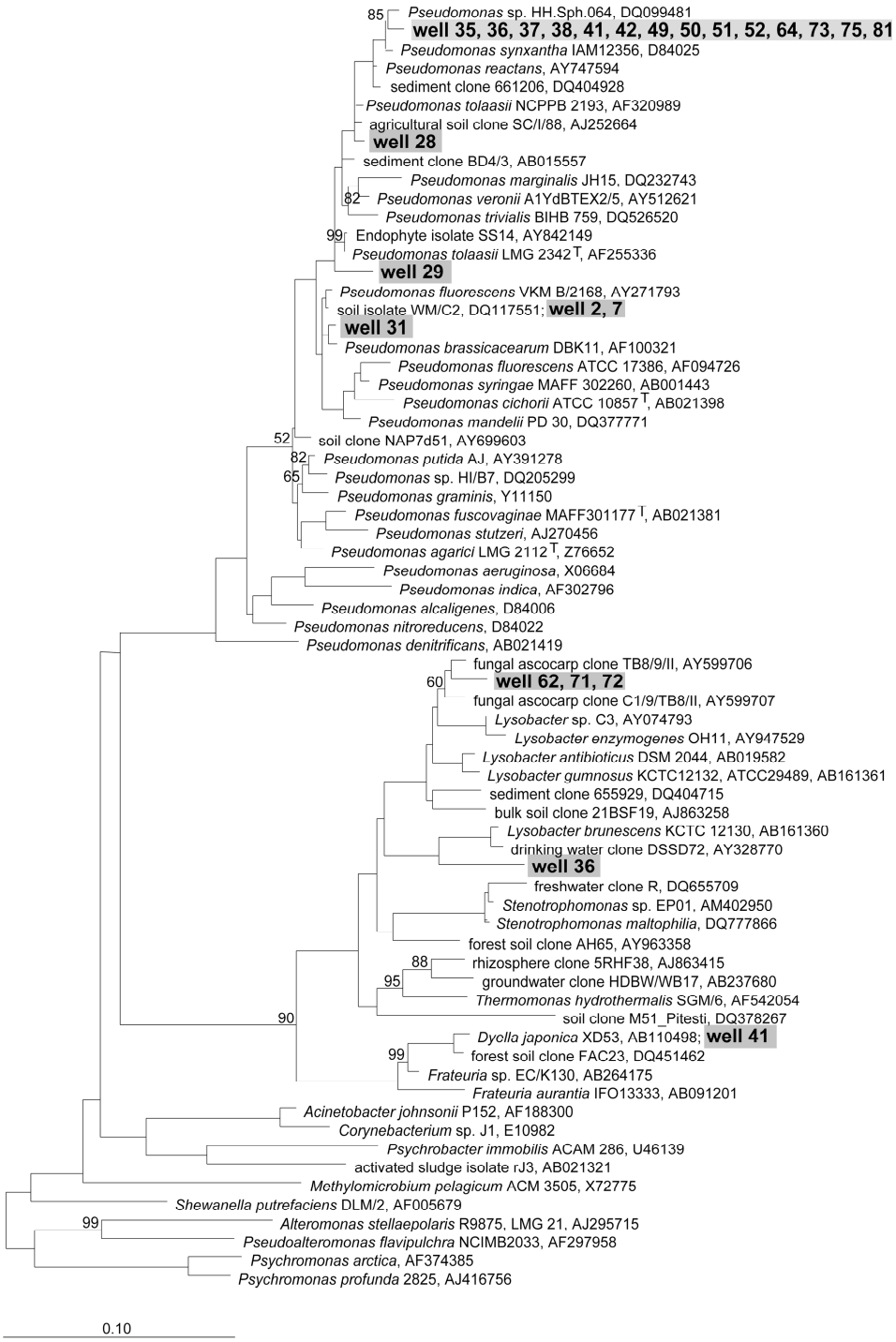

Flavobacterium sp. Tibet-S721, DQ177492
clone 107
clone 102
permafrost isolate Tibet-IIU27, DQ177480
Flavobacterium sp.WB 4.4-73, AM177638; well 1, 4, 5, 6, 7, 9, 10, 13, 15, 16, 20, 39
soil clone TIIH4, DQ297959
soil clone TIIH4, DQ297959
leaf clone MEB085, EF154128
clone 86
clone 31
Sporocytophaga cauliformis 9726R, AY550033
Flavobacterium sp. WB 3.1-83, AM177615
Flavobacterium sp. Wuba46, AF336355
clone 75
Flavobacterium johnsoniae DSM 425, AM230488
clone 76
grassland soil clone FCPT731, EF516404
Flavobacterium sp. PR01, DQ640006
Flavobacterium sp. WB 4.3-15, AM177628
soil clone K20-54, AF145849
clone 50
soil clone Amb_16S_845, EF018577
clone 73
clone 17
clone 85
clone 42
Flavobacterium sp. EP030, AF493635
groundwater clone BANW527, DQ265405
clone 48
Flavobacterium sp. 0499, DQ515962
freshwater clone W-Btb7_09, DQ017917
clone 18
Flavobacterium succinicans DSM 4002T, AM230492
Flavobacterium granuli Kw05, AB180736
Flavobacterium sp. WB 2.3-35, AM167562
clone 52
Flavobacterium sp. WB 2.1-80, AM167560
agricultural soil clone SC-I-03, AJ252688
Flavobacterium sp. SOC A4(51), DQ628945
clone 29
Flavobacterium sp. WB 2.4-4, AM167564
Flavobacterium limicola ST-82, AB075230
freshwater clone SF63, AJ697703
clone 56
Flavobacterium sp. icc-oil-522, DQ521308
Flavobacterium xinjiangensis As1.2748, AF433172
Flavobacterium xanthum R-9010, AJ601392
Flavobacterium sp. GOBB3-C101, AF321008
Flavobacterium frigidarium A2i, ATCC 700, AF162266
saltwater clone TLM12, AF534436
freshwater clone IRD18C02, AY947917
clone 16
soil clone Elev_16S_629, EF019396
clone 19
Flavobacterium aquatile ATCC 11947T, M26236 M62797
freshwater clone FukuN36, AJ289998
freshwater clone IRD18G02, AY947964
freshwater clone IRD18H08, AY947982
freshwater clone ST11 10, DQ501329
soil clone Elev_16S_950, EF019769
clone 36
clone 98
clone 24
Flavobacterium saliodium AS 1.3801, DQ021903
clone 71
Flavobacterium torrae R2A1 13, EF117329
Flavobacterium columnare LP-8, AB015480
Flavobacterium terrigena DS-20, DQ889742
clone 12
biofilter clone Rlhi40, AJ318141
clone 22
environmental isolate A0853, AF236016
environmental isolate SS1, AJ223454
environmental clone 302, DQ482736
Coenonia anatina LMG 14382, Y17612
Capnocytophaga sputigena CIP 100 897, AF133536
environmental clone Arctic96AD-4, AF354619
deep-sea clone BD2-17, AB015545
marine isolate SCRIPPS_413, AF359548
Golidibacter algens IC147, AF001367
Salegentibacter salegens DSM 5424, M92279
marine isolate A16s, AF300973
Cellulophaga uliginosa ATCC 14397T, M28238 M62799
Cytophaga fucicola NNO15860 T AJ005973
Cytophaga marinoflava NCMB 397T, M58770
Leeuwenhoekella aequorea LMG22501, AJ278780
Polaribacter sp. IC063, U85885
environmental clone Arctic96B-11, AF354621
sediment clone CS_B009, AF419681
environmental clone Arctic96B-24, AF354616
Chryseobacterium sp. LDA39 G1966 12a, AY468467
Chryseobacterium indologenes SB1, AY050493
soil isolate BW2, DQ985274
Chryseobacterium sp. FR2, AF217562
Riemerella columbina LMG11607T, AF181448
Chryseobact meningosepticum CIP 79.5, AF207074; well 21
ground water clone S15D-MN4, AJ583192
plant clone ER-102, AB114609
blood isolate NML no.99-0049, AF414116
Empedobacter brevis ATCC 14234, M59052
cold seep clone JTB250, AB016264
hot spring clone YJQ-9, AY569286
rape rhizosphere clone wr0012, AJ295473
fungal ascocarp clone C1-3-TB8-II, AY599660
soil isolate CHNCT12, DQ337558
Sphingoterrabacterium pocheensis Gsoil 032, AB267718
well 10, 11, 13, 14, 15, 16, 17, 18, 19, 28, 29, 31, 32, 34, 35, 36, 37, 38, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58
agricultural soil isolate LC9, AY337604
well 72
permafrost isolate Tibet-IIK55, DQ177471
clone 60
grassland soil clone FCPU660, EF516280
Endosymbiont of Acanthamoeba sp. ATCC 30871 (AC010), AY549545
freshwater clone FSW11-3, DQ501323
Sphingoterrabacterium koreensis Gsoil 3017, AB267721
forest soil clone FAC33, DQ451472
sediment clone GL182.29, AY038609
Pedobacter jejuensis JS11-06T, AM279216
soil isolate Elln108, AF408950
Pedobacter panaciterrae Gsoil 042, AB245368
clone 88
plant clone TM15_56, DQ279367
Pedobacter africanus DSM1216T, AJ438171
Pedobacter steynii WB2.3-45 T, AM491372
clone 13
Pedobacter ginsengisoli Gsoil 104, AB245371
well 68
Pedobacter westerhofensis WB3.3-22T, AM491369
clone 1 , clone 7
soil clone UA8, DQ297969
Pedobacter ginsengarvi TR6-06 T, AB267720
Flexibacter canadensis IFO 15130, AB078046
clone 97
farm soil clone AKYG1670, AY921888
clone 47
clone 92
soil clone UD12, DQ298005
grassland soil clone FCPT641, EF516038
environmental clone AA26, AF275921
Hymenobacter roseosalivarius AA688, Y18834
Taxeobacter sp. clone KL-2-5-12, AF408272
Hymenobacter group clone KL-27-1-11, AF408277
Taxeobacter ocellatus Txo1, Y18838
deep-sea clone BD7-14, AB015587
Rhodothermus marinus PRQ-34, AF217496
environmental clone LA7-R21N, AF513957
Cyclobacterium marinum ATCC 43824, M27801 M62788
Microscilla sericea ATCC 23182, M58794
environmental clone NAC60-3, AF246645
Endosymbiont of Acanthamoeba, AF215634
Flexibacter cf. sancti 1, AF181568
well 12, 60
isolate 14C13, DQ298787
Flexibacter sancti IFO 16034, AB078068
Chitinophaga sp. Gsoil 052, AB245374
environmental Flexibacter sp. clone TM7_33, DQ279363
Flavobacterium ferrugineum ATCC 13524T, M28237 M62798
environmental clone AT425_EubA6, AY053480
environmental clone JTB251, AB015265
Sayrsnyira grandis ATCC 23119T, M58795
environmental clone SHA-54, AJ249107
sediment isolate C1_B008, AF419690
Anaerophaga thermohalophila Fru22, AJ418048
Rikenella microfusus ATCC 29728 T, L16498
oral clone DA064, AY005071
Bacteroides sp. / Porphyromonas sp.
Bacteroides sp.
Prevotellaceae
0.10

Supplementary Figure 3. Neighbour joining phylogenetic tree of the *Bacteroidetes* recovered by cultivation of Jochberg soil samples collected in February 2002 and by cloning of environmental sequences obtained by real-time PCR from the same sample. Cultured phylotypes are shaded in grey and denoted by the suffix 'well' followed by the serial culture number. Cloned environmental sequences are shown in boxes and denoted by the suffix `clone´. See legend to Supplementary Fig. 1 for further explanations. Bar indicates 0.1 fixed point mutations per nucleotide base.

Supplementary Figure 4. Neighbour joining phylogenetic tree of the *Alphaproteobacteria* recovered by cultivation of Jochberg soil samples collected in February 2002. See legend to Supplementary Fig. 1 for further explanations. Bar indicates 0.1 fixed point mutations per nucleotide base.



Supplementary Figure 5. Neighbour joining phylogenetic tree of the *Betaproteobacteria* recovered by cultivation of Jochberg soil samples collected in February 2002. See legend to Supplementary Fig. 1 for further explanations. Bar indicates 0.1 fixed point mutations per nucleotide base.



Supplementary Figure 6. Neighbour joining phylogenetic tree of the *Gammaproteobacteria* recovered by cultivation of Jochberg soil samples collected in February 2002. See legend to Supplementary Fig. 1 for further explanations. Bar indicates 0.1 fixed point mutations per nucleotide base.

Chapter 4

Edaphobacter modestus gen. nov., sp. nov., and *Edaphobacter aggregans* sp. nov., two novel acidobacteria isolated from alpine and forest soils

4 *EDAPHOBACTER MODESTUS* GEN. NOV., SP. NOV., AND *EDAPHOBACTER AGGREGANS* SP. NOV., TWO NOVEL ACIDOBACTERIA ISOLATED FROM ALPINE AND FOREST SOILS

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4.1 Summary

The phylum *Acidobacteria* consists mostly of environmental 16S rRNA gene sequences and so far comprises only the four validly described species *Holophaga foetida*, *Geothrix fermentans*, *Acidobacterium capsulatum* and *Terriglobus roseus*. In the present study two different novel strains of acidobacteria were isolated. High-throughput enrichments were set up with the MicroDrop technique using an alpine calcareous soil sample and a mixture of polymeric carbon compounds supplemented with signal compounds. This approach yielded a novel, previously unknown acidobacterium strain Jbg-1. The second strain Wbg-1 was recovered from a coculture with a methanotrophic bacterium established from calcareous forest soil. Both strains represent members of subdivision 1 of the phylum *Acidobacteria* and are closely related to each other (98.0 % 16S rRNA gene sequence similarity). At a sequence similarity of 93.8-94.7 %, strains Jbg-1 and Wbg-1 are only distantly related to the closest described relative, *Terriglobus roseus*, and accordingly are described as members of the novel genus *Edaphobacter* gen. nov. Based on the DNA-DNA-similarity between strains Jbg-1 and Wbg-1 of 11.5-13.6% and their chemotaxonomic and phenotypic characteristics, the two strains are assigned to two separate species, *Edaphobacter modestus* sp. nov. with strain Jbg-1^T (= ATCC BAA-1329^T = DSM 18101^T) as the type strain, and *E. aggregans* sp. nov. with strain Wbg-1^T (= ATCC BAA-1497^T = DSM 19364^T) as the type strain. The two novel species are adapted to low carbon concentrations and to neutral to slightly acidic conditions.

4.2 INTRODUCTION

Based on analyses of 16S rRNA gene clone libraries, members of the phylum *Acidobacteria* on average represent a fraction of 20% of typical soil bacterial communities but in some cases can even contribute up to 51 or even 80% (Dunbar *et al.*, 1999; Janssen, 2006; Chan *et al.*, 2006). These culture-independent studies indicate that the diversity of this phylum is nearly as great as the diversity of the phylum *Proteobacteria* (Ludwig *et al.*, 1997; Hugenholtz *et al.*, 1998). In pronounced contrast to the high overall phylogenetic diversity, only four species, *Acidobacterium capsulatum* DSM 11244^T (strain 161^T) (Kishimoto *et al.*, 1991), *Holophaga foetida* DSM 6591^T (Liesack *et al.*, 1994), *Geothrix fermentans* DSM 14018^T (Coates *et al.*, 1999) and *Terriglobus roseus* KBS63^T (Eichorst *et al.*, 2007) have been validly described to date.

Within the acidobacteria, eight phylogenetic subdivisions were previously recognized (Hugenholtz *et al.*, 1998). Recently, the number of subdivisions was extended to 26 (Barns *et al.*, 2007). *Geothrix fermentans* and *Holophaga foetida* are representatives of subdivision 8. *G. fermentans* is a strictly anaerobic bacterium that oxidizes acetate and other simple organic acids with F(III) as the sole electron acceptor (Coates *et al.*, 1999). *H. foetida* is a strictly anaerobic demethylating homoacetogen that degrades aromatic compounds to acetate and is capable of transferring methyl groups from phenylmethylethers to sulfide, thus forming methanethiol and dimethyl sulfide (Bak *et al.*, 1992). *Acidobacterium capsulatum* and *Terriglobus roseus* are the sole validly described representatives of subdivision 1, additional aerobic chemoorganotrophic strains have been isolated, however (Sait *et al.*, 2002; Joseph *et al.*, 2003; Stevenson *et al.*, 2004; Eichorst *et al.*, 2007). In the soil environment, members of subdivisions 1, 4 and 6 are the most abundant groups of acidobacteria (Janssen, 2006). However, since *A. capsulatum* is an acidophile, which grows between 3.0 to 6.0, its physiology may not be representative of many other acidobacteria. Still, subdivision 1 acidobacteria are more abundant in clone libraries from acidic soils and members of this subdivision can be selectively cultivated on solid laboratory media at a low pH (Sait *et al.*, 2006). In the present study, two novel strains of acidobacteria were isolated from two different soil types. Both isolates are aerobic chemoheterotrophs, but phylogenetically only distantly related to *Terriglobus roseus* and *A. capsulatum*.

4.3 MATERIAL AND METHODS, RESULTS AND DISCUSSION

One soil sample was obtained from an alpine rendzina (mollisols: rendolls) located at an altitude of 1400 m on Jochberg (close to Kochel in southern Germany). The upper organic-rich A_h horizon extended over a depth of 22 cm. The top 3 cm were sampled in February 2002 at an *in*

situ temperature of -3°C. Most-probable-number cultures were set up in soil solution equivalent (SSE; pH 6.3; Angle *et al.*, 1991) buffered with 10 mM HEPES at a pH of 6.3, employing the high-throughput MicroDrop technique (Bruns *et al*., 2003b). As a carbon source, the medium was supplemented with a polymer mixture (Chin *et al*., 1999) containing pectin, chitin, soluble starch, cellulose, xylan and curdlan at a concentration of 0.1% (w/v) each. The medium was spiked with the inducer molecules cyclic AMP (cAMP), *N*-(oxohexanoyl)-DL-homoserine lactone (OHHL) and *N*-(butyryl)-DL-homoserine lactone (BHL), each at a final concentration of 10 μM (Bruns *et al*., 2003a). Each culture was inoculated with 50 bacterial cells and incubated at 15°C. Enrichments were screened for the presence of acidobacteria by group-specific PCR after lysis of the cells by six consecutive freeze and thaw cycles, employing primers 31F and 341r and the conditions described previously (Zul *et al.*, 2007). One of the positive enrichments was chosen to isolate strain Jbg-1 on agar solidified HD-medium (1:10 diluted; 0.05% casein peptone, 0.01% glucose, 0.025% yeast extract, w/v).

The second novel acidobacterium strain Wbg-1 was isolated from a protorendzina (leptosol) in a deciduous forest near Würzburg (Germany). The upper 8 to 13 cm of the A_h horizon were sampled in July 2001 (Knief *et al*., 2003) and soil crumbs were placed on agar plates of dilute ammonium mineral salts medium containing per liter: 0.1 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2$, 0.001 g sequestrene Fe (ethylenediaminedi(*o*-hydroxyphenylacetic) acid (Fe EDDHA)), 0.1 ml trace elements (Whittenbury *et al*., 1970) and 10 ml of sterile-filtered 100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0). Incubation at 20°C in a gas-tight chamber containing 20 % (v/v) methane in air yielded large, slimy, semitransparent masses composed primarily of the methane oxidizing proteobacterium *Methylocella silvestris* around the soil crumbs on the plates (Dunfield *et al*., 2003). After several transfers, colonies were streaked on R2A agar (Oxoid) for the isolation of accompanying bacteria. Among the latter the isolated strain Wbg-1 was identified as an acidobacterium by 16S rRNA gene sequencing.

Strains Jbg-1 and Wbg-1 grew on agar-solidified media as well as in liquid media. Similar to other subdivision 1 isolates, strains Jbg-1 and Wbg-1 grew rather slowly, with visible colonies appearing on agar plates only after 2-3 weeks of incubation. Purity of the cultures was checked by phase contrast microscopy. In addition, 16S rRNA gene fragments were amplified with primers GC357f and 907r and the fragments were subsequently separated by denaturing gradient gel electrophoresis (Muyzer *et al.*, 1997) to check for the presence of additional bacterial phylotypes in the cultures. In all cases, only a single band was detected, however.

In order to improve the growth rate of the isolates, different media were tested. In comparison to the SSE containing polymeric carbon compounds, which was used for isolation of strain Jbg-1, the growth rate could be doubled in SSE (pH 6.3) supplemented with 0.0025%

yeast-extract, 0.1% glucose and trace element solution SL10 (1ml·l^{-1}) (Widdel *et al.*, 1983) or in HD medium (1:10 diluted, containing 0.05% casein peptone, 0.01% glucose, 0.025% yeast extract, pH 7.0). No growth occurred in undiluted HD medium (0.5% casein peptone, 0.1% glucose, 0.25% yeast extract), LB medium (Miller, DIFCO), or *Planctomycetes* medium (Marine broth 2216; DIFCO). Therefore, 1:10 diluted HD-medium was applied for subsequent growth tests except for the investigation of carbon substrate utilization (see below). *A. capsulatum* strain 161^T was grown in DSM medium 269 (http://www.dsmz.de/microorganisms/media_list.php).

On plates solidified with agar (1.5%, w/v) or gellan gum (8 g·l^{-1} gellan gum; Sigma), strains Jbg-1 and Wbg-1 formed circular beige colonies. Colonies of strain Wbg-1 were highly cohesive. During exponential growth, cells of strain Jbg-1 were 1.0 - 1.8 μm long and 0.5 - 0.7 μm wide; those of strain Wbg-1 measured 1.5 - 2.1 μm in length and 0.7 - 0.9 μm in width (Fig. 1). Cells of *Terriglobus roseus* KBS63^T and *A. capsulatum* strain 161^T have a similar morphology (Table 1). Whereas cells of strain Jbg-1 were nonmotile at neutral pH, the majority of cells were found to be motile in cultures growing below a pH of 5.5. In contrast, cells of strain Wbg-1 were always nonmotile and formed cell aggregates in liquid media. Like *T. roseus* but unlike *A. capsulatum*, cells of strains Jbg-1 and Wbg-1 did not form capsules as tested by negative staining with India ink (Bast, 2001). The presence of poly-β-hydroxybutyrate (PHB) granules was investigated by epifluorescence microscopy after Nile blue A staining according to Ostle and Holt (1982). PHB could neither be detected in strains Jbg-1 and Wbg-1 nor in *Acidobacterium capsulatum*.

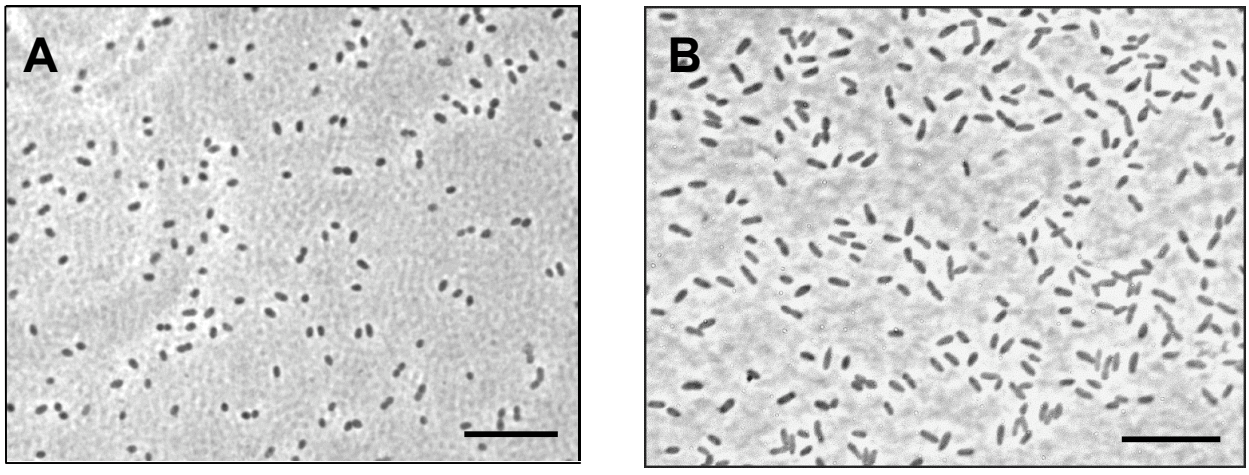

Figure 1. Phase-contrast photomicrographs of exponentially growing cells of **A.** strain Jbg-1^T and **B.** strain Wbg-1^T. Bars, 10 μm.

Table 1. Morphological, biochemical and physiological characteristics of strains Jbg-1^T and Wbg-1^T as compared to *Acidobacterium capsulatum* 161^T and *Terriglobus roseus* KBS63^T. Data for *Terriglobus roseus* taken from Eichorst *et al*. (2007) and data for *Acidobacterium capsulatum* taken from Kishimoto *et al*. (1991) and Eichorst *et al*. (2007).

Characteristic	Jbg-1^T	Wbg-1^T	*T. roseus* KBS63^T	*A. capsulatum* 161^T
Source of isolation	alpine soil	forest soil	agriculture soil	acidic mineral environments
Cell shape and size (µm)	short ovoid rods (1.0-1.8 x 0.5-0.7)	short ovoid rods (1.5-2.1 x 0.7-0.9)	short ovoid rods (0.9-1.3 x 0.5-0.7)	short ovoid rods (1.1-2.3 x 0.3-0.8)
Capsule formation	-	-	-	+
Motility	+	-	-	+
Cytochrome oxidase	+	-	-	-
pH range	4.5 – 7.0	4.0 – 7.0	5.0 – 7.0	3.0 – 6.0
pH optimum	5.5	5.5	6.0	5.0
Temperature range	15–30°C	15–37°C	12°C, 23°C	25-37°C
Temperature optimum	30°C	30°C	23°C	30°C
Major fatty acids	16:1 ω7c;15 iso 2OH 15:0 iso 16:0	17:1 ω8c 16:0 iso 17:0	15:0 iso 16:1 ω7c; 15 iso 2OH 16:0	15:0 iso 18:1 ω9c
G + C content of DNA (mol%)	55.8	56.9	59.8±0.5	59.7 - 60.8
Pigment	-	-	pink	orange
Utilization of				
Succinate	-	-	+	-
L(+)-Aspartate	-	+	ND	-
L(+)-Glutamate	+	+	ND	-
L(+)-Glutamine	+	+	ND	-
L(+)-Ornithine	-	+	ND	-
L(+)-Arabinose	+	-	+	+
D(+)-Cellobiose	-	-	+	+
D(-)-Fructose	+	-	+	+
D(+)-Galactose	-	-	+	+
D(+)-Glucosamine	+	-	ND	+
D(-)-Lactose	+	+	ND	+
D(-)-Lyxose	+	-	ND	-
D(+)-Maltose	-	-	+	+
D(+)-Mannose	-	-	+	+
D(+)-Trehalose	+	-	ND	+
D(+)-Raffinose	-	-	+	ND
D(+)-Xylose	+	-	+	+
D-Gluconic acid	-	-	+	ND
D-Glucuronic acid	-	+	+	+
L(+)-Rhamnose	+	-	ND	+
D-Sorbitol	+	-	-	-
L(+)-Lyxitol	+	-	ND	-
D-Mannitol	+	-	-	-
myo-Inositol	+	-	ND	-
Xylitol	+	-	ND	-
Casamino acids (0.01%, w/v)	+	+	ND	+
Yeast extract (0.01%, w/v)	+	+	ND	+
Peptone (0.01%, w/v)	+	+	ND	+

Cell shape and size were determined in HD (1:10) medium. All strains tested gram-negative and positive for catalase.
If not indicated otherwise, substrates were tested at concentrations of 5 mM. Substrates utilized by all four strains: D(+)-glucose. Substrates tested but not utilized for growth by Jbg-1 and Wbg-1: acetoin (10 mM), N-acetyl glucosamine, adipate, L(+)-alanine, D(-)-arabinose, L(+)-ascorbate, L(+)-asparagine, benzoate (2 mM), 1,2-butandiol, 2,3-butandiol, butanol, butyrate (2.5 mM), caproate, caprylate, crotonate, L(+)-cysteine, dulcitol, ethylene glycol, D(-)-erythrose, erythrulose, D(+)-fucose, fumarate, glycollate, glyoxylate, heptanoate, L(+)-histidine, α-hydroxybutyrate (2.5 mM), β-hydroxybutyrate (2.5 mM), γ-hydroxybutyrate (2.5 mM), isobutyrate (2.5 mM), L(+)-isoleucine, isovalerate (2.5 mM), isocitrate, L(+)-leucine, levulinate, L(+)-lysine, malate, malonate, D(+)-melizitose, methanol, L(+)-methionine, α-oxoisocaproate, α-oxo-D-gluconate, α-oxoglutarate, α-oxovalerate, oxaloacetate, L(+)-phenylalanine, L(+)-proline, 1,2-propandiol, propanol, propionate, protocatechuate, ribitol, L(+)-serine, L-sorbose, shikimate, tartrate (2 mM), L(+)-threonine, trimethoxybenzoate (2 mM), L(+)-tryptophane, L(+)-tyrosine, valerate, L(+)-valine, fermented rumen extract. Substrates tested but not utilized by all three strains: acetate, L(+)-arginine, citrate (2 mM), ethanol, glycerol, L(+)-glycine, lactate (10 mM). Substrates tested but not utilized by Jbg-1, Wbg-1 and *Terriglobus roseus* KBS63^T: pyruvate, formate, maleic acid. Substrates tested but not utilized by Jbg-1, Wbg-1 and *A. capsulatum* 161^T: L(+)-arginine, glycerol, L(+)-glycine. Substrates tested but not utilized by all four strains: acetate, citrate, lactate. ND, not determined.

Cells of strains Jbg-1 and Wbg-1 stained Gram-negative (Gerhardt, 1994) like *T. roseus* and *A. capsulatum*. The Gram-negative cell-wall structure was confirmed by transmission electron microscopy of cells harvested in the exponential growth phase (Fig. 2). Cells were fixed with 2.5% glutardialdehyde in 75 mM cacodylate / 2 mM $MgCl_2$ (pH 7.0) post-fixed with 1% osmium tetroxide buffer and then stained *en bloc* with uranyl acetate in 20% acetone. Dehydration was performed with a graded acetone series. Samples were then infiltrated, embedded in Spurr's low viscosity resin (Spurr, 1969) and ultrathin sections (50 and 70 nm) were cut with a diamond knife and mounted on uncoated copper grids. The sections were post-stained with aqueous lead citrate (100 mM, pH 13.0). All micrographs were taken with an EM 912 Zeiss electron microscope equipped with an integrated OMEGA energy filter operated with zero loss mode. In addition to the structure of the cell envelope, electron microscopy revealed the presence of thin extracellular fibres and tubular structures and an electron-dense center of the bacterial cells (Fig. 2).

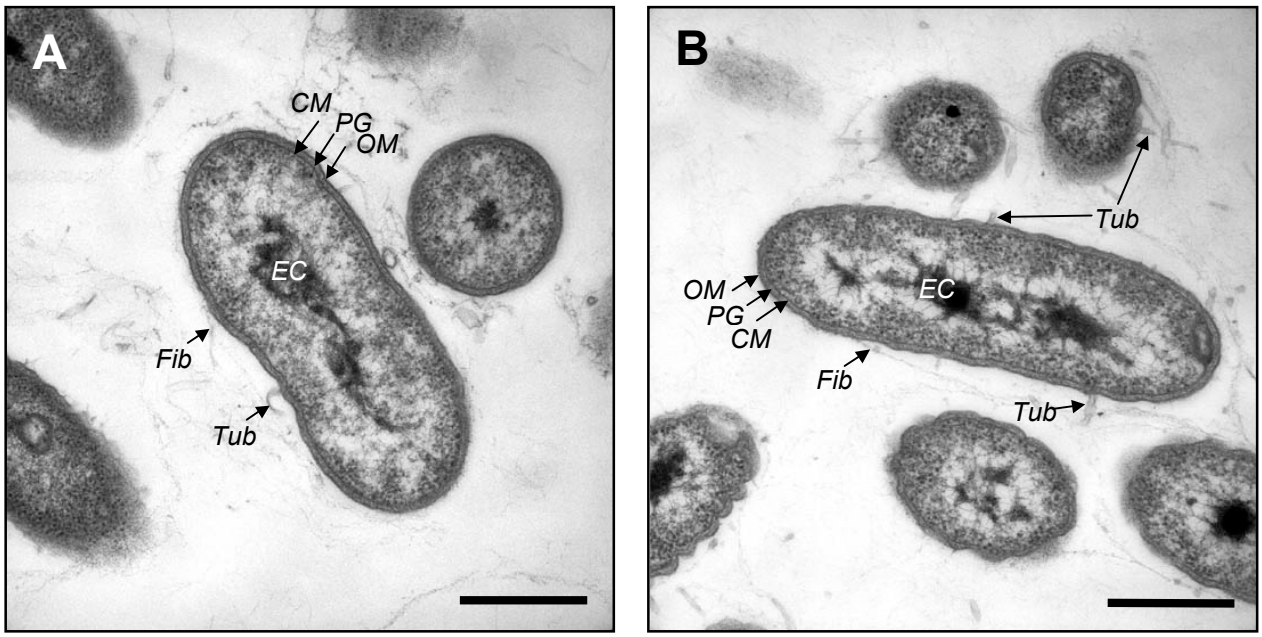

Figure 2. Electron micrographs of longitudinal and transverse thin sections of cells of **A** strain Jbg-1^T and **B** strain Wbg-1^T. The outer membrane (*OM*), cytoplasmic membrane (*CM*) and peptidoglycan layer (*PG*) can be distinguished. In addition, cells bear extracellular tubular structures (*Tub*), small fibres (*Fib*) and an electron dense center (*EC*). Bars indicate 0.5 μm.

For the analysis of the cellular fatty acid composition, both strains Jbg-1 and Wbg-1 were grown at 25°C on agar solidified HD medium (1:10 diluted), which ensured optimum growth conditions (see above). Forty mg (wet weight) of cells were scraped from Petri dishes and the fatty acid methyl esters were extracted using the method of Miller (1982) and Kuykendall *et al*. (1988). Analyses were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Strain Jbg-1 contained summed feature 1 (16:1 ω7c and 15 iso 2OH) as major fatty acids (Table 1, Supplementary Table 1). 15:0 iso and 16:0 were the secondary fatty acids. In the case of strain Wbg-1, 17:1 ω8c was the dominant fatty acid, and 16:0 iso and 17:0 were less common. Based on their fatty acid profiles, a chemotaxonomic classification of the isolates Jbg-1 and Wbg-1 to a known taxon was not possible. The major fatty acids of *A. capsulatum* strain 161^T are 15:0 iso and, in addition, 18:1 ω9c. Furthermore, the fatty acid profile of the recently described *Terriglobus roseus* showed distinct differences especially to strain Wbg-1.

The capability to utilize different organic carbon substrates was tested in two parallels in SSE (pH 6.3) supplemented with 0.0025% (w/v) yeast-extract and with the trace element solution SL10 (1 ml·l^{-1}), and buffered with 10 mM HEPES. A spectrum of 104 different carbon sources, comprising organic acids, amino acids, sugars, complex substrates, ketoacids and alcohols were tested at concentrations between 2.5 to 10 mM (Table 1). Strains Jbg-1 and Wbg-1 as well as *A. capsulatum* clearly preferred sugars as growth substrates and utilized glucose and

lactose as sole carbon and energy source. The capacity to utilize amino acids was very restricted. Growth substrates specific for strain Jbg-1 were the sugar lyxose and the sugar alcohols sorbitol, mannitol, myo-inositol, lyxitol and xylitol. Strain Wbg-1 exhibited the most narrow substrate spectrum (Table 1), but unlike Jbg-1 and *Acidobacterium capsulatum* utilized aspartate and ornithine. Strains Jbg-1 and Wbg-1 were capable of growing with glutamate and glutamine in contrast to *A. capsulatum*. None of the organic acids and oxo acids tested was utilized. Finally, complex carbon sources like peptone and casamino acids promoted growth, albeit only at low concentrations. By their inability to utilize cellobiose, galactose, maltose and mannose, our two isolates can be readily distinguished from *Terriglobus roseus* and *A. capsulatum*. Furthermore, strains Jbg-1 and Wbg-1 differ from *Terriglobus roseus* by their inability to grow on succinate, raffinose, and gluconic acid (Table 1).

Catalase and oxidase tests were carried out by standard methods (Gerhardt, 1994). Strains Jbg-1, Wbg-1, *T. roseus* and *A. capsulatum* tested positive for catalase whereas only strain Jbg-1 contained a cytochrome *c* oxidase (Table 1). Anaerobic growth was tested using 1:10 diluted HD-medium prepared under a nitrogen atmosphere in serum flasks. Before autoclaving, the medium and the headspace were flushed for 10 min with dinitrogen to replace the air. Nitrate respiration was examined in the same medium by adding 5 mM KNO_3 to the serum bottles. These growth experiments indicated that both novel isolates, like *T. roseus* and *A. capsulatum*, are obligately aerobic and incapable of nitrate respiration.

The optima for temperature were investigated by growth experiments in 1:10 diluted HD-medium at pH values between 3.0 and 9.0 and temperatures between 4 and 45°C. Growth was monitored measuring optical density at 580 nm. Strain Jbg-1 did not grow above 30°C whereas strain Wbg-1 did not grow above 37°C. The temperature optimum was at 30°C. Dependence on the pH was determined in media buffered with MES (≤6.5) or HEPES (≥6.5). The pH range of growth was 4.5 to 7.0 for strain Jbg-1 and 4.0 to 7.0 for strain Wbg-1, with an optimum of both strains at pH 5.5. Our results are in line with the recent suggestion that moderately acidic pH-values in general may favor the growth of members of subdivision 1 of the phylum *Acidobacteria* (Sait *et al*., 2002, 2006). In contrast to our present data, however, results of the previous study indicated that other acidobacteria of subdivision 1 exhibit a somewhat lower pH optimum between pH 4.0 and 5.0 and an upper limit of growth at a pH of 6.5.

We tested the growth of strains Jbg-1 and Wbg-1 in Hungate-tubes under an atmosphere of synthetic air supplemented with 5% CO_2. No difference in growth rates could be observed compared to medium saturated with synthetic air alone, indicating that our isolates do not require elevated CO_2 partial pressures.

The activity of 19 different enzymes was tested employing API ZYM galleries (API systems; bioMérieux) in two replicates (Supplementary Table 2). For comparison, *A. capsulatum* strain 161^T was tested along with the two new isolates. Whereas earlier studies (Kishimoto *et al*. 1991) had indicated that *A. capsulatum* strain 161^T contains most of the enzymes tested with the exception of cystyl arylamidase, our API ZYM tests yielded a significantly lower number of enzyme activities for this strain (Supplementary Table 2). *A. capsulatum* strain 161^T exhibits acid phosphatase activity but not alkaline phosphatase activity. Strains Jbg-1 and Wbg-1 possess alkaline phosphatase activity in addition to acid phosphatase activity, which might represent an adaptation to soils with neutral pH. Whereas all three strains tested positive for most glycosidases, cells of strains Jbg-1 and Wbg-1 synthesize a wider variety of esterases and amidases than does A. *capsulatum* strain 161^T. These include leucyl amidase, valyl amidase and α-chymotrypsin (Supplementary Table 2).

The G + C content of the genomic DNA was analyzed by the DSMZ according to Mesbah *et al*. (1989). The molecular G + C content of genomic DNA of strain Jbg-1 was 55.8 mol% and that of strain Wbg-1 56.9 mol%. These values are distinctly different from those of four *Terriglobus* strains (59.8 ± 0.5 mol%) (Eichorst *et al.*, 2007) and those of eight strains of *A. capsulatum* (59.9 - 60.8 mol%) (Kishimoto *et al*., 1991) (Table 1).

For phylogenetic classification, the nearly complete 16S rRNA genes of strains Jbg-1 and Wbg-1 were sequenced after amplification with the primer set 8f/1492r (Lane, 1991) and employing the seven primers 8f, 341f, 517r, 907r, 926f, 1055r and 1492r (Lane, 1991; Amann *et al*., 1995; Muyzer *et al*., 1997) for double pass sequencing. The 16S rRNA gene sequences of the closest relatives were retrieved by a BLAST search (Altschul *et al.*, 1997) and the sequences were added to the ARB database (Ludwig *et al*., 2004), using only sequences longer than 1300 bp. The Fast Aligner V1.03 tool was used for automated alignment. Pairwise phylogenetic distances were calculated using the DNADIST program of the PHYLIP package (Felsenstein, 1989). The resulting alignments were then corrected based on 16S rRNA secondary structure information. Positions at which the majority of the sequences contained gaps or uncertainties were filtered out. The final alignment comprised *E. coli* positions 44 through 1477 with a total of 1411 valid positions. A phylogenetic tree was constructed using neighbor joining (Fig. 3) and confirmed using the maximum likelihood algorithm (Fast DNA_ML, not shown). Both treeing methods yielded similar results. Bootstrap values were calculated from 100 bootstrap resamplings generated with the SEQBOOT and CONSENSE programs of the PHYLIP package.

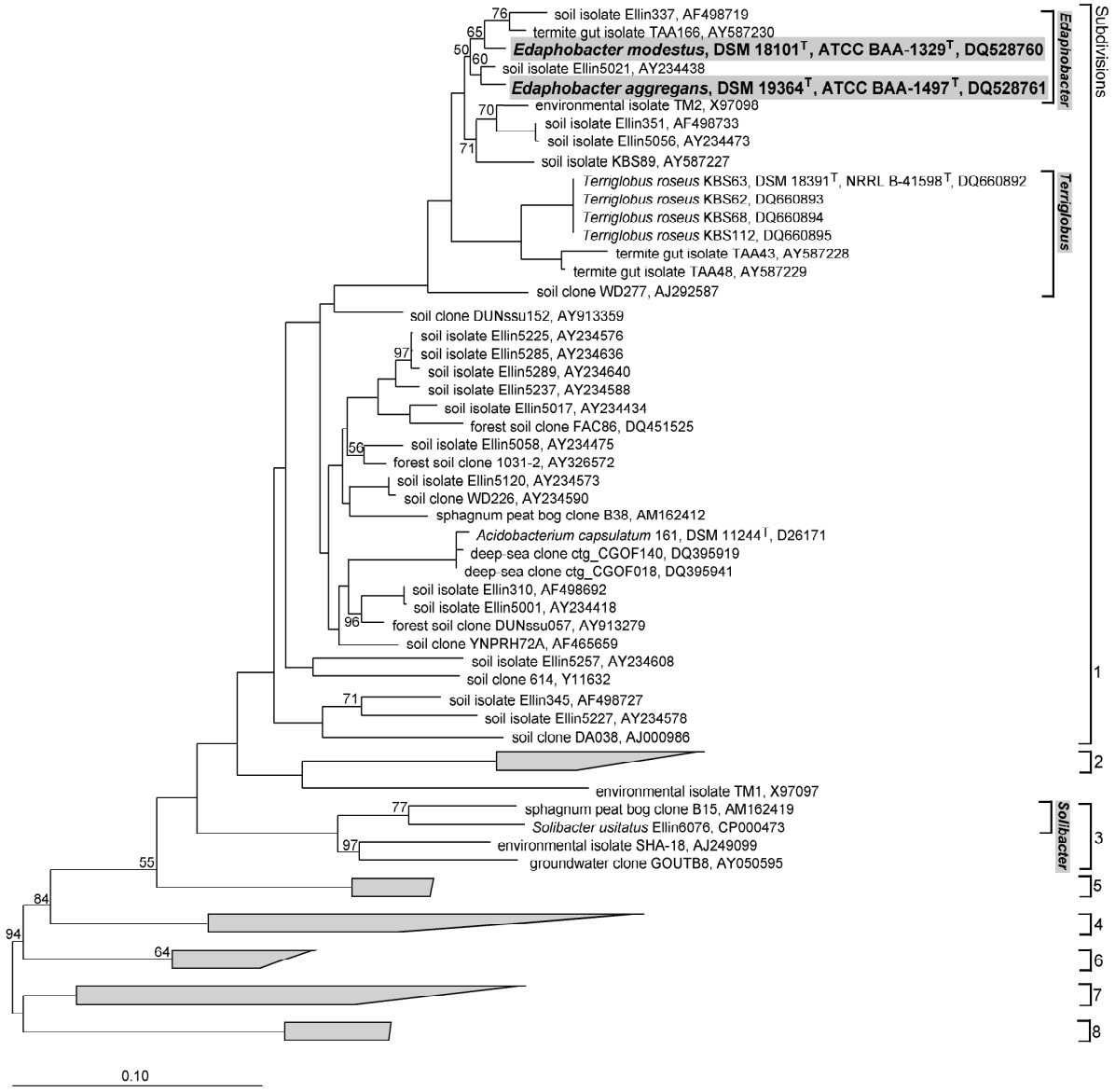

Figure 3. Affiliation of strains Jbg-1^T and Wbg-1^T calculated on the basis of 16S rDNA sequence information using the neighbour joining algorithm. Subdivisions of acidobacteria as defined by Hugenholtz *et al*. (1998) are given by arabic numerals on the right. For better clarity, only the phylogenetically most divergent members of each of the subdivisions 2 to 7 are depicted in this tree. Bootstrap values were calculated from 100 bootstrap resamplings; only values ≥50% are depicted. Bar indicates 0.1 fixed point mutations per nucleotide.

Numerous 16S rRNA gene sequences of environmental clones of closely related acidobacteria have accumulated in the Genbank database over the past years and the isolation of several strains of acidobacteria has been reported (Sait *et al.*, 2002; Joseph *et al.*, 2003; Stevenson *et al.*, 2004; Chan *et al.*, 2006; Eichorst *et al.*, 2007). To date, however, only the distantly related *A. capsulatum* strain 161^T and, recently, the novel genus *Terriglobus* with *T. roseus* as the type species and KBS63^T as the type strain (Eichorst *et al.*, 2007) have been validly described for subdivision 1 of the phylum *Acidobacteria*. 16S rRNA gene sequence comparisons between strains Jbg-1, Wbg-1, and the four available strains of *Terriglobus roseus*, revealed

phylogenetic distances between 93.8 and 94.7% (Fig. 3). Sequence similarities of *A. capsulatum* strain 161^T to strains Jbg-1 and Wbg-1 are only 91.1 and 92.8% (Fig. 3). Finally, the genus *Solibacter* has recently proposed (http://jgi.doe.gov). Our analyses demonstrated that strains Jbg-1 and Wbg-1 are phylogenetically very distantly related to *S. usitatus* Ellin6076 (Fig. 3) with sequence similarities of only 81.7 – 82.6%. Although the phylogenetic definition of a genus has been a matter of debate (Wayne *et al.*, 1987), a value of 95% sequence similarity has previously been suggested to delineate different prokaryotic genera (Ludwig *et al.*, 1998). Furthermore, bootstrap support for the separate cluster encompassing strains Jbg-1 and Wbg-1 and 3 additional sequences was high (Fig. 3, Supplementary Fig. 1). Based on our phylogenetic analysis of all related environmental clones and isolated strains, our novel isolates represent a distinct lineage which is well separated from members of the genus *Terriglobus*, *Acidobacterium* or *Solibacter* (Supplementary Fig. 1). The phylogenetic distance of strains Jbg-1 and Wbg-1 to members of the genus *Terriglobus* is commensurate with the differences in the GC-content and substrate utilization patterns. Based on these characteristics (Table 1, Supplementary Tables 1 and 2), the two strains Jbg-1 and Wbg-1 represent a new bacterial genus, which is described as *Edaphobacter* gen. nov. In addition to the two novel isolates, the genus *Edaphobacter* at present comprises the two soil isolates Ellin5021 (Joseph *et al.*, 2003) and Ellin337 (Sait *et al.*, 2002) as well as isolate TAA166 from a termite hindgut (Stevenson *et al.*, 2004). Two other sequence clusters are suggested to represent the currently known phylogenetic breadth of the two genera *Terriglobus* and *Solibacter* (marked in Fig. 3).

According to our analysis, both novel isolates obtained in this study represent members of the subdivision 1 of the phylum *Acidobacteria*. The 16S rRNA gene sequence similarity between the two isolates Jbg-1 and Wbg-1 is 98.0%. Based on our phylogenetic analysis (Fig. 3), the other close relatives (Ellin5021, Ellin337, TAA166) exhibited sequence similarities of 98.6% to 97.5% to strains Jbg-1 and Wbg-1, indicating that additional members of the genus *Edaphobacter* exist in soil but also other environments like the termite hindgut.

Since the 16S rRNA gene sequence similarity between the two isolates was 98.0%, DNA-DNA-hybridization studies were performed in order to determine whether both isolates should be assigned to one species. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA-DNA-hybridization was carried out as described by De Lye *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-themostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA-DNA-similarity determined for strains Jbg-1 and Wbg-1 was 11.5-13.6%. Although the similarity of the 16S

rRNA gene sequences of strains Jbg-1 and Wbg-1 surpasses the value, which is commonly used for the delineation of a bacterial species (≥ 97% 16S rRNA gene sequence similarity) (Rosselló-Mora and Amann, 2001), the value of the DNA-DNA-similarity ranges significantly below the accepted criterion (≥ 70%) (Rosselló-Mora and Amann, 2001). Similarly, the chemotaxonomic (Supplementary Table 1) and phenotypic (Table 1) characteristics of the two isolated strains differ significantly, supporting an assignment of the two strains to two separate species which are described as *Edaphobacter modestus* gen. nov., sp. nov. with strain Jbg-1^T as the type strain, and *E. aggregans* sp. nov. with strain Wbg-1^T as the type strain. *Edaphobacter modestus* is described as the type species of the novel genus *Edaphobacter*.

4.4 DESCRIPTION ON THE GENUS AND SPECIES LEVEL

4.4.1 Description of *Edaphobacter* gen. nov.

Edaphobacter (Ed'a.fo.bac'ter. Gr. neut. n. *edaphos* soil; *bacter* N. L. masc. n. (equivalent of Gr. neut. n. *baktron*), a short rod; *Edaphobacter* N.L. masc. n. rod-shaped soil bacterium)

Cells are non-spore-forming short ovoid rods that do not form a capsule and stain Gram-negative. One of the known strains is motile at pH values ≤ 5.5. Cells multiply by binary fission and form circular beige colonies on agar plates. Do not form polyhydroxybutyrate granules. Catalase positive. Fermentation or anaerobic growth with nitrate is not detected. Typical growth substrates are glucose, lactose, glutamate and glutamine. Grow well in media containing 0.05% casein peptone, 0.01% glucose and 0.025% yeast extract. 16S rRNA gene sequence information places the genus within subgroup 1 of the acidobacteria. The G+C content of the genomic DNA is 55.8 -56.9% (determined by HPLC). Occurs typically in soils, but also in other environments like termite hindgut microbial communities.

The type species is *Edaphobacter modestus*.

4.4.2 Description of *Edaphobacter modestus* sp. nov.

Edaphobacter modestus (mo.des'tus. *modestus* L. masc. adj. moderate, adaptated to low substrate concentrations).

Rods are 1.0 - 1.8 μm long and 0.5 - 0.7 μm wide. General characteristics are those given in the description of the genus. Additional growth substrates are different sugars like arabinose, fructose, rhamnose, lyxose, xylose, trehalose, glucosamin, and the sugar alcohols sorbitol, lyxitol, mannitol, myo-inositol and xylitol. Do not use organic acids for growth. Cells exhibit enzymic activities for alkaline and acid phosphatases, naphthol-AS-BI-phosphohydrolase, esterases (C4 and C8), leucyl and valyl arylamidases, α-chymotrypsin, α- and β-glucosidase and α- and β-

galactosidase. Oxidase positive. Optimum conditions for growth are 30°C and a pH of 5.5. Major cellular fatty acids are summed feature 1 (16:1 ω7c and/or 15 iso 2OH) and 15:0 iso. The G+C content of the genomic DNA is 55.8 % (determined by HPLC).

The type strain is Jbg-1^T (= ATCC BAA-1329^T = DSM 18101^T), which was isolated from an alpine rendzina near Kochel, southern Bavaria, Germany.

4.4.3 Description of *Edaphobacter aggregans* sp. nov.

Edaphobacter aggregans (ag'gre.gans. L. v. *aggregare*, to flock or band together; L. pres. part. *aggregans* assembling, aggregating).

Rods are 1.5-2.1 μm long and 0.7-0.9 μm wide. General characteristics are those given in the description of the genus. Additional growth substrates are aspartate, ornithine and glucuronic acid. Does not grow on arabinose, fructose, rhamnose, lyxose, xylose, trehalose, glucosamin, sorbitol, lyxitol, mannitol, myo-inositol and xylitol or organic acids. The type strain grows in coculture with the methane oxidising proteobacterium *Methylocella silvestris*. Cells exhibit enzymic activities for alkaline and acid phosphatases, naphthol-AS-BI-phosphohydrolase, esterases (C4 and C8), leucyl and valyl arylamidases, α-chymotrypsin, α- and β-glucosidase and α- and β-galactosidase. Oxidase negative. Optimum conditions for growth are 30°C and a pH of 5.5. Major cellular fatty acids are 17:1 ω8c and 16:0 iso. The G+C content of the genomic DNA is 56.9 % (determined by HPLC).

The type strain is Wbg-1^T (= ATCC BAA-1497^T = DSM 19364 ^{T}), which was isolated from a protorendzina in a deciduous forest near Würzburg, northern Bavaria, Germany.

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4.7 SUPPLEMENTARY MATERIAL

Supplementary Table 1. Composition of fatty acids of strains Jbg-1^T and Wbg-1^T as compared to *Acidobacterium capsulatum* 161^T and *Terriglobus roseus* KBS63^T. Data for *A. capsulatum* and *T. roseus* were taken from Eichorst *et al*. (2007). The latter two were grown in a glucose-yeast extract medium, which contains similar constituents as the HD medium used for cultivation of Jbg-1^T and Wbg-1^T.

Fatty acids	Jbg-1^T	Wbg-1^T	KBS63^T	161^T
Saturated				
14 : 0	2.20	-	3.42	1.58
15 : 0	-	1.02	0.64	0.57
16 : 0	**6.26**	0.34	**7.77**	4.29
16 : 0 N alcohol	-	-	0.52	4.61
17 : 0	-	**8.10**	0.70	1.85
18 : 0	0.91	-	0.38	2.34
19 : 0	-	0.87	-	-
20 : 0	-	-	0.76	-
Unsaturated				
14 : 1 ω5c	2.76	-	1.07	-
15 : 1 ω6c	-	0.22	0.83	-
16 : 1 ω5c	-	-	0.43	-
16 : 1 iso H	-	1.22	-	-
17 : 1 ω6c	-	1.11	-	-
17 : 1 ω8c	-	**48.91**	-	3.97
18 : 1 ω5c	-	-	1.05	2.24
18 : 1 ω7c	-	-	-	0.45
18 : 1 ω9c	-	4.40	-	**21.21**
18 : 1 iso H	-	0.92	-	-
20 : 2 ω6,9c	-	-	0.67	-
Methyl-branched				
13 : 0 iso	-	-	4.02	-
14 : 0 iso	-	0.50	-	-
15 : 0 iso	**30.82**	0.96	**43.88**	**40.28**
15 : 0 anteiso	-	-	0.48	-
16 : 0 iso	-	**19.02**	-	-
17 : 1 iso ω9c	0.64	-	-	0.90
17 : 1 iso ω5c	-	-	4.24	7.40
17 : 0 iso	1.27	0.69	0.53	2.15
17 : 0 anteiso	-	-	0.27	0.49
17 : 0 10 methyl	-	3.49	-	-
18 : 0 iso	-	2.23	-	-
Hydroxy				
17 : 0 iso 3-OH	-	-	-	0.77
Summed feature[1]				
1	**55.14**	1.15	**27.08**	4.30
2	-	4.85	-	-
3	-	-	0.59	0.32

[1] Summed feature represents a group of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contained one or more of the following fatty acids: 16:1 ω7c / 15 iso 2OH. Feature 2 contained one or more of the following fatty acids: 19:1 ω11c / 19:1 ω9c. Feature 3 contained 15:1 iso H / 13:0 3-OH. -, not detected.

Supplementary Table 2. Enzymes detected in strains Jbg-1^T, Wbg-1^T and *Acidobacterium capsulatum* 161^T.

Results of API ZYM test. +, strong positive reaction; (+) weakly positive reaction; -, no enzyme activity detected.

Enzyme assay	Jbg-1^T	Wbg-1^T	161^T
Alkaline phosphatase	+	+	-
Acid phosphatase	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+
Esterase (C 4)	+	+	+
Esterase Lipase (C 8)	+	+	-
Lipase (C 14)	-	-	-
Leucyl arylamidase	+	(+)	-
Valyl arylamidase	+	(+)	-
Cystyl arylamidase	-	-	-
Trypsin	-	-	-
α-Chymotrypsin	+	(+)	-
α-Galactosidase	+	+	(+)
β-Galactosidase	+	+	+
β-Glucuronidase	+	+	+
α-Glucosidase	+	(+)	(+)
β-Glucosidase	+	(+)	+
N-Acetyl-β-glucosaminidase	-	+	+
α-Mannosidase	-	-	-
α-Fucosidase	+	(+)	-


soil isolate Ellin337, AF498719
termite gut isolate TAA166, AY587230
Edaphobacter modestus, DSM 18101T , ATCC BAA-1329T , DQ528760
soil isolate Ellin5021, AY234438
Edaphobacter aggregans, DSM 19364T, ATCC BAA-1497T , DQ528761
soil clone WD243, AJ292579
soil clone cw7, AY850302
environmental isolate TM2, X97098
soil isolate Ellin351, AF498733
soil isolate Ellin5056, AY234473
soil isolate KBS89, AY587227
Terriglobus roseus KBS63, DSM 18391T, NRRL B-41598T, DQ660892
Terriglobus roseus KBS62, DQ660893
Terriglobus roseus KBS68, DQ660894
Terriglobus roseus KBS112, DQ660895
termite gut isolate TAA43, AY587228
termite gut isolate TAA48, AY587229
termite gut clone Rs-M39, AB089126
acid mine clone TRB82, AF047646
soil clone WD228, AJ292578
soil clone WD277, AJ292587
soil clone DUNssu152, AY913359
soil isolate Ellin5225, AY234576
soil isolate Ellin5285, AY234636
soil isolate Ellin5289, AY234640
soil isolate Ellin5237, AY234588
forest soil clone BS66, AY963502
soil clone WD213, AJ292574
soil isolate Ellin5017, AY234434
forest soil clone FAC86, DQ451525
forest soil clone DUNssu124, AY913329
soil clone UA2, AF200698
sphagnum peat bog clone B89, AM162411
soil isolate Ellin5058, AY234475
forest soil clone 1031-2, AY326572
forest soil clone AS21, AY963390
sphagnum peat bog clone B116, AM162408
sphagnum peat bog clone B140, AM162409
soil isolate Ellin5120, AY234573
soil clone WD226, AY234590
sphagnum peat bog clone K-5b10, AF524859
sphagnum peat bog clone B45, AM162410
sphagnum peat bog clone B38, AM162412
forest soil clone DUNssu021, AY913242
forest soil clone BS25, AY963463
soil clone WD226, AJ292577
Acidobacterium capsulatum 161, DSM 11244T, D26171
deep-sea clone ctg_CGOF140, DQ395919
deep-sea clone ctg_CGOF018, DQ395941
soil isolate Ellin310, AF498692
soil isolate Ellin5001, AY234418
forest soil clone DUNssu057, AY913279
soil clone YNPRH72A, AF465659
forest soil clone DUNssu190, AY913396
forest soil clone BS67, AY963503
soil clone DUNssu017, AY913238
soil isolate Ellin5257, AY234608
soil clone 614, Y11632
pasture soil clone EB1005, AY395324
soil clone UA3, AF200699
forest soil clone FAC9, DQ451448
forest soil clone BS51, AY963488
peat bog clone K-5b2, AF524860
soil isolate Ellin345, AF498727
soil isolate Ellin5227, AY234578
forest soil clone 1209-2, AY326560
alpine soil clone C10-WMSP1, DQ450710
soil clone DA038, AJ000986
environmental isolate TM1, X97097
sphagnum peat bog clone B15, AM162419
Solibacter usitatus Ellin6076, CP000473
environmental isolate SHA-18, AJ249099
groundwater clone GOUTB8, AY050595
0.10
Edaphobacter
Terriglobus
Solibacter
Subdivisions
1
2
3
5
4
6
7
8

Supplementary Figure 1. Affiliation of strains Jbg-1^T and Wbg-1^T calculated on the basis of 16S rDNA sequence information using the neighbour joining algorithm. Subdivisions of acidobacteria as defined by Hugenholtz *et al*. (1998) are given by arabic numerals on the right. For better clarity, only the phylogenetically most divergent members of each of the subdivisions 2 to 7 are depicted in this tree. Bootstrap values were calculated from 100 bootstrap resamplings; only values ≥50% are depicted. Bar indicates 0.1 fixed point mutations per nucleotide.

Chapter 5

Significance of inducers and bacterial interactions for the cultivation of soil bacteria

5 SIGNIFICANCE OF INDUCERS AND BACTERIAL INTERACTIONS FOR THE CULTIVATION OF SOIL BACTERIA

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5.1 SUMMARY

The cultivation approach of an alpine soil sample revealed an unexpected high percentage of interspecific interactions in the cultures. Therefore, the effect of different treatments on the number of bacteria in the co-cultures and their phylogenetic composition was investigated in the present study. In order to estimate the number of bacteria capable of dividing in a particular medium, the high-throughput MicroDrop cultivation technique was combined with universal and phylogenetic group-specific PCR/DGGE fingerprinting. Different types of carbon compounds, including humic acid analogs, a mixture of polymers and artificial root exudates were used as substrates in the media. A total of 81 bacterial cultures were obtained. Subsequent phylogenetic analyses revealed that most of the cultures obtained were in fact co-cultures, whereas only 16 of the 70 cultures with artificial root exudates consisted of single cultivated strains. Due to the high frequency of occurrence of different bacteria in the same cultures, the actual cultivation success was 4.9 fold higher than the value calculated from the abundance of positive cultures. In order to differentiate between free-living and attached cells, bacteria were detached from soil particles and used to set up parallel incubations. The detachment from soil particles prior to inoculation had no effect on the cultivation success. However, addition of signal compounds (cyclic AMP and *N*-butyryl homoserine lactone) yielded different types of activated bacteria and enhanced the total number of phylotypes per co-culture towards 4, 5, 6, and 7 different bacteria. The major part of the single cultivated strains represented a single phylotype, which was related to *Sphingoterrabacterium pocheensis*. In contrast, most co-cultures contained members of the *Alpha-* and *Betaproteobacteria* whereas relatives of *Phyllobacterium brassicacearum*, *Rhodospirillum rubrum*, *Inqulinus ginsengisoli*, *Delftia tsuruhatensis*, and *Rhodocyclus tenuis* were the most abundant ones.

5.2 INTRODUCTION

Genetic analyses have shown a large diversity of soil bacteria. Per 10 or 30 g of soil DNA, reassociation experiments revealed between 4,000 to 10,000,000 genome equivalents (Dykhuizen, 1990; Torsvik *et al*., 1990; Øvreås *et al.*, 1998; Torsvik and Øvreås, 2002; Sandaa *et al*., 1999; Gans *et al*., 2005). However, traditional methods of bacterial cultivation typically recovered less than 1% of the total bacterial species present in soil (Amann *et al*., 1995). As follows, only a tiny amount about their functional diversity is known to date. Considering the extent of functional diversity already described for microbes and the numerous applications of their secondary metabolites, the biotechnological potential hidden among 99% of the bacteria in soil that are nonculturable is immense. Therefore, novel approaches to culture these nonculturable bacteria are necessary because the isolation of tangible microorganisms is still a prerequisite for a functional understanding of soil bacteria (Kamagata and Tamaki, 2005).

Usually it is assumed that each bacterial cell can multiply independently of other bacteria in a pure culture. However, there is increasing evidence that bacteria need to interact with each other for growth (Kaprelyants and Kell, 1996). Consequently, the typically low cultivation success in artificial media may largely be due the lack of cell-to-cell interaction (Mukamolova *et al*., 1998; Bruns *et al*., 2002; Bruns *et al*., 2003a). Particular signal molecules, like the well-studied acyl homoserine lactones (acyl-HSLs) function as autocrine molecules in the quorum sensing of numerous Gram-negative bacteria (D. Kirke, The quorum sensing site [http://www.nottingham.ac.uk/quorum/ table.htm], 2001) and trigger processes like resuscitation from the lag phase (Batchelor *et al*., 1997), bioluminescence (Kaplan and Greenberg, 1985), and virulence (Swift *et al*., 1999). Interestingly, recent studies already determined that the addition of acyl-HSLs to growth media significantly enhance the cultivation success of natural bacterial communities (Bruns *et al*., 2002; Bruns *et al*., 2003a).

Cyclic AMP (cAMP) represents another signal molecule, which has also been shown to enhance culturability of freshwater and marine bacterioplankton (Bruns *et al*., 2002; Bruns *et al*., 2003a). cAMP is involved in the regulation of different genes, such as those for chemotaxis (Feit *et al*., 2007), catabolite repression (Botsford and Harman, 1992) and starvation (Schultz *et al*., 1988). It has been suggested that cAMP enhances the cultivation success by preventing substrate-accelerated death in starved cultures (Calcott and Postgate, 1972). So far, the effects of signal molecules as BHL and cAMP on the cultivation success of soil bacteria have not been investigated in a systematic manner.

Therefore, the present study assesses the relevance of different cultivation conditions on the cultivation success of an alpine soil bacterial community. The factors tested were (i) free-

living versus attached bacteria, (ii) different carbon substrates, (iii) different signal compounds, and (iv) co-cultivation with accompanying phylogenetically different bacteria.

5.3 MATERIAL AND METHODS

5.3.1 Sampling site and sampling processing

Soil cores were collected on February 8, 2002 from an alpine rendzina (mollisols: rendolls) located on Jochberg (close to Kochel in southern Germany) on a north-facing cliff at 1,400 m altitude as outlined before (Koch *et al*., 2008; Chapter 3). Soil homogenates were stored field moist at 4°C in the dark until used for cultivation. Soil parameters were determined as described previously (Chapter 3).

5.3.2 Total cell counts

Soil subsamples were suspended in sterile filtered (0.1-μm-pore-size polycarbonate filters; Millipore, Eschborn, Germany) tap water and fixed in 2% (vol/vol) glutaraldehyde. After fixation, soil slurries were stained with SYBR-Green-II (MoBiTec, Göttingen, Germany) and filtered onto black 0.1-μm-pore-size polycarbonate filters (Millipore). Total cell numbers were determined by epifluorescence counting as described previously (Bruns *et al*., 2002).

5.3.3 Cultivation of soil bacteria

The cultivation efficiency of the Jochberg soil bacteria was determined with three different carbon sources and was described previously (Chapter 3). SSE-medium (buffered with 10 mM HEPES; pH 6.7) was supplemented with either artificial root exudates (RO) containing a variety of sugars, alcohols, organic acids and amino acids at different concentrations (Kozdrój and van Elsas, 2000), or a mixture of humic acids (HA) containing abietin, 9,10-anthraquinone-2,6-disulfonic acid, benzoic acid, coumestrol, 3,5-dimethoxy-4-hydroxycinnamic acid, quercetin, sodium salicilate, and solanadine (each at 500 μM), or a polymer mixture (POL) containing cellulose, chitin, curdlan, pectin, soluble starch, and xylan (0.1% [wt/vol] each).

Signal molecules (Inducers, IND) were added in half of the cultures (homoserine lactones, HSL): cyclic AMP (cAMP) and *N*-(butyryl)-DL-HSL (BHL), 10 μM final concentration each (Bruns *et al*., 2003a). As a control (CON), a mixture of AMP, hexanoate, butyrate and HSL was used (each at 10 μM). Aliquots of 20 μl of the carbon sources and aliquots of 180 μl of the growth medium were distributed into sterile 96-well polysterene microtiter plates (Greiner, Frickenhausen, Germany), as described earlier (Chapter 3).

The MicroDrop microdispenser system (Bruns *et al*., 2003b; Gich *et al*., 2005) was used to inoculate large arrays of small cultures in microtiter plates, whereas the system provided a high number of replicates at a high dilution. At higher dilutions, the numerically dominant bacteria of the original sample are selected whose growth is inhibited at smaller dilutions (Jackson *et al*., 1998). Therefore, we estimated highly enriched or even pure cultures, at a much higher frequency than with conventional most probable number (MPN) dilutions (Bruns *et al*., 2003b). Employing the MicroDrop technique for inoculation, the soil suspensions had to be filtered (12-μm-pore-size nitrocellulose filters; Sartorius, Göttingen, Germany) prior to inoculation in order to avoid clogging of the microdispenser pipette of the MicroDrop apparatus with soil particles. Prior to filtration, prokaryotic cells were detached from soil particles to get as many as possible soil bacteria in the soil suspensions. Therefore, soil subsamples were suspended in SSE-medium and supplemented with 10 mM sodium pyrophosphate (PP) (Schinner *et al*., 1993) and Tween 80 (0.05 % (v/v)). Total cell counts of detached and non-detached soil bacteria were determined by epifluorescence counting. In order to obtain a sufficient number of positive wells containing highly enriched or even pure cultures of soil bacteria, each well was inoculated with diluted soil suspensions containing only 50 cells.

One half of the microtiter plate wells was inoculated with filtered soil suspensions, which contained detached soil bacteria and the other half with only filtered soil suspensions. A total set of 12 microtiter plates was included in the present study, representing 1008 individual growth tests. After 6 weeks of incubation at 15°C in the dark, growth was monitored by turbidity.

5.3.4 High-resolution phylogenetic fingerprinting of MicroDrop cultures

In order to analyze the large number of isolates generated efficient, rapid, and sensitive screening techniques are required. Fingerprinting of universal and phylogenetic group-specific 16S rRNA gene fragments by denaturing gradient gel electrophoresis (DGGE) was used, which has been proven to be an efficient approach for the rapid comparison of isolates with environmental sequences (Bruns *et al.*, 2002, 2003a, b; Jaspers and Overmann, 2004, Gich *et al*., 2005).

Cultures were initially screened by PCR with the conventional universal primer pair GC341f / 907r (Muyzer *et al*., 1995). For the subsequent detailed phylogenetic analyses of the cultivated bacteria, the cultures were also screened with group-specific primer-sets targeting six taxonomic groups of prokaryotes, which are known to be abundant in soil samples (Janssen, 2006): the Gram-positive bacteria with low G+C content (*Firmicutes*), Gram-positive bacteria with high G+C content (*Actinobacteria*), *Cytophaga-Flavobacterium-Bacteroides* (*Bacteroidetes*), *Alpha-* and *Betaproteobacteria*, and *Acidobacteria* (Chapter 3). The 16S rRNA

gene sequences were amplified in step-down PCR reactions as described previously (Overmann *et al*., 1999; Gich *et al*., 2005; Zul *et al*., 2007). The PCR products were separated with denaturing gradient gel electrophoresis (DGGE) as outlined before (Gich *et al*., 2005; Chapter 3).

5.3.5 Sequencing and phylogenetic analyses

For subsequent phylogenetic analyses of the cultivated prokaryotes, DGGE-bands of interest were excised. At least 1 µl of the eluate was reamplified using corresponding primers without a GC-clamp. After reamplification of the excised bands, the PCR products were purified with QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced as described earlier (Gich *et al*., 2005). Bands, which resulted in ambiguous sequences, were analyzed after cloning employing the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) previously described (Gich *et al*., 2005).

Finally, 16S rRNA sequences were added to an existing phylogenetic tree of the program package ARB (Ludwig *et al.*, 2004), together with the closest relatives according to the GenBank database employing BLAST 2.0.4 (Altschul *et al*., 1997). Sequences longer than 1,300 bp were used to perform the alignment. Phylogenetic trees were constructed with neighbor-joining as described previously (Gich *et al*., 2005). The tree topologies were evaluated with bootstrap resamplings (100 x) and maximum likelihood. The shorter sequences obtained from DGGE-bands were added with an *Escherichia coli* filter using the parsimony tool and the branch lengths were recalculated. Percentages of similarity of the shorter sequences to the closest relatives were calculated in a distance matrix using the neighbor-joining algorithm.

The culturability of the phylogenetic groups was calculated based on a binomial distribution (Button *et al*., 1993):

$$x = - \ln(1 - p)$$

$$CI_{95\%} = \pm 1.96 \cdot \sqrt{\frac{p}{n(1-p)}}$$

5.4 RESULTS AND DISCUSSION

5.4.1 Co-cultivation increases the cultivation success

A total of 81 bacterial cultures were obtained using the MicroDrop technique for cultivation of the Jochberg soil bacteria. The RO have been shown to be the best substrate for our cultivation approach, since the RO-cultures represented 87.7% (71 cultures) of the total number of overgrown wells. In contrast to previous results, neither HA nor POL showed a high number of overgrown wells (Davis *et al*., 2005). Only two and eight cultures were obtained using HA and POL, respectively, as carbon source. Based on the high cultivation success with RO, the present analysis focused on co-cultivation of soil bacteria and the effect of signal molecules on the cultivation success for the RO-cultures. The number of RO-cultures corresponded to a value of cultivation efficiency of about 0.44% (±0.05%).

In order to analyze the large number of isolates generated with the MicroDrop technique, the cultures were initially screened with the conventional eubacterial primer pair by PCR/DGGE (Fig. 1). Interestingly, it was shown that 18 of the cultures, corresponding to 25.4% of the positive wells, contained up to three different fingerprints. Most of these cultures were obtained using signal compounds as growth additives. Theoretically, one and the same bacterium may contain multiple rRNA operons and yield different fingerprints on DGGE-gels (Nübel *et al*., 1996). In our case, however, some of the fingerprints, which were observed to correlate with other fingerprints, were also found as sole fingerprints in other wells. Therefore it appeared more likely that different fingerprints represented co-cultures of different bacteria occurring together in one microtiter plate well as already previously observed (Bruns *et al*., 2003b). However, the previous study investigated the cultivation success of a bacterioplankton sample and different fingerprints were detected only in 6% of the total number of cultures generated with the MicroDrop technique (Bruns *et al*., 2003b). Therefore, one may assume that cultivation of soil bacteria with the MicroDrop technique using signal compounds as growth additives generally yield in cultures containing more than one bacterium.

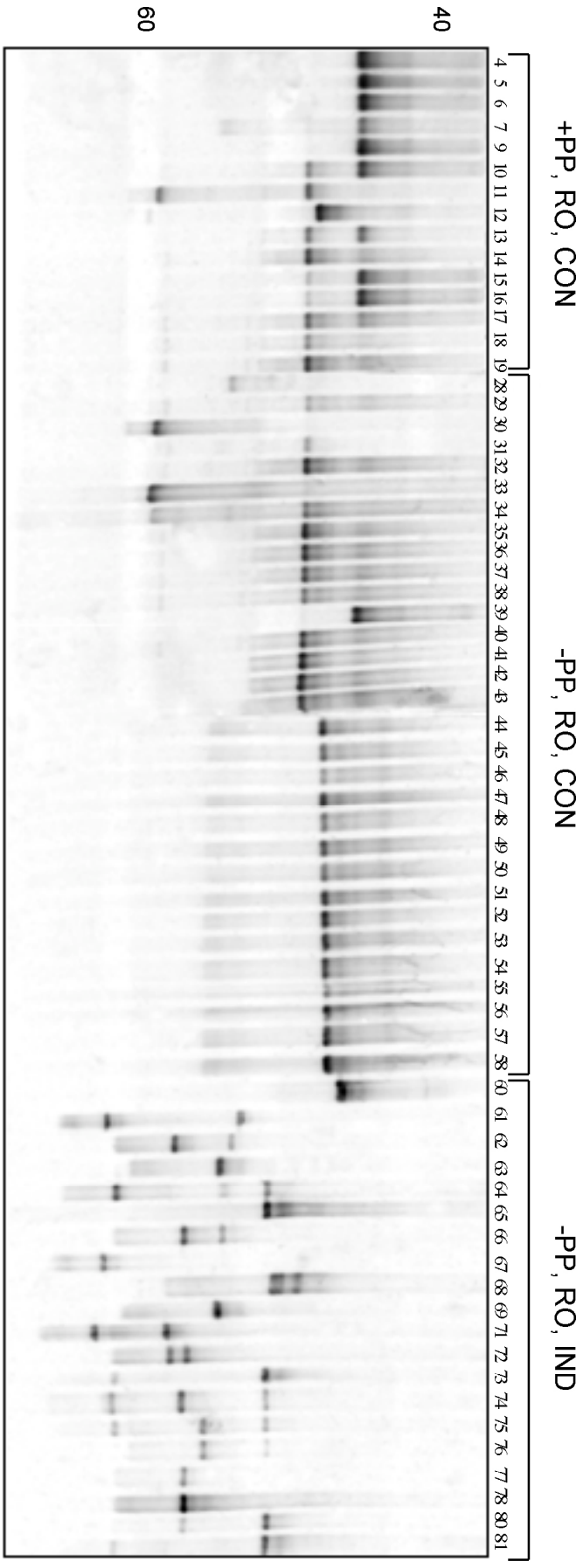
60
40
4 5 6 7 9 10 11 12 13 14 15 16 17 18 19
28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58
60 61 62 63 64 65 66 67 68 69 71 72 73 74 75 76 77 78 80 81
+PP, RO, CON
-PP, RO, CON
-PP, RO, IND

Figure 1. 16S rDNA gene fragments obtained in this study separated by universal DGGE-fingerprinting from different wells (numbers) exhibiting growth after inoculation with the MicroDrop technique (PP, pyrophosphate and Tween 80; RO, artificial root exudates; IND, inducer molecules; CON, control without inducer molecules. A negative image of a SYBRGold-stained gel is shown. Numbers on the left margin give percentages of concentrations of denaturant. Cultures of wells no. 8, 20, 59, 70, and 79 have not been analyzed with universal primers.

For subsequent phylogenetic analyses of the cultures, 67 bands of the eubacterial DGGE-gel were excised and sequenced. It was confirmed that most of the cultures, which initially showed more than one melting type actually contained more than one bacterium. A total of ten cultures were observed as so-called co-cultures, however, consisting of two different phylotypes at most. Most of these cultures contained two different phylotypes of the *Bacteroidetes*, close related to *Sphingoterrabacterium pocheensis* Gsoil 032 and *Flavobacterium* sp. WB 4.4-73 (well 10, 11, 13, 15; Chapter 2). The media with the signal compounds represented in fact no higher amount of co-cultures, but showed a higher variability within their composition (wells 61, 62, 71, 72, 74). The phylogenetic analyses showed that all cultures were differently composed of members of the *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria* and *Actinobacteria* whereas bacteria with the close relatives *Sinorhizobium* sp. TB2-10, *Lysobacter enzymogenes* OH11, *Variovorax* sp. C59, and *Agromyces cerinus* DSM 8595 were the most abundant ones (Chapter 3). These findings suggested that more bacteria were grown as primarily assumed, which increased the initially calculated cultivation success from 0.44% (±0.05%) to 0.51% (±0.06%).

In order to compare the resolution attained by the conventional DGGE method with eubacterial primers, fingerprint patterns were also generated from the cultures employing phylogenetic group-specific primers. By sequencing and analyzing the representative and discrete DGGE-bands, a more detailed database was generated for the identification the representative members of the soil microbial community among the cultured isolates. A total of 47 different phylotypes were detected for the Jochberg soil bacteria in media containing RO with phylogenetic group-specific primers (Table 1). Compared to the analysis with the eubacterial primers, the total number of phylotypes was 2.6 times higher. Hence, the phylogenetic diversity detected with the group-specific PCR/DGGE was considerably higher compared to the diversity detected by conventional fingerprinting. Initially, this indicated that the eubacterial primer-set underestimated the bacterial diversity. Already previous studies assumed that most species escape detection with eubacterial fingerprinting (Gich *et al*., 2005) because bacteria that constitute ≤9% of a microbial community are not detectable by conventional DGGE (Straub and Buchholz-Cleven, 1998). While it is proven that DGGE is useful in examining abundant populations, it may not detect minor populations (Jackson *et al*., 1998).

Table 1. Phylogenetic analyses of the co-cultures and the most frequent phylotypical interaction obtained from the February 2002 soil sample

(Sub)phylum		Total no. of phylotypes	Total no. of fingerprints	Frequency of single and co-cultivated fingerprints								Max. phylotypical interaction of total no. fingerprints (%)	
				1	2	3	4	5	6	7	8		
Firmicutes	(L)	5	16	0	0	1	1	5	5	3	1	A	33.3
Actinobacteria	(H)	8	15	0	0	3	2	5	2	3	0	B	40.4
Bacteroidetes	(C)	5	51	15	14	9	1	7	2	2	1	A, B	27.8
Alphaproteobacteria	(A)	14	64	0	16	8	6	11	13	8	2	B	28.3
Betaproteobacteria	(B)	6	49	1	4	5	7	19	5	6	2	A	29.1
Gammaproteobacteria	(G)	8	23	0	2	6	3	3	3	4	2	A	23.3
Acidobacteria	(Ac)	1	3	0	0	1	0	0	0	2	0	H, A	30.8
Sum		47	221	16	36	33	20	50	30	28	8	-	-
No. of cultures		-	-	16	18	11	5	10	5	4	1	-	-

-, not applicable

The total number of the co-cultures initially observed by conventional fingerprinting was confirmed by group-specific fingerprinting. However, the group-specific analysis revealed that most of the co-cultures were composed of an even higher number phylotypes. This indicated that the universal primer-set did not only underestimate the bacterial diversity but also falsely identified single cultivated strains. Whereas the maximum number of different phylotypes per cultures was determined as three when screened with universal primers, group-specific fingerprinting revealed the presence of up to eight different phylotypes in the same culture (Table 1). Based on group-specific fingerprinting, only 16 of the 71 cultures growing in the presence of RO consisted of single cultivated strains. The majority of the cultures, however, was assessed as 2-, 3- and 5-phylotype-cultures (sum, 39), whereas fewer cultures represented 4-, 6-, 7- and 8-phylotype cultures (sum, 15; Table 1). This indicated that co-cultivation was advantageous compared to single cultivation in the present study. The higher number of bacteria detected in the same wells with the group-specific analysis increased the already calculated cultivation success once again. A 1.2 fold increase was determined due to the initial conventional detection of co-cultures and the more detailed analysis of the cultures with group-specific fingerprinting induced a 4.0 fold increase to 2.14% (±0.15%).

Accordingly, our group-specific primer-sets have been shown to be more efficient recovering the composition of bacterial cultures, similar to previous studies with sediment and freshwater samples (Coolen and Overmann, 1998; Overmann *et al*., 1999; Gich *et al*., 2005). A high percentage of 88.7% of the totally detected phylotypes was determined only using the group-specific analysis, whereas the universal primer-set simply detected 35.3%.

5.4.2 Co-cultivation is independent of detachment

Different factors might have caused and influenced the dominant co-cultivation event and therefore the total cultivation success. Initially, the natural conditions in the soil sample, for example the close contact among the microorganisms in soil aggregates, together with the unavailability of major part of organic matter as recalcitrant material (Gestel *et al.,* 1996) might select towards the development of beneficial microbial interactions. However, incubation conditions during cultivation also might have an effect on co-cultivation efficiency and were investigated in the present analysis.

For example, it is possible that the detachment of bacteria from soil particles previous to inoculation has influenced the development of co-cultures. To date, it is known that 80 to 90% of the microorganisms inhabiting soil occur on solid surfaces (Nannipieri *et al*., 2003). They produce extracellular polymeric substances (EPS), which promote the adhesion. The adhesion to

particles is advantageous for microbial growth and survival in soils, since particles are capable to buffer the nutrient supply to microorganisms closely associated to their surfaces or offer protection from protozoan (Gestel *et al*., 1996). This adhesion may be dissolved with chemical substances such as pyrophosphate (Böckelmann *et al*., 2003) and Tween 80 (Kuczynska and Shelton, 1999), which were used in the present study for detachment.

The detachment increased the number of cells in the <12µm fraction from 2.2% (±0.02%) to 8.5% (±0.5%). However, no effect was determined on the total cultivation success and on the co-cultivation efficiency. Therefore, we compared the microtiter plates, which only differ in the presence of detachment, +PP,RO,CON and –PP,RO,CON (Table 2, Supplementary Table 1). Significant variation was neither detected in the number of co-cultures per plate nor in the number of single-cultivated strains. A similar fraction of co-cultures was observed, 68.8% and 67.7% respectively. Hence, co-cultivation probability of loosely attached bacteria was as high as from free-living bacteria. However, this finding contradicted previous studies, which investigated antagonistic interactions among marine pelagic bacteria (Long and Azam, 2001). They found that isolates from marine particles were more effective than free-living bacteria in inhibiting the growth of two human-pathogenic bacteria.

Table 2. Efficiency of co-cultivation according to the media compounds.

Number of phylotypes	Number of wells	+PP,RO,CON [1]	+PP,RO,IND [1]	-PP,RO,CON	-PP,RO,IND
1	16	5	0	10	1
2	18	7	0	5	6
3	11	1	0	8	2
4	6	0	1	2	3
5	10	2	0	4	4
6	5	0	0	1	4
7	4	1	0	0	3
8	1	0	0	1	0
Sum	71	16	1	31	23
Co-cultivation (%)	-	68.8	100	67.7	95.7

[1] PP, pyrophosphate and Tween 80; RO, artificial root exudates; CON, without inducer molecules; IND, with inducer molecules

5.4.3 Signal compounds influence the co-cultivation efficiency

For the cultivation experiment different growth media were used in parallel since due to the high diversity of soil bacterial communities it is unlikely that one set of incubation condition will permit growth of the majority of bacteria (Balestra and Misgahi, 1997; Davis *et al*., 2005). Hence, the influence of the signal molecules BHL and cAMP on the cultivation success of alpine soil bacteria and their effect on the number of co-cultures and their composition was also

determined since they are already known to significantly enhance the cultivation success of marine and freshwater bacterioplankton (Bruns *et al*., 2002; Bruns *et al*., 2003a).

As a result, the addition of signal compounds yielded increased numbers of cultures consisting of more than one bacterium, already apparent with conventional DGGE fingerprinting (Fig. 1). For further analysis, the wells were characterized with the group-specific primer-sets, which determined the highest percentage of co-cultivation for the medium with the inducer molecules (Fig. 2). Almost each culture with inducer molecules showed interacting bacteria (100% and 95.7%), in contrast to +PP,RO,CON with only 68.8% co-cultures and –PP,RO,CON with only 67.7% (Table 2). This means that the addition of these molecules enhanced the observed total cultivation success compared to the expected culturability (Fig. 2). Interestingly, signal compounds also stimulated the growth of co-cultures with more than three members. Thus, the fraction of co-cultures with four, five, six, and seven different bacteria was significantly increased (Table 2, Supplementary Table 1).

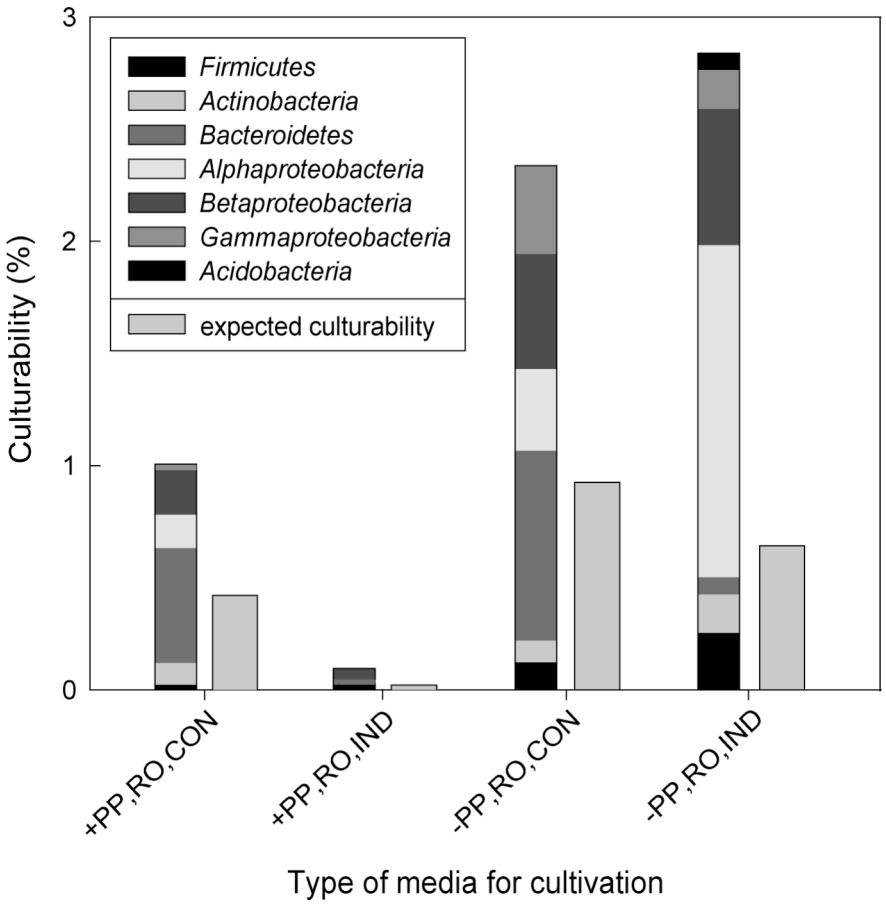

Figure 2. Observed (stacked bars) and expected (grey bars) culturability of the phylogenetic groups in the cultures obtained from the Jochberg 2002 soil sample.

In conclusion, BHL and cAMP had an effect on the number and composition of the co-cultures. This finding contradict recent studies, which showed that cAMP, HSL, or peptides represented no growth factors for the co-cultivation of two different *Alphaproteobacteria* strains (Tanaka *et al*., 2004). However, it is not known whether both signal compounds were responsible for the increased number of co-cultures. In previous studies the positive effect on cultivation of BHL, for example, was not observed for all tested natural samples (Bruns *et al*., 2002; Bruns *et al*., 2003a). Therefore, cAMP may be the active inducer.

5.4.4 Co-cultivation pattern

The high frequency of co-cultures among bacteria in the present study was unexpected for two reasons; (i) there was not that high evidence of co-cultures in previous cultivation studies using the MicroDrop technique, for bacteria from freshwater lakes (Bruns *et al*., 2003b; Gich *et al*., 2005), and (ii) if the frequency of culturable bacteria is low, inoculation of culturable bacteria in the same well should occur very rarely. Assuming a Poisson distribution, if 92 % of the wells are empty, the expected number of cells per well is –ln 0.92 = 0.083. The probability of a well containing more than one cell is $1 - 0.92 - ((e^{-0.083}) \cdot (0.083^{1}))/1!) = 0.00361$. Consequently, at maximum, only 4.3% of all positive wells would be expected to contain more than one type of bacterium. In pronounced contrast, all bacteria have been cultivated as co-cultures, except 15 *Bacteroidetes* cultures and 1 *Betaproteobacteria* culture (Table 1, Supplementary Table 1). The major part of the single cultivated strains, presumably pure cultures, was closely related to the same phylotype, namely *Sphingoterrabacterium pocheensis* Gsoil 032 (AB267718), which already represented the most abundant relative in the previous analysis (Chapter 3). However, this strain was not obligately single cultivated since it was also detected in co-cultures together with all other investigated phylogenetic groups (Supplementary Table 2). The ability to grow individually and in co-cultures might have caused the high cultivation success of the *Bacteroidetes*. They represent almost one fourth of the total number of fingerprints (51 of 221; Table 1).

Members of the *Alpha-* and *Betaproteobacteria* seemed to be especially adapted to co-cultivation since approximate one third of the fingerprints of each phylum was detected together with fingerprints of the *Alpha-* and *Betaproteobacteria* (23.3-40.4%; Table 1, Supplementary Table 1). This indicated that co-cultivation was advantageous and might have also caused their high cultivation success. They both represent 51.1% of the total number of fingerprints (64, 49, respectively; Table 1, Supplementary Table 1). Phylogenetic analyses of the cultures revealed that relatives of *Phyllobacterium brassicacearum* STM 196 (AY785319), *Rhodospirillum rubrum* DSM 107 (X87278), *Inquilinus ginsengisoli* Gsoil 080 (AB245352), *Delftia*

tsuruhatensis 180282 (DQ864991), and *Rhodocyclus tenuis* DSM 110 (D16209) were the most abundant ones with 21.4%, 15.7%, 15.7%, 25.7%, and 22.8% respectively, of the total number of fingerprints.

The fact that co-cultures occurred mostly among the same phylogenetic groups suggested specific interactions related to phylogenetic identity of the co-cultured partners. For example, the presence of *Bacillus cereus* has been recently described as necessary to specific co-isolate strains of the *Bacteroidetes* (Peterson *et al*., 2006). The authors indicated that peptidoglycan produced by *B. cereus* stimulates growth of *Bacteroidetes* species by serving as a source of carbon and energy. It is also possible that organisms require growth factors produced by other organisms. For example, a bacterium isolated from activated sludge, *Cattelibacterium nectariphilum*, did not show significant growth on nutrient broth. However, the growth was significantly stimulated by the addition of supernatants from another bacterial strain related to the genus *Sphingomonas* (Tanaka *et al*., 2004). Since some of our co-cultures contain *Bacillus* species (well 7, 31; Supplementary Table 2) and mostly contain *Alphaproteobacteria* and even members of *Sphingomonas* (well 63, 69; Supplementary Table 2), similar interactions might have taken place.

In addition, media compounds may influence co-cultivation events. Previous studies already showed that stimulation of bacterial growth was only detected in medium with artificial root exudates and not in a rich medium (Peterson *et al*., 2006). This indicated that artificial root exudates might have stimulated the co-cultivation efficiency in the present study, together with the addition of signal compounds.

In conclusion, it is supposed that cell-to-cell interaction routinely occurs between different species of microorganisms. While synthrophic association is a common and well-documented way of life for anaerobes and much is known about antagonistic interactions involving antibiotics (Long and Azam, 2001; Brinkhoff *et al*., 2004), the way, how these aerobic microorganisms beneficially interact remain to be shown. It has been stated that the mechanisms by which microorganisms interact are as diverse as the environments in which these interactions occur (Peterson *et al*., 2006). The elucidation of such interactions seems to be the most successful approach to enhance the culturability of interesting soil bacteria to promote their growth in pure or defined co-cultures. This can be achieved by means of dialysis chambers (Overmann, 2006), or diffusion chambers, which already allowed the isolation of bacteria from marine sediments (Kaeberlein *et al*., 2002).

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5.7 SUPPLEMENTARY MATERIAL

Supplementary Table 1. Result of the phylogenetic analysis of the cultures with universal and group-specific primer-sets.

+PP, RO, CON			
well number	only universal primer-set[a]	also universal primer-set[b]	only specific primer-sets[c]
4	C[d]	-	-
5	C	-	-
6	C	-	A
7	C	G	L+H+A+2B
8	ND[e]	ND	2B
9	C	-	-
10	C	C	H+2B
11		H+C	A+2B
12	C	-	-
13	C	C	-
14	-	C	H+A
15	C	C	-
16	C	C	-
17	-	C	A
18	-	C	A
19	-	C	-

+PP,RO,IND			
20			L+C+2B

–PP, RO, CON			
well number	only universal primer-set	also universal primer-set	only specific primer-sets
28	G	-	L+H+C+2B
29	-	C	G
30	-	H	L+H+2B
31	-	C	L+2B+G
32	-	C	A
33	H	-	3B
34	-	C	L+A+2B
35	-	C	G
36	-	C	A+G+G
37	-	C	A+G
38	-	C	A+G
39	C	-	2B
40	-	C	A
41	-	C	L+2A+2B+2G
42	-	C	A+G
43	-	C	-
44	-	C	-
45	-	C	-
46	-	C	-
47	-	C	-

48	-	C	-
49	-	C	A+G
50	-	C	A+G
51	-	C	G+A+2B
52	-	C	A+G
53	-	C	-
54	-	C	B+B
55	-	C	-
56	-	C	A
57	-	C	-
58	-	C	-

-PP,RO,IND			
well number	only universal primer-set	also universal primer-set	only specific primer-sets
59	ND	ND	2A+3B
60	C	-	2A+2B
61	-	H+A	2B
62	-	A+G	L+B
63	A	-	A
64	-	A	L+2A+2B+G
65	A	-	A
66	-	B	A
67	H	-	H+Ac
68	-	C	2L+2A+B
69	-	A	L+2A+B
70	ND	ND	L+A+B
71	-	G+H	L+A+B
72	A	B+G	L+H+C+Ac
73	A	-	H+2A+B+G+Ac
74	A+H	-	L+2A+B
75	-	B	L+3A+G
76	-	B	A
77	A	-	A
78	ND	ND	B
79	A	-	A
80	A	-	2A+B
81	A	-	4A+G

[a] only universal primer-set; result of the phylogenetic analysis only detected with the universal primer-set
[b] also universal primer-set; result of the phylogenetic analysis detected with the universal and specific primer-sets
[c] only specific primer-sets; result of the phylogenetic analysis only detected with the specific primer-set
[d] L; Gram positive bacteria with Low G+C content (*Firmicutes*)
H; Gram positive bacteria with High G+C content (Actinobacteria)
C; *Cytophaga-Flavobacterium-Bacteroides* (*Bacteroidetes*)
A; *Alphaproteobacteria*
B; *Betaproteobacteria*
G; *Gammaproteobacteria*
Ac; *Acidobacteria*
[e] ND; not detected

Supplementary Table 2. Close relatives of cultivated bacteria in the co-cultures.

1-phylotype cultures		
Bacteroidetes:		
well 4, 5, 9, 12, 19, 43, 44, 45, 46, 47, 48, 53, 55, 57, 58		
well 4	*Flavobacterium* sp. (AM177638)	
well 5	*Flavobacterium* sp. (AM177638)	
well 9	*Flavobacterium* sp. (AM177638)	
well 12	*Flexibacter* cf. *sancti* (AF181568)	
well 19	*Sphingoterrabacterium pocheensis* (AB267718)	
well 43	*Sphingoterrabacterium pocheensis* (AB267718)	
well 44	*Sphingoterrabacterium pocheensis* (AB267718)	
well 45	*Sphingoterrabacterium pocheensis* (AB267718)	
well 46	*Sphingoterrabacterium pocheensis* (AB267718)	
well 47	*Sphingoterrabacterium pocheensis* (AB267718)	
well 48	*Sphingoterrabacterium pocheensis* (AB267718)	
well 53	*Sphingoterrabacterium pocheensis* (AB267718)	
well 55	*Sphingoterrabacterium pocheensis* (AB267718)	
well 57	*Sphingoterrabacterium pocheensis* (AB267718)	
well 58	*Sphingoterrabacterium pocheensis* (AB267718)	
Betaproteobacteria:		
well 78	*Variovorax paradoxus* (AJ420329)	
2-phylotypes co-cultures		
C[a]+C	well 13	*Sphingoterrabacterium pocheensis* (AB267718) *Flavobacterium* sp. (AM177638)
	well 15	*Sphingoterrabacterium pocheensis* (AB267718) *Flavobacterium* sp. (AM177638)
	well 16	*Sphingoterrabacterium pocheensis* (AB267718) *Flavobacterium* sp. (AM17763)
A+A	well 63	*Bradyrhizobium japonicum* (AF417541) *Sphingomonas rhizogenes* (AY962684)
	well 65	*Phyllobacterium brassicacearum* (AY785319) *Brevundimonas variabilis* (AJ227783)
	well 77	*Phyllobacterium brassicacearum* (AY785319) *Inquilinus ginsengisoli* (AB245352)
	well 79	*Phyllobacterium brassicacearum* (AY785319 *Inquilinus ginsengisoli* (AB245352)
B+B	well 8	*Delftia tsuruhatensis* (DQ864991) *Rhodocyclus tenuis* (D16209)
C+A	well 6	*Flavobacterium* sp. (AM177638) *Methylobacterium* sp. (AY364020)
	well 17	*Sphingoterrabacterium pocheensis* (AB267718) *Rhodospirillum rubrum* (X87278)
	well 18	*Sphingoterrabacterium pocheensis* (AB267718) *Sinorhizobium* sp. (AY505135)
	well 32	*Sphingoterrabacterium pocheensis* (AB267718) *Caulobacter henricii* (AJ227758)
	well 40	*Sphingoterrabacteriu pocheensis* (AB267718) *Rhodospirillum rubrum* (X87278)
	well 56	*Sphingoterrabacterium pocheensis* (AB267718) *Inquilinus ginsengisoli* (AB245352)
C+G	well 29	*Sphingoterrabacterium pocheensis* (AB267718) *Pseudomonas tolasii* (AF255336)
	well 35	*Sphingoterrabacterium pocheensis* (AB267718) *Pseudomonas synxantha* ((D84025)
A+B	well 66	*Phyllobacterium brassicacearum* (AY785319) *Sinobacter albidoflavus* (AY965999)
	well 76	*Phyllobacterium brassicacearum* (AY785319) *Variovorax* sp. (AB167220)

	3-phylotypes co-cultures	
C+A+G	well 37, 38, 42, 49, 50, 52	
		Sphingoterrabacterium pocheensis (AB267718)
		Rhodospirillum rubrum (X87278)
		Pseudomonas synxantha (D84025)
C+B+B	well 39	*Flavobacterium* sp. (AM177638)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
	well 54	*Sphingoterrabacterium pocheensis* (AB267718)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
L+A+B	well 70	*Staphylococcus schleiferi* (D83372)
		Phyllobacterium brassicacearum (AY785319)
		Herbaspirillum frisingense (AJ238359)
H+C+A	well 14	*Rhodococcus* sp. (AF046885)
		Sphingoterrabacterium pocheensis (AB267718)
		Phyllobacterium brassicacearum (AY785319)
H+H+Ac	well 67	*Agromyces cerinus* (X77448)
		Nocardioides sp. (AF210769)
		Edaphobacter modestus (DQ58760)
	4-phylotypes co-cultures	
C+A+2G	well 36	*Sphingoterrabacterium pocheensis* (AB267718)
		Rhodospirillum rubrum (X87278)
		Pseudomonas synxantha (D84025)
		Lysobacter brunescens (AB161360)
H+A+2B	well 61	*Agromyces cerinus* (X77448)
		Sinorhizobium sp. (AY505135)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
3B+H	well 33	*Delftia tsuruhatensis* (DQ864991)
		Rhodocyclus tenuis (D16209)
		Herbaspirillum frisingense (AJ238359)
		Streptomyces griseus (DQ359266)
L+A+B+G	well 62	*Bacillus aminovorans* (X62178)
		Phyllobacterium brassicacearum (AY785319)
		Delftia tsuruhatensis (DQ864991)
		Lysobacter enzymogenes (AY947529)
L+C+2B	well 20	*Bacillus aminovorans* (X62178)
		Flavobacterium sp. (AM177638)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
3A+B	well 80	*Brevundimonas variabilis* (AJ227783)
		Inquilinus ginsengisoli (AB245352)
		Phyllobacterium brassicacearum (AY785319)
		Delftia tsuruhatensis (DQ864991)
	5-phylotypes co-cultures	
2A+3B	well 59	*Phyllobacterium brassicacearum* (AY785319)
		Bradyrhizobium japonicum (AF417541)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
		Herbaspirillum frisingense (AJ238359)
L+3A+1B	well 69	*Staphylococcus schleiferi* (D83372)
		Phyllobacterium brassicacearum (AY785319)
		Bradyrhizobium japonicum (AF417541)
		Sphingomonas rhizogenes (AY962684)
		Herbaspirillum frisingense (AJ238359)
C+2A+2B	well 60	*Flexibacter sancti* (AF181568)

		Brevundimonas variabilis (AJ227783)
		Inquilinus ginsengisoli (AB245352)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
H+C+A+2B	well 11	*Rhodococcus* sp. (AF046885)
		Sphingoterrabacterium pocheensis (AB267718)
		Caulobacter henricii (AJ227758)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
L+C+A+2B	well 34	*Staphylococcus schleiferi* (D83372)
		Sphingoterrabacterium pocheensis (AB267718)
		Phenylobacterium immobile (Y18216)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
G+C+A+2B	well 51	*Pseudomonas synxantha* (D84025)
		Sphingoterrabacterium pocheensis (AB267718)
		Rhodospirillum rubrum (X87278)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
L+C+2B+G	well 31	*Bacillus mojavensis* (AB02119)
		Sphingoterrabacterium pocheensis (AB267718)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
		Pseudomonas brassicacearum (AY271793)
H+2C+2B	well 10	*Agromyces cerinus* (X77448)
		Sphingoterrabacterium pocheensis (AB267718)
		Flavobacterium sp. (AM177638)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
L+2H+2B	well 30	*Staphylococcus schleiferi* (D83372)
		Rhodococcus sp. (AF046885)
		Steptomyces griseus (M76388)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
L+H+A+B+G	well 71	*Staphylococcus schleiferi* (D83372)
		Steptomyces humidus (AB184339)
		Phyllobacterium brassicacearum (AY785319)
		Herbaspirillum frisingense (AJ238359)
		Lsyobacter enzymogenes (AY947529)
		6-phylotypes co-cultures
L+H+C+2B+G	well 28	*Staphylococcus schleiferi* (D83372)
		Mycobacterium flavescens (AF174289)
		Sphingoterrabacterium pocheensis (AB267718)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
		Pseudomonas tolasii (AF320989)
2L+C+2A+B	well 68	*Staphylococcus schleiferi* (D83372)
		Staphylococcus hominis (AY167805)
		Pedobacter ginsengisoli (AB245371)
		Phyllobacterium brassicacearum (AY785319)
		Ochrobactrum cytisi (AY776289)
		Herbaspirillum frisingense (AJ238359)
L+3A+B+G	well 75	*Staphylococcus schleiferi* (D83372)
		Agrobacterium tumefaciens (D01262)
		Brevundimonas diminuta (X87274)
		Inquilinus ginsengisoli (AB245352)
		Herbaspirillum frisingense (AJ238359)
		Pseudomonas synxantha (D84025)

5A+G	well 81	*Sinorhizobium* sp. (AY505135)
		Phyllobacterium brassicacearum (AY785319)
		Brevundimonas variabilis (AJ227783)
		Inquilinus ginsengisoli (AB245352)
		Rhodospirillum rubrum (X87278)
		Pseudomonas synxantha (D84025)
L+3A+B+H	well 74	*Staphylococcus schleiferi* (D83372)
		Phyllobacterium brassicacearum (AY785319)
		Inquilinus ginsengisoli (AB245352)
		Brevundimonas variabilis (AJ227783)
		Herbaspirillum frisingense (AJ238359)
		Agromyces cerinus (X77448)
		7-phylotypes co-cultures
L+H+C+A+2B+G	well 7	*Bacillus aminovorans* (X62178)
		Propionibacterium propionicus (AJ003058)
		Flavobacterium sp. (AM177638, AM177615)
		Roseomonas lacus (AJ786000)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
		Pseudomonas fluorescens (AY271793)
L+H+C+A+B+G+Ac	well 72	*Granulitacella elegans* (Y15413)
		Mycobacterium goodii (AF513815)
		Endosymbiont of *Acanthamoeba* (AY549545)
		Brevundimonas variabilis (AJ227783)
		Variovorax sp. (AB167220)
		Lsyobacter enzymogenes (AY947529)
		Edaphobacter modestus (DQ58760)
H+3A+B+G+Ac	well 73	*Nocardioides kribbensis* (AY835926)
		Phyllobacterium brassicacearum (AY785319)
		Inquilinus ginsengisoli (AB245352)
		Brevundimonas variabilis (AJ227783)
		Herbaspirillum frisingense (AJ238359)
		Pseudomonas synxantha (D84025)
		Edaphobacter modestus (DQ58760)
L+3A+2B+G	well 64	*Staphylococcus schleiferi* (D83372)
		Phyllobacterium brassicacearum (AY785319)
		Inquilinus ginsengisoli (AB245352)
		Brevundimonas variabilis (AJ227783)
		Herbaspirillum frisingense (AJ238359)
		Ralstonia eutropha (AF027407)
		Pseudomonas synxantha (D84025)
		8-phylotypes co-cultures
L+C+2A+2B+2G	well 41	*Staphylococcus schleiferi* (D83372)
		Sphingoterrabacterium pocheensis (AB267718)
		Phyllobacterium brassicacearum (AY785319)
		Rhodospirillum rubrum (X87278)
		Delftia tsuruhatensis (DQ864991)
		Rhodocyclus tenuis (D16209)
		Pseudomonas synxantha (D84025)
		Dyella japonica (AB110498)

[a] **L**, Gram positive bacteria with low G+C content (*Firmicutes*); **H**, Gram positive bacteria with high G+C content (*Actinobacteria*); **C**, *Cytophaga-Flavobacterium-Bacteroides* (*Bacteroidetes*); **A**, *Alphaproteobacteria*; **B**, *Betaproteobacteria*; **G**, *Gammaproteobacteria*; **Ac**, *Acidobacteria*

Chapter 6

Discussion

6 DISCUSSION

6.1 BACTERIAL CULTURABILITY AND ABUNDANCE IN ALPINE SOIL

The aim of this work was the characterization of the bacterial culturability and abundance in an alpine soil sample in detail. To date, culture-independent analysis of the 16S rRNA gene sequence showed that the majority of bacteria thriving in the soil environment represent not-yet-cultured *Bacteria* and *Archaea* with unknown genetic and physiological properties. Particular functions have been detected using culture-independent metagenomic approaches (Quaiser *et al*., 2003; Leininger *et al*., 2006). Due to the large complexity of soil bacterial communities, however, the study of bacterial genetics and physiology using laboratory cultures often remains a prerequisite for the functional understanding of not-yet-cultured bacteria in the soil environment.

The culture-independent analysis of the February alpine soil bacterial community (Chapter 3) with the RT-PCR approach revealed that the major part was composed of the *Alphaproteobacteria* (49.2%). This value exceeds the upper limit of previous analyses (Janssen, 2006) but a similar high abundance was recently reported for other alpine soils (Labbé *et al*., 2007). Hence, the high abundance of *Alphaproteobacteria* may be a common characteristic of high altitude ecosystems. In contrast, the values for the *Betaproteobacteria* and *Actinobacteria* are at the lower limit reported for other soils (Janssen, 2006). The abundance of the *Acidobacteria* was similar to the values of other lowland soils (20.1%) but significantly lower than the value of another alpine soil at a higher elevation (Lipson and Schmidt, 2004).

Employing high-throughput cultivation and screening approach, a total of 251 different cultivated bacteria were analyzed, which represented 53 distinct phylotypes. The number of cultivated bacteria significantly surpasses that of most previous culture collections (McCaig *et al*., 2001; Sait *et al*., 2002; Lipson and Schmidt, 2004; Shrestha *et al*., 2007). A high fraction of 73% previously unknown phylotypes were obtained in the present study, which indicates that the potential of the cultivation approach so far has not been exploited to its full extent. The repeated recovery of identical phylotypes in all target groups revealed that the culture collection covers a significant fraction of all the phylotypes, which are capable of growing in these media.

Most of the cultures were closely related to the *Alphaproteobacteria* (46% of all cultured bacteria) with the largest number of different phylotypes and the highest evenness value. The *Alphaproteobacterium* most frequently recovered by cultivation was *Phyllobacterium brassicacearum* and the second most frequent isolate a distant (99.8% sequence similarity) relative of *Rhodospirillum rubrum*. Although this (sub)phylum dominated the cultivated fraction, its cultivation success was hundredfold lower than its abundance in the natural community (0.4% of total cell numbers). Hence, *Alphaproteobacteria* should represent the target group of future

cultivation attempts. In addition, the *Betaproteobacteria* were most frequently cultured whereas distant relatives of *Delftia tsuruhatensis* and *Rhodocyclus tenuis* dominated the cultivated fraction. Also the *Bacteroidetes* represent one of the most frequent cultured groups with the highest relative culturability, however, they showed the lowest evenness value since the cultures were dominated (70% of the *Bacteroidetes* cultures) by one phylotype (*Sphingoterrabacterium pocheensis*). All cultivated members and clones were affiliated with the two classes *Sphingobacteria* and *Flavobacteria,* which is in line with previous analyses of soil clone libraries (Janssen *et al*., 2006). In contrast to other soil studies, our alpine soil sample harbored a particularly high diversity of previously unknown *Bacteroidetes* bacteria. Studies of additional samples are required to test whether it is typical for alpine soils. In contrast, the *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* were found in significantly fewer cultures whereas relatives of a member of the genus *Pseudomonas*, *Staphylococcus schleiferi* and *Rhodococcus erythropolis* were the most abundant ones.

Despite of their high abundance in the soil sample, the culturability of the *Acidobacteria* was the lowest since only one single phylotype grew in our media (0.12%; *Edaphobacter modestus*, Chapter 3 and 4) with the polymer mixture and artificial root exudates. Studies with another alpine soil showed similar results (Lipson and Schmidt, 2004), which may indicate that *Acidobacteria* in high altitude soil environments require specific cultivation conditions.

The ATP content was used as a sensitive parameter to follow the effects of substrate addition and of soil homogenization on the physiological state of the soil bacteria. The initial ATP content was similar to other values reported for other soils ((4.5 nmol · (g dry weight)$^{-1}$ compared to 1.2-11.1 nmol · (g dry weight)$^{-1}$); Tsai *et al*., 1997; Contin *et al*., 2002; Joergensen and Raubuch, 2002). In contrast, the intracellular ATP concentration was lower than the average reported for other soils (0.03 mM compared to 2.4 mM; Contin *et al*., 2001) and the substrate addition did not cause an increase of ATP content but a drop over the first 72 hours. This may be explained due to continuous mixing of the soil, which has been shown to decrease the cellular ATP content (Joergensen and Raubuch, 2003) and may initially supersede the stimulating effect of substrate addition, possibly by destroying the microenvironments of the bacterial cells. Subsequently, only the cultivation approach revealed that monomeric organic carbon compounds as substrates are superior to humic analogs or a mixture of polymers for optimization of the cultivation success. These results, however, contradicted those of other soil cultivation approaches (Sait *et al*., 2002; Davis *et al*., 2005), which indicated that the media composition need to be adopted on a case-by-case basis for the study of particular soil ecosystem.

In general, the cultivation success of the February Jochberg soil sample was very low compared to the April and August soil cultivation approaches. Out of the 1008 cultivation

assays, only 81 wells displaced growth after 6 weeks of incubation. Unexpectedly, we observed a high number of co-cultures (75%) although at maximum only 4.3% of all positive wells would be expected to contain more than one type. This suggests that most bacteria grew only if accompanied by unrelated bacteria of other phyla and that this dependence on interspecific interaction was the major reason for the low cultivation success (Chapter 5).

6.2 TWO NOVEL ACIDOBACTERIA ISOLATED FROM ALPINE AND FOREST SOILS

As already described above, the investigation of the correlation of bacterial culturability and abundance in the February Jochberg soil (Chapter 3) revealed a high abundance of members of the phylum *Acidobacteria* in the natural community. Up to 20.1% of the totally detected *Eubacteria* belonged to this phylum, which is in line with other soil bacterial communities but in some cases *Acidobacteria* can even contribute up to 51 or even 80% (Dunbar *et al*., 1999; Chan *et al*., 2006; Janssen, 2006). In pronounced contrast to a high overall phylogenetic diversity, only four species, *Acidobacterium capsulatum* DSM 11244^T (Kishimoto *et al*., 1991), *Holophaga foetida* DSM 6591^T (Liesack *et al*., 1994), *Geothrix fermentans* DSM 14018^T (Coates *et al*., 1999), and *Terriglobus roseus* KBS63^T (Eichorst *et al*., 2007) have been validly described to date. Hence, the very low culturability of *Acidobacteria* in the present study was not surprising. The single phylotype, which grew in our media with a polymer mixture or artificial root exudates as carbon source, represented a novel strain in subdivision 1 (strain Jbg-1), only distantly related to *T. roseus* and *A. capsulatum* (Chapter 4). The novel strain represents the first case of a cultivated acidobacterial phylotype, which corresponds to a sequence detected in the same sample by culture-independent methods (Chapter 3). Furthermore, a second novel acidobacterial phylotype (strain Wbg-1) of subdivision 1 has been isolated from a deciduous forest soil sample near Würzburg (Germany), sampled in July 2001 by another working group. Following, many chemotaxonomic and phenotypic characteristics have been determined in the present study to compare and demarcate both strains of the remaining described *Acidobacteria* (Chapter 4).

While strain Jbg-1 was isolated in SSE medium at a pH of 6.3 with the polymer mixture as carbon source and inducer molecules (cAMP and BHL), strain Wbg-1 was obtained on diluted ammonium mineral salts medium and isolated after several streaks on R2A agar. In order to improve their growth rates, further media were tested since both strains grew rather slowly on agar-solidified media as well as in liquid media. Their growth rates could be enhanced using various but only 1:10 diluted full strength media. Therefore, 1:10 diluted HD medium was applied for most of the subsequent growth tests. To elucidate further properties, many

morphological and chemotaxonomic characteristics have been determined, necessary for the valid description of both strains.

Cells of strain Jbg-1 and Wbg-1 were non-spore forming short ovoid rods, which stained Gram-negative whereas cells of *T. roseus* and *A. capsulatum* had a similar morphology. Cells of strain Jbg-1 were nonmotile at a neutral pH but the majority was found to be motile in cultures growing below a pH of 5.5. In contrast, cells of strain Wbg-1 were always nonmotile and formed cell aggregates in liquid media. Like *T. roseus* but unlike *A. capsulatum*, cells of strain Jbg-1 and Wbg-1 did not form capsules and the presence of PHB could neither be detected in strains Jbg-1 and Wbg-1 nor in *A. capsulatum*. The subsequent analysis of the major fatty acids gave us a hint that strains Jbg-1 and Wbg-1 generate two different species since they have been shown to differ in their profiles. Based on the comparison of their fatty acid profiles with the Microbial ID database, a chemotaxonomic classification of the isolates to *A. capsulatum* and *T. roseus*, or any other known taxon was not possible.

The capability to utilize different organic carbon substrates was tested for both strains in order to recover more differences to *A. capsulatum* and *T. roseus*. Strains Jbg-1 and Wbg-1 as well as *A. capsulatum* clearly preferred sugars as growth substrates whereas the capability to utilize amino acids was very restricted. In contrast to *A. capsulatum*, however, both strains were capable of growing with glutamate and glutamine and could be further distinguished from *A. capsulatum* and *T. roseus* by their inability to utilize cellobiose, galactose, maltose and mannose. Growth substrates specific for strain Jbg-1 were lyxose and some sugar alcohols whereas strain Wbg-1 utilized aspartate and ornithine.

Furthermore, strains Jbg-1, Wbg-1, *T. roseus*, and *A. capsulatum* were determined to be obligately aerobic and incapable of nitrate respiration. Hence, all strains were tested positive for catalase whereas only strain Jbg-1 contained a cytochrome *c* oxidase.

The results of the investigated temperature optimum (30°C) and pH optimum (pH 5.5) for both strains were in line with the recent suggestion that moderately acidic pH-values in general may favor the growth of subdivision 1 *Acidobacteria* (Sait *et al.*, 2002, 2006). In contrast to our present data, however, results of the previous study indicated that other acidobacteria of subdivision 1 exhibit a somewhat lower pH optimum between pH 4.0 – 5.0.

The values of the molecular GC content further indicated the demarcation of the acidobacterial strains. Strains Jbg-1 and Wbg-1 showed variation of only 1.1% among themselves, but 2.9-4.5% to *A. capsulatum* (Kishimoto *et al.*, 1991) and *T. roseus* (Eichorst *et al.*, 2007).

Referring to the API ZYM galleries, *A. capsulatum* was tested along with the two novel isolates in the present study. All three strains were tested positive for most glycosidases in the

API ZYM test, which confirmed that all validly described acidobacteria of subdivision 1 clearly preferred sugars as growth substrates. Additionally, it was shown that their enzyme activities were significantly different, correlated to the natural environment they have been isolated from. *A. capsulatum* exhibits the acid phosphatase activity but lacks the alkaline phosphatase. Strains Jbg-1 and Wbg-1 possess alkaline phosphatase activity in addition to acid phosphatase activity, which might represent an adaptation to soils with neutral pH.

To elucidate further adaptations, the growth of the newly isolated acidobacterium strain Jbg-1 was investigated in media varying in their DOC concentration (Chapter 3). The increased cultivation success in media containing reduced concentrations of organic carbon (Davis *et al*., 2005) would indicate that a major fraction of soil *Acidobacteria* is oligotrophic. Strain Jbg-1 exhibited only moderately oligotrophic characteristics directly after its isolation, being able to grow up to 0.4 mg $DOC \cdot l^{-1}$, but adapted to 10fold higher concentrations after prolonged subculturing. Additionally, the adaptation to long-term survival was investigated for the new strain Jbg-1. Our results revealed that this strain is extraordinarily well adapted to long-term survival in organic carbon-free medium since the culturability of a rather large subpopulation is maintained even over a period of more than half a year. By contrast, *Pseudomonas* sp., *Micrococcus luteus*, some *Enterobacteriaceae*, *Campylobacter jejuni*, *Xanthomonas campestris*, and *Shewanella algae* show significantly higher declines of culturability (Morita, 1993; Mukamolova *et al*., 1995; Bogosian *et al*., 1998; Federighi *et al*., 1998; Ghezzi and Steck, 1999; Gram *et al*., 1999).

For phylogenetic classification, the nearly complete 16S rRNA genes of strains Jbg-1 and Wbg-1 were sequenced. Although numerous 16S rRNA gene sequences of environmental clones of closely related acidobacteria have accumulated over the past years and the isolation of several strains of acidobacteria has been reported (Sait *et al*., 2002; Joseph *et al*., 2003; Stevenson *et al*., 2004; Chan *et al*., 2006; Eichorst *et al*., 2007), only *A. capsulatum* and *T. roseus* have been validly described for the subdivision 1. 16S rRNA gene sequence comparisons between strains Jbg-1, Wbg-1 and *T. roseus* are 93.8% and 94.7% whereas sequence similarities of *A. capsulatum* to both strains are only 91.1% and 92.8%. The phylogenetic definition of a genus has been a matter of debate (Wayne *et al*., 1987) but a value of 95% sequence similarity has previously been suggested to delineate different prokaryotic genera (Ludwig *et al*., 1998). Hence, our novel isolates represent a distinct lineage, which is well separated from members of the genus *Terriglobus* and *Acidobacterium*. Based on the differences to *Terriglobus* in the GC-content, substrate utilization pattern, and phylogenetic analyses, the two strains represent a new bacterial genus, which is described as *Edaphobacter* gen. nov.

Since the 16S rRNA gene sequence similarity between the two isolates was 98.0%, DNA-DNA-hybridization studies were performed in order to determine whether both isolates should be assigned to one species. The DNA-DNA-similarity determined for strains Jbg-1 and Wbg-1 was 11.5-13.6%. Although the similarity of the 16S rRNA gene sequences of strains Jbg-1 and Wbg-1 surpasses the value, which is commonly used for the delineation of a bacterial species (≥ 97% 16S rRNA gene sequence similarity; Rosselló-Mora and Amann, 2001), the value of the DNA-DNA-similarity ranges significantly below the accepted criterion (≥70%) (Rosselló-Mora and Amann, 2001). Similarly, the chemotaxonomic and phenotypic characteristics of the two isolated strains differ significantly, supporting an assignment of the two strains to two separate species which are described as *Edaphobacter modestus* gen. nov., sp. nov. with strain Jbg-1^T as the type strain, and *E. aggregans* sp. nov. with strain Wbg-1^T as the type strain. *Edaphobacter modestus* is described as the type species of the novel genus *Edaphobacter*.

6.3 SIGNIFICANCE OF INDUCERS AND BACTERIAL INTERACTIONS FOR THE CULTIVATION OF SOIL BACTERIA

We already suggested that the dependence of interspecific bacterial interaction was the reason for the low cultivation success of the cultivation approach with the February Jochberg soil sample (Chapter 3). Hence, the present thesis was also carried out to characterize the bacterial interaction in detail and to determine the significance of the inducer molecules for the cultivation success (Chapter 5).

Previous studies already proved that bacteria need to interact with each other for growth (Kaprelyants and Kell, 1996) and that the typically low cultivation success in artificial media may largely be due to the lack of cell-to-cell interaction (Mukamolova *et al*., 1998; Bruns *et al*., 2002; Bruns *et al*., 2003a). Hence, particular signal molecules like the acyl-HSLs, which function as autocrine molecules in the quorum sensing of numerous Gram-negative bacteria (D. Kirke, The quorum sensing site [http://www.nottingham.ac.uk/quorum /table.htm], 2001), are known to enhance the cultivation success of natural bacterial communities (Bruns *et al*., 2002; Bruns *et al*., 2003b) to date. Moreover, cAMP represents another signal molecule, which has also been suggested to enhance the cultivation success, by preventing substrate-accelerated death in starved cultures (Calcott and Postgate, 1972). At the beginning of this thesis, however, the effects of signal molecules as BHL and cAMP on the cultivation success of soil bacteria have not been investigated in a systematic manner, as well as the factors ‘free-living versus attached bacteria’ and ‘co-cultivation with accompanying phylogenetically different bacteria’. Based on

the high cultivation success with artificial root exudates (Chapter 3), the present analysis focused on these cultures, which represented 71 of the totally detected 81 cultures and corresponded to a value of cultivation efficiency of about 0.44%.

The cultures were initially screened with conventional eubacterial PCR/DGGE fingerprinting. Interestingly it was shown that 25.4% of the cultures contained up to three different fingerprints and that most of these cultures were obtained using signal molecules as growth additives. It appeared more likely that the fingerprints represented co-cultures of different bacteria occurring together in one microtiter plate well. Already in previous cultivation approaches with a bacterioplankton sample, also generated with the MicroDrop technique, the occurrence of different fingerprints was observed but only in 6% of the total number of cultures (Bruns *et al*., 2003b). Therefore, one may assume that cultivation approaches of soil bacteria particularly induce co-cultivation.

In order to compare the resolution attained by the conventional method, fingerprint patterns from the cultures were also generated employing phylogenetic group-specific primers. Interestingly it was shown, that the eubacterial primer-set underestimated the bacterial diversity (18 phylotypes compared to 47 phylotypes) and falsely identified single cultivated strains. It was shown that 77.5% of the total number of cultures was actually cultivated with accompanying bacteria, which also contained an even higher number of phylotypes (up to eight different phylotypes) as primarily assumed. While the majority of the cultures were assessed as 2-, 3- and 5-phylotype cultures, the analysis indicated that co-cultivation was advantageous compared to single cultivation and induced a 4.0fold increase to 2.14% of the total cultivation success.

Different factors might have caused and influenced the dominant co-cultivation event and therefore the total cultivation success. The natural conditions in the soil sample, for example the close contact among the microorganisms in the soil aggregates, together with the unavailability of major part of organic matter as recalcitrant material might have selected towards the development of beneficial microbial interaction (Gestel *et al*., 1996). The present study focused on the effect of the different incubation conditions during the cultivation approach. Firstly, it was shown that the detachment of soil bacteria from particles had neither an effect on the co-cultivation efficiency, nor on the total cultivation success. Although 80 to 90% of the microorganisms inhabiting soil occur on solid surfaces (Nannipieri *et al*., 2003) because this adhesion is advantageous for microbial growth and survival in soils (Gestel *et al*., 1996), co-cultivation probability of loosely attached bacteria was as high as from free-living bacteria. This finding contradicted previous studies, which determined that isolates from marine particles were more effective in inhibiting growth of two human-pathogenic bacteria (Long and Azam, 2001). However, the addition of signal compounds yielded increased numbers of cultures consisting of

more than one bacterium, already apparent with DGGE-fingerprinting. Almost each culture with inducer molecules showed interacting bacteria (95.7-100%) in contrast to the decline in co-cultivation for cultures, which lack these molecules (67.7-68.8%). Interestingly, signal compounds also increased the bacterial diversity within the co-cultures, since the growth of co-cultures with more than three members was stimulated. These findings, however, also contradict previous studies, which showed that cAMP, HSL and peptides represented no growth factors for the co-cultivation of two different *Alphaproteobacteria*-strains (Tanaka *et al*., 2004).

The high frequency of co-cultures among bacteria in the present study was unexpected for two reasons; (i) there was not that high evidence of co-cultures in previous cultivation studies using the MicroDrop technique for bacteria from freshwater lakes (Bruns *et al*., 2003b; Gich *et al*., 2005), and (ii) if the frequency of culturable bacteria is low, inoculation of culturable bacteria in the same well should be very rarely. In pronounced contrast, all investigated bacteria have been cultivated as co-cultures, except 15 *Bacteroidetes* and 1 *Betaproteobacteria* bacteria, which almost were close related to the same phylotype, namely *Sphingoterrabacterium pocheensis*, although this strain was not obligately single cultivated. Members of the *Alpha*- and *Betaproteobacteria* seemed to be especially adapted to co-cultivation, which were observed in up to 40.4% co-cultures. This indicated that co-cultivation was advantageous for those (sub)phyla and may have caused their high cultivation success (Chapter 3). Further phylogenetic analyses revealed that relatives of *Phyllobacterium brassicacearum*, *Rhodospirillum rubrum*, *Inquilinus ginsengisoli*, *Delftia tsuruhatensis*, and *Rhodocyclus tenuis* were the most abundant ones.

The fact that co-cultures occurred mostly among the same phylogenetic groups suggested specific interactions related to the phylogenetic identity of the co-cultured partners as described for *Bacillus cereus*, that co-isolate strains of the *Bacteroidetes* (Peterson *et al*., 2006), or *Catellibacterium nectariphilum*, which growth is stimulated by a strain related to *Sphingomonas* (Tanaka *et al*., 2004). The authors indicated that the peptidoglycan produced by *B. cereus* serves as carbon and energy source, or media compounds, like artificial root exudates, might have stimulated the co-cultivation efficiency (Peterson *et al*., 2006). Furthermore, supernatants were shown to be involved in a co-cultivation event (Tanaka *et al*., 2004). Since some of our co-cultures with artificial root exudates contain *Bacillus* species and mostly contain *Alphaproteobacteria* and even members of *Sphingomonas*, similar interactions might have taken place.

In conclusion, it is supposed that cell-to-cell interaction routinely occurs between different species of microorganisms. While synthrophic association is a common and well-documented way of life for anaerobes and much is known about antagonistic interactions

involving antibiotics (Long and Azam, 2001; Brinkhoff *et al*., 2004), the way, how these aerobic microorganisms beneficially interact remain to be shown. It has been stated that the mechanisms by which microorganisms interact are as diverse as the environments in which these interactions occur (Peterson *et al*., 2006). The elucidation of such interactions seems to be the most successful approach to enhance the culturability of interesting soil bacteria to promote their growth in pure or defined co-cultures. This can be achieved by means of dialysis chambers (Overmann, 2006), or diffusion chambers, which already allowed the isolation of bacteria from marine sediments (Kaeberlein *et al*., 2002).

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