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ISOLATION AND CHARACTERIZATION OF MEMBERS OF THE PHYLUM *ACIDOBACTERIA* FROM SOILS

presented by

STEPHANIE A. EICHORST

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ISOLATION AND CHARACTERIZATION OF MEMBERS OF THE PHYLUM *ACIDOBACTERIA* FROM SOILS

by

Stephanie A. Eichorst

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ABSTRACT

Isolation and characterization of members of the phylum *Acidobacteria* from soils

by

Stephanie A. Eichorst

The soil environment is an abundant source of microbial life; recent studies have estimated that in one gram of soil, there are over one million species. These microbes are essential to the environment as major contributors to biogeochemical cyles (e.g. carbon and nitrogen). Unfortunately only 0.1 to 1% of the total microbial community has been cultivated, leaving a wealth of unexplored diversity; one such phylum is the *Acidobacteria.* The phylum *Acidobacteria* is an ubiquitous group of microorganisms found in various soil and sediment environments. Historically, it contains eight monophyletic subdivisions and only three cultivated representatives. This dissertation explored members of this unknown phylum by using molecular and growth-based approaches to increase our understanding of acidobacteria ecology in the soil environment.

A phylogenetic survey of soil taken from the KBS LTER revealed the presence of subdivisions 1, 3, 4, 5, and 6, with members of subdivision 4 being more dominant in the conventional agriculture treatment and subdivisions 1 and 6 being more dominant in the successional community treatment. Additionally, the acidobacteria community composition changed in relation to edaphic properties such as soil moisture (subdivision 3), carbon concentration (subdivision 4), soil pH (subdivision 1), methane fluxes (subdivision 1), and nitrous oxide fluxes (subdivision 4). These trends were used to isolate strains of acidobacteria and helped to provide insight into their physiology.

Successful attempts were made to cultivate members of this phylum with soil collected from the KBS LTER using cultivation strategies designed to mimic the native soil environment. After ca. 30 days of growth, total recoveries ranged from ca. 3 to 6% of the total microbial community; soils containing high moisture content had significantly increased total recovery. In order to screen for the presence of acidobacteria on cultivation plates, a facile high-throughput method called Plate Wash PCR (PWPCR) was developed to rapidly screen enrichment plates using phylum-specific 16S rRNA gene primers. Additionally, PWPCR revealed that acidobacteria were more frequently detected with elevated levels of carbon dioxide (significantly), the presence of the catalase enzyme, low nutrients, and low oxygen concentrations.

The use of these cultivation strategies along with PWPCR was instrumental in isolating more than a dozen members of the phylum *Acidobacteria* from subdivisions 1 and 3. Colonies of these strains were approximately 1 mm in diameter and either white, pale yellow or pink in color, the latter due to a carotenoid that was synthesized preferentially under 20% as compared to 2% oxygen. Strains were Gram negative, aerobic, chemoorganotrophic, nonmotile rods that produced an extracellular matrix causing cells to clump in liquid culture. All strains contained either 1 or 2 copies of the 16S ribosomal RNA encoding gene, which along with a relatively slow doubling time suggests an oligotrophic lifestyle. Genotypic, physiological, and morphological data revealed the presence of a novel genus in subdivision 1, *Terriglobus*, which contained pigmented strains of acidobacteria. The physiological and nutritional characteristics of these acidobacteria are consistent with their potential widespread distribution in soil.

DEDICATION

This work is dedicated to my dearly beloved grandparents and great aunts: Edward J. Schultz, Constance B. Schultz, Anna Grien, Leonarda Ciesla, John H. Eichorst, and Rose M. Eichorst.

ACKNOWLEDGEMENTS

Throughout this journey, I have grown as a scientist, with the guidance and support of my co-advisors, John Breznak and Tom Schmidt. I am forever grateful to them for their support, encouragement, intellectual challenges, opportunities, and intoxicating enthusiasm for teaching and science. I am truly thankful. I would like to thank members of my graduate thesis committee, Terry Marsh, Mike Klug, Frank Dazzo, and Phil Robertson, for their advice and insight into my project during our rewarding meetings. I would like to thank members of the Microbiology & Molecular Genetics department who have all made this experience memorable, and the staff at the Kellogg Biological Station, especially Drew Corbin who was extremely helpful in the soil sampling process. Lastly, I would like to thank the College of Natural Science and the Lyman Bridges School for giving me the opportunities to teach. My most rewarding teaching experience was Honors Biology with John Urbance. He has been a true teaching mentor to me and has led by example.

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CHAPTER 1

MICROBIAL DIVERSITY IN SOIL AND THE PHYLUM *ACIDOBACTERIA*

INTRODUCTION

Soil is a dynamic, three-dimensional environment containing an abundant source of life. This environment encompasses an area of ca. 1.23×10^{14} m^2 on Earth (93), which is home to a multitude of our planet's biodiversity. It is also the foundation of our society supporting our way of life primarily as a growth medium for food such as corn, soybean, and wheat to sustain both animal and human life. It is ironic that we know and understand very little about this diverse environment, since we are highly dependent on the community living within the soil environment.

In this work I explored a major, but poorly understood group of soil bacteria called the *Acidobacteria*. Acidobacteria are in high abundance in the soil environment based on rRNA studies suggesting that they are essential to the soil environment. My underlying goal was to understand the ecological role(s) of the phylum *Acidobaceria* in soil. I addressed this goal by combining molecular and growth–based approaches. This research is necessary to build a strong foundation of knowledge and understanding about this bacterial group. It justifies and demands the need for further research addressing the impact the *Acidobacteria* have on nutrient cycling, biogeochemistry, and agricultural practices.

The soil environment

Soil is a complex habitat of living and non-living components. Air and water make up the pore space, and mineral and organic matter make up the solid space (7). The organic matter includes nutrients, living microbiota, and organic matter in various stages, whereas the mineral fraction is classified as being either larger sized particles of sand, silt, or smaller sized particles of clay (86). Depending on the soil, the relative proportions of these components vary which in turn can affect the composition of the microbial communities (29, 90).

Solid soil particles are in association with eachother due to interactions with factors such as humic substances, bacterial polysaccharides, plant roots, and fungal hyphae (67) which together form soil aggregates. The formation of these aggregates and the ability of these particles to withstand change defines the overall soil structure (32). Soil aggregates are divided up into two main categories based on composition and size: microaggregates and macroaggregates. Microaggregates, defined as having a diameter of < 250 μm, are composed of humic molecules, clay minerals, microbes, and amorphous Al and Fe oxides, whereas macroaggregates typically have a diameter of > 250 μm and are composed of the smaller sized microaggregates often adhering to plant roots (32, 67). The organic matter in the soil allows these different sized aggregates to withstand external forces, and often there is a linear relationship observed between aggregates stability and organic matter content of the soil (32). Within soil aggregates exists a gradient of oxygen (7, 67, 91), creating anoxic microsites, which are ideal for metabolic processes such as denitrification and fermentation as well as providing transition zones between oxic and anoxic conditions (37, 79). Methods have been developed to study the

communities of these aggregates by separating them based on class size (81) as well as analyzing the outer- and inner- microaggregate communities (64). In general, microbial diversity increases with soil aggregate size which is in turn influenced by soil management (57, 62); in this way aggregate size affects the overall microbial community composition (63).

Aggregates create a three-dimensional structure amidst gradients of carbon, nitrogen, phosphorous, sulfur, oxygen, carbon dioxide, pH, moisture, and temperature (Figure 1.1). The foremost gradient in this environment is the soil profile made up of different horizons with the O horizon at the soil-air interface followed by the A, B, and C horizons atop the underlying bedrock. The soil content changes with depth from the surface (O horizon), which primarily consists of organic matter, to clays, oxides, and carbonates (A, B, and C horizons), and eventually transforms into salts and minerals (bedrock) (7).

Soil is one of the major reservoirs of organic carbon on the planet (93) with most of the carbon resulting from the degradation leaves, roots, fauna, and microbes; however it is typically defined as carbon-limiting since the carbon is not present in a readily usable form. Microbes are responsible for the breakdown of this recalcitrant carbon, which is an astronomical feat taking into account that this community comprises only ca. 5% of the overall available space in soil (39). Organic carbon in soil is typically composed of high molecular weight organic molecules known as humic substances; for example, in mineral soils humic substances account for ca. 70 to 80% of the total organic matter (32). The

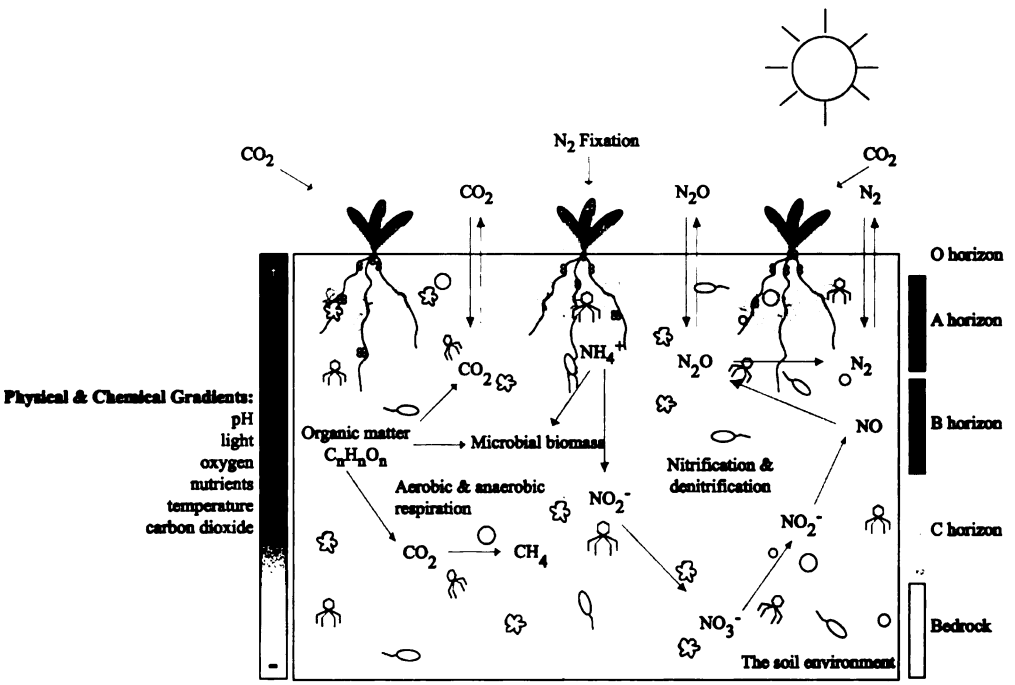


Figure 1.1. Representation of the soil environment. Gradients of pH, light, oxygen, nutrients, temperature, and carbon dioxide are indicated on the left-hand side, and the soil profile on the right-hand side. An outline of the carbon and nitrogen cycle are represented. Microbial and viral communities are depicted pictorially.

amount of soil organic matter in a particular soil is influenced by soil management. Soil organic matter is of the utmost importance to this environment as it contributes to soil fertility by acting as a reservoir for nutrients such as nitrogen, sulfur, and phosphorus; source of water; maintenance of proper cation exchange; and ultimately is essential in the formation and stability of the soil structure (32).

Microbial diversity and function in soil

The soil has an abundant wealth of life including members of the Domains *Archaea*, *Bacteria*, and *Eukarya*. Eukaryotic life found in soil includes, but is not limited to, algae, nematodes, protozoans, lichens, earthworms, insects, arthropods, plants, and fungi. Fungi, an often overlooked group, are responsible for maintaining the cycle of nutrients through the decomposition of plant material such as root exudates, lignin, and cellulose; soil stabilization; and balancing of other microbial populations through predation on certain species (18). There is a great deal of untapped fungal diversity, as seen by a recent survey of basidiomycetes in Michigan agricultural soils which revealed multiple, unidentified species (58).

Another poorly studied group in the soil environment is viruses. Viruses, typically lytic phages, are a source of mortality for soil microbes (13), causing a release of nutrients as well as genetic material into the surrounding environment. Determination of virus diversity is typically based on morphotype as viewed by electron microscopy with such groups as tailless, *Podoviridae*, filamentous, *Siphoviridae*, and *Myoviridae*. Viruses are influenced by land use, moisture, water content, and soil organic matter and

have been observed with a high abundance, ca. 1.9×10^9 virus-like particles/gram soil (dry weight), in forested and agricultural soils (94).

The bacteria and archaea encompass a great wealth of diversity and importance. One study estimated that the worldwide soil environemnt contains ca. 2.6×10^{29} bacteria and archaea (93). The importance of these communities becomes clear when estimating their enormous carbon contributions of ca. 5 to 7% to the planet (93). Additionally their contributions to the total amount of nitrogen and phosphorous are similar to that of plants (93).

Bacteria and archaea play a major role in the carbon and nitrogen cycles. The majority of the carbon in the soil environment results from the bacterial and archaeal decomposition of plant polymers and sugars. Plant polymers are hydrolysed and degraded into simple sugars by a variety of microorganisms, oftentimes by a collaborative effort of different microbial groups. This carbon is converted into microbial biomass, and ultimately degraded into carbon dioxide. Carbon dioxide can be used to produce biomass via autotrophic pathways, methane by the methanogenic archaeal communities which is further oxidized by nearly methanotrophic communities, or released into the atmosphere.

Nitrogen, another important, limiting nutrient in the soil environment, is introduced to the soil environment in one of three ways: (i) nitrogen fixation exclusively performed by bacterial and archaeal communities, (ii) addition of fertilizer or deposition of nitrogen, and (iii) decomposition of plant and microbial biomass. Microbial decomposition of plant and microbial biomass releases various forms of nitrogen, such as ammonium, into the surrounding community. Bacteria oxidize ammonium to nitrite and

nitrate through a process known as nitrification. Nitrification is predominantly performed by the γ-*Proteobacteria* and β-*Proteobacteria* in soil, however it was recently discovered that members of the Kingdom *Crenarchaeota* have this capacity (46). Nitrate and nitrite, under anoxic conditions, can be used as a terminal electron acceptor whereby these forms of nitrogen are sequentially reduced to nitric oxide, nitrous oxide, and dinitrogen gas which is referred to as denitrification. This is a heterotrophic, facultative trait common to organisms including the proteobacteria, gram positive bacteria, archaea, and fungi (99).

The community of soil bacteria is very diverse based on 16S rDNA studies; some have even described this unique environment as being a "microbial rain forest" (9), containing exotic species of bacterial life. Members of the phyla *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Cytophagales*, *Actinobacteria*, *Firmicutes*, and *Planctomycetes* are dominant members in the soil environment with their abundance varying depending on the environmental condition. In addition to these bacterial phyla, nonthermophilic members of the Kingdom *Crenarchaeota* are diverse and abundant members of the soil environment accounting for as much as 1.42 ± 0.42% of the total community (10).

Over the years, numerous methods have been developed to study the soil microbial diversity. Common techniques include rDNA (typically the 16S/18S small subunit rRNA) based analyses such as terminal restriction fragment length polymorphism, sequencing of the 16S or 18S rRNA gene, amplified ribosomal DNA restriction analysis (ARDRA) or restriction fragement length polymorphism (RFLP), while other techniques used are determination of the G + C content, BIOLOG™ profiles, stable isotope probing, fatty acid analysis, nucleic acid reassociation curves, and DNA

microarrays (44, 87). Depending on the application and question, a basic picture of the structure and/or function of specific microbial community can be obtained with these methods; however these techniques yield no new cultivated representatives.

Cultivating the uncultivables

Soil is a plentiful source of life. Based on total microscopic counts, the soil environment contains approximately 10^9 to 10^{10} microorganisms per gram of soil dry weight. The soil community is estimated to contain over a million species per gram of soil (27); as a result soil harbors more genera and species of microorganisms than any other habitat on the planet (91).

However, only a small percentage 0.1 to 1% of the total soil microbial community has been cultivated (88, 89) based on direct counts. This phenomenon is referred to as the "great plate count anomaly" (83) owing to the observation that there are oftentimes orders of magnitude differences between counts from cultivation plates and direct counts from the native environment. The lack of cultivated representatives of common soil bacterial phyla becomes evident when one compares the number of cultivated sequences to the total number of sequences (Figure 1.2). It has been suggested that these cells have not been cultivated not because they are uncultivable; rather microbiologists are not providing the proper growth conditions for these bacteria. Cells are viable but as a result of various environmental stresses, they enter a dormant state where they cannot be cultivated under standard conditions (6); oftentimes referred to as the viable but non-culturable state (VBNC). This argument has been used to explained the uncultivability of such organisms as *Enterococcus faecalis* (33) and *Vibrio vulnificus* (66).

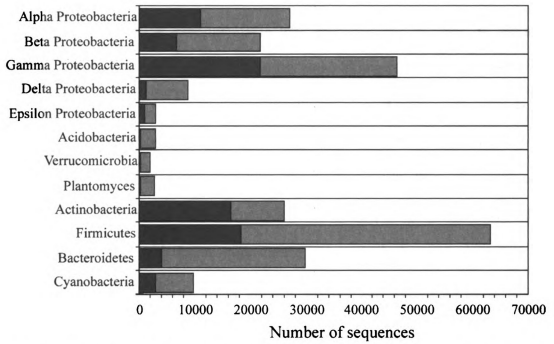


Figure 1.2. Common phyla of bacteria found in the soil environment. Each gray bar represents the number of clones in each phylum; the black, shaded region represents the percentage of the phylum that has been cultivated. Data was obtained from the Ribosomal Database Project-II in January 2007.

Recalcitrant microorganisms such as members in the phyla *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Plantomyces*, *Actinobacteria*, *Firmicutes*, and *Bacteriodetes* continue to evade our cultivation experiments, and as a result our understanding of these microorganisms' roles is remaining obscure. The need to retrieve this "not-yet-cultured majority" (8, 69) has become evident with the footprint of these microbial communities becoming more and more defined with molecular techniques. Our poor understanding of microorganisms' biochemistry and a lack of patience to grow these presumed, slow growing microbes (52) is to blame for our low cultivation recovery, since typical cultivation experiments include short incubation times on nutrient rich medium, under oxic atmospheres. Recently, microbiologists have attempted to address the "great plate count anomaly" by developing new, novel cultivation methods such as diffusion chambers to isolate aerobic organoheterotrophs from intertidal marine sediments (43), extinction culturing with low-nutrient media from oligotrophic marine environments (14), encapsulation of cells in gel microdroplets (96), and novel cultivation strategies combined with facile, high throughput methods for screening such as plate wash PCR (PWPCR) (85).

In addition to these growth-based techniques, the innovative field of metagenomics (31) has developed in recent years as a culture-independent means to gain insight into the physiology of novel groups of microorganisms by discovering novel genes and revealing their physiology from such environments as soil (23, 34, 35, 70), seawater (4, 5, 15, 84), and marine sponges (77). Data obtained from metagenomic projects in conjunction with the patience and desire to isolate microorganisms in pure culture will be the synergism needed to push the scientific community forward in the 21st

century. The combination of patience with the satisfying reward of isolating novel groups of microorganisms was summarized best by Peter H. Janssen:

> *"Successful approaches to culturing these microorganisms require patience, but the outcomes are immensely satisfying to microbiologists who enjoy the challenge and savor the reward of observing colony on the plate, seeing the cells of the pure culture under the microscope, elucidating the bacterium's physiology, or releasing its genome sequence into public databases"* (40).

The phylum *Acidobacteria*

A group of bacteria that are prevalent in the soil environment, but have few cultivated representatives are members of the phylum *Acidobacteria*. This phylum is defined by a large collection of 16S rRNA gene sequences (>3,000 in the Ribosome Database Project) (12) retrieved from diverse environments including: soils and sediments (3, 21), soil crusts of sand dunes (82), wastewater (16, 50), water distribution systems (60), peat bogs (19), acid mine drainage (45), hot springs (36), shallow submarine hydrothermal vents (80), and on the surface of Paleolithic cave paintings and catacombs (74-76, 97, 98) (Table 1.1). I*n situ* hybridization with acidobacteria-specific probes has also confirmed the presence of intact acidobacteria in many environments, and revealed multiple cellular morphotypes, including cocci, short rods, and thin filaments (56).

Historically, the phylum *Acidobacteria* has been comprised of three organisms, *Acidobacterium capsulatum*, *Holophaga foetida*, and *Geothrix fermentans*. The name *Acidobacterium* was first introduced in the early 1990's after a group of Japanesse

Table 1.1. Location, description, and sample type of various environments in which members of the phylum *Acidobacteria* reside.

Table 1. Distribution of members of the phylum *Acidobacteria* from environmental samples

Location	Description	Sample Type	Reference
Soils			
Sunset Crater, Ariz.	Volcanic	Cinders	(3, 21, 48)
Sunset Crater, Ariz.	Pinyon rhizosphere	Cinders	(3, 21, 48)
Cosnino, Ariz.	Pinyon woodland	Sandy loam	(3, 21, 48)
Cosnino, Ariz.	Pinyon rhizosphere	Sandy loam	(3, 21, 48)
Las Cruces, N. Mex.	Chile Field	Sandy loam	(3, 21, 48)
Raleigh, N.C.	Meadow	Clay loam	(3, 21, 48)
Elk River, Minn.	Garden black soil	Organic	(3, 21, 48)
Paineville, Ohio	Agricultural	Sandy loam	(3, 21, 48)
Elk River, Minn.	Aged cow manure	Organic	(3, 21, 48)
Oslo, Norway	Boreal forest	Organic	(3, 21, 48)
Antelope Valley, Calif.	Citrus grove	Sandy loam	(3, 21, 48)
Dugway, Utah	Semiarid desert	Sandy clay loam	(3, 21, 48)
Elk River, Minn.	Gravel Pit "white peat"	Silt organic	(3, 21, 48)
Niceville, Fla.	Pine Forest	Sandy	(3, 21, 48)
Poncha Pass, Colo.	Roadside Hill	Sandy	(3, 21, 48)
Berkeley Heights, N.J.	Hardwood forest	Organic	(3, 21, 48)
Ohio	Cranberry bog	Muck; rifle peat	(3, 21, 48)
Cologne, Germany	Oak-hornbeam forest	no data	(71)
Drentse A, Netherlands	Dutch Grasslands	loamy fine sand	(24-26)
Ellinbank, Victoria, Australia	Dairy Research Institute	basaltic clay loam	(17, 40-42, 72, 73)
Kellogg Biological Station	Long Term Ecological Research Site	Kalamazoo sandy loam	(22, 85)
Colorado Rocky Mountains	Niwot Ridge Long Term Research Site	Skeletal-loamy pergaelic	(55)
Cape Cod National Seashore, Massachusetts	Sand dunes	Crusted sands	(82)
Yellowstone National Park	Ragged Hills, Norris Geyser Basin	No data	(65)
Madison, Wisconsin	West Madison Agricultural Station	No data	(54)
Canyonlands National Park, Utah	Arid grasslands	Calcareous loamy sand	(49)
Western, Morroco	Institut National de la Recherche Agronomique experimental station, Settate	Vertisol	(1)
Yorkshire, England	Gisburn Forest Experiment	Acidic oak forest soil	(68)
Ultuna, Sweden	Ultuna long-term field experiment	No data	(78)
Eschikon, Switzerland	Monoculture soil	Clay loam	(59)
Border Region, Scotland	Sourhope Research Station	Rhizosphere soil	(61)
Sediments and mats			
Los Alamos, N. Mex.	Marsh (anaerobic)	Organic	(3, 21, 48)
Abiquiu, N. Mex.	River (anaerobic)	Organic	(3, 21, 48)

Table 1.1 ***continued***

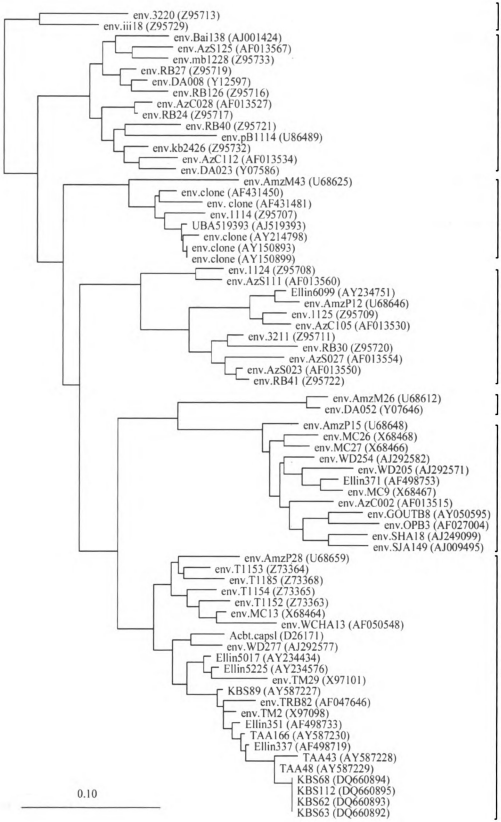
Location	Description	Sample Type	Reference
Sediments and mats ***continued***			
Los Alamos, N. Mex.	Shallow Lake	Organic	(3, 21, 48)
Santa Rosa I., Fla.	Shallow Marine	Sandy	(3, 21, 48)
Pine Barrens, N.J.	Cedar Swamp	Organic	(3, 21, 48)
Pine Barrens, N.J.	Hardwood forest	Streambed	(3, 21, 48)
Yellowstone National Park	71^{o}C hot spring	Sediment	(3, 21, 48)
Yellowstone National Park	67^{o}C hot spring	Mat	(3, 21, 48)
Yellowstone National Park	64^{o}C hot spring	Mat	(3, 21, 48)
Aiken, S.Carolina	Rainbow Bay	no data	(95)
Vercelli, Italy	Rice paddies (anoxic)	no data	(92)
Other			
Santillana del Mar, Spain	Altamira Cave	no data	(74)
Ribadesella, Spain	Tito Bustillo Cave	no data	(75)
Rome, Italy	Saint Callixtus Catacombs	Epilithic biofilms	(97)
Aegean Sea, Milos, Greece	Hydrothermal vent	no data	(80)
Wastewater , Indiana	Bioreactors	no data	(51)
Queensland, Australia	Wastewater batch reactors	no data	(16)
West Siberia, Russia	Peat samples Bakchar, Plotnikovo	*Sphagnum* peat bog	(19)
North Wales, United Kingdom	Mynydd Parys & Cae Coch mines	Acidic, metal-rich mine waters	(30)

scientists isolated eight novel strains (termed, Biogroup 5) from an acidic mineral environment. This was the first report of an isolation of an acidophilic heterotrophic bacteria inhabiting acidic mineral environments other then *Acidiphilium* (45, 47). The phylum was named *Acidobacteria* after the only validated species at the time (56).

Shortly after the isolation and characterization of *A. capsulatum*, *H. foetida* was isolated and described as a new homoacetogenic bacterium capable of degrading methoxylated aromatic compounds (53). Given the high sequence similarity of its 16S rRNA gene to *A. capsulatum* (ca. 81.6%), it was placed in the phylum *Acidobacteria.* Additionally, *G. fermentans,* a bacterium capable of Fe (III) reduction, was added to this new phylum based on the high sequence similarity of its 16S rRNA gene (ca. 94%) to *H. foetida* (11).

The phylum *Acidobacteria* is now officially recognized in the Bergey's Manual of Systematic Bacteriology (28) and includes three genera with cultured representatives: *Acidobacterium* (45), *Geothrix (11)*, and *Holophaga (53).* Currently, there are three proposed genera in this phylum; *Solibacter* (www.jgi.doe.gov), *Terriglobus* (22), and *Edaphobacter* (www.ncbi.nih.gov). This phylum contains eight recognized monophyletic subdivisions (38) (Figure 1.3) that encompass the molecular diversity originally recognized as *Acidobacteria* (48), along with additionally unnamed and mostly uncharacterized cultivars in subdivisions 1, 2, 3, and 4 (17, 19, 20, 30, 40-42, 71-73, 85). Recently, a survey of 16S rRNA genes in microbial communities associated with uranium contaminated subsurface sediment uncovered additional novel acidobacteria, expanding the number of subdivisions to as many as twenty-six (2).

Figure 1.3. Maximum-likelihood tree of the *Acidobacteria* subdivisions 1, 2, 3, 4, 5, 6, and 7 (indicated to the right of the group) based on the 16S rRNA gene using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). The scale bar indicates 0.10 changes per nucleotide.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env.T1185 (Z73368)
env.T1154 (Z73365)
env.T1152 (Z73363)
env.MC13 (X68464)
env.WCHA13 (AF050548)
Acbt.capsI (D26171)
env.WD277 (AJ292577)
Ellin5017 (AY234434)
Ellin5225 (AY234576)
env.TM29 (X97101)
KBS89 (AY587227)
env.TRB82 (AF047646)
env.TM2 (X97098)
Ellin351 (AF498733)
TAA166 (AY587230)
Ellin337 (AF498719)
TAA43 (AY587228)
TAA48 (AY587229)
KBS68 (DQ660894)
KBS112 (DQ660895)
KBS62 (DQ660893)
KBS63 (DQ660892)
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Thesis Outline

The biodiversity of the soil environment is poorly understood, although we rely on it quite extensively as a society. The exploration of this untapped microbial diversity allows us to understand the role microorganisms play in the environment, which will in turn enhance our understanding of nutrient cycling, biogeochemistry, and the effect of agricultural practices. This research was a union of both molecular and growth-based approaches, which generated information pertaining to a ubiquitous and abundant group of soil bacteria called the *Acidobacteria*.

My underlying, long-range objective was to understand the ecological role(s) members of the phylum *Acidobacteria* have in the soil. In order to address this long-range objective, I have divided my thesis into three main goals:

1. Determine the composition of the phylum *Acidobacteria* in the soil environment, particularly assessing the influence of edaphic properties on the distribution of the different monophyletic subdivisions.

2. Develop novel cultivation strategies and screening methods to cultivate members of the phylum *Acidobacteria*.

3. Characterize novel members of the phylum *Acidobacteria* from a genotypic, physiological, and morphological perspective as a first step towards understanding this interesting and diverse group of bacteria to ultimately understand their ecological role(s) in the soil environment.

Experimental Approach

These goals were addressed with soil collected from Michigan State University's W.K. Kellogg Biological Station Long Term Ecological Research Site (KBS LTER). The KBS LTER is a 48-hectare research site established in 1989 to study ecological processes in agroecosystems. The dominant soil series are Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs). A detailed description of this site can be accessed at http://lter.kbs.msu.edu/.

Chapter 2 describes a phylogenetic survey of the phylum *Acidobacteria* from various treatments at the KBS LTER as well as novel cultivation strategies and screening methods used to isolate members of this phylum. The phylogenetic survey revealed information linking the different subdivisions with various edaphic properties. In addition, trends linking members of subdivision 1 to soil pH were consistent with data obtained during the characterization experiments in Chapters 3 and 4. PWPCR, a rapid, high-throughput method (85), as well as novel cultivation strategies employed with particular attention to the conditions found in the native soil environment (22), were developed and instrumental in the successful isolation of members of subdivisions 1 and 3.

In Chapters 3 and 4, the newly isolated members of subdivisions 1 and 3 were characterized with particular attention to genotypic, physiological, and morphological properties which helped to provide insight into their presumed ecology in the soil environment. During these experiments, data revealed that a group of newly isolated members of subdivision 1 warranted the creation of a new genus, *Terriglobus*.

Finally, in Chapter 5, I explored the capacity of acidobacteria to oxidize one-carbon compounds. Previous work with the use of stable isotope probing with ^{13}C-methanol indicated that members of the phylum *Acidobacteria* from oak-forest soil samples were potentially capable of oxidizing methanol, since a portion of the ^{13}C DNA isolated was acidobacterial (68). Molecular and growth–based approaches were used to explore this interesting physiology in acidobacteria.

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CHAPTER 2

ACIDOBACTERIA COMPOSITION FROM MIGHIGAN SOILS IN CONJUNCTION WITH NOVEL CULTIVATION AND SCREENING METHODS FOR THEIR SUCCESSFUL ISOLATION

Some of these results have been published in the article: Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt, and J. A. Breznak. 2004. New Strategies for Cultivation and Detection of Previously Uncultured Microbes. Appl. Environ. Microbiol. Vol. 70, No. 8, pgs. 4748-4755.

The raw data for the cultivation experiments from the KBS LTER can be found in Appendix B.

INTRODUCTION

Soil is a heterogeneous environment containing over one million different species per gram (26), harboring more species of microorganisms than any other habitat on the planet (72). Additionally, this community of soil microbes is very diverse based on 16S rDNA studies; some have even described this unique environment as being a "microbial rain forest" (10), containing exotic species of microbial life. Members of the phyla *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Cytophagales*, *Actinobacteria*, *Firmicutes*, and *Planctomycetes* are dominant members in the soil environment with their abundance varying depending on the environmental condition.

Unfortunately, only a small percent, 0.1 to 1%, of the total microbial community has been cultivated from soil (39, 49), revealing a glimpse of the diversity in culture. Excitingly, the soil microbial diversity continues to grow with every 16S rRNA gene analysis uncovering additional novel, uncultivated phyla such as OP11 (5, 42), TM7 (5), and WS6 (75), subsequently reducing our cultivation percentage more and more. These recalcitrant microorganisms continue to evade our cultivation experiments primarily because of our poor understanding of their biochemistry and a lack of patience to grow these presumed, slow-growing microorganisms (46). Recently, microbiologists have attempted to address our inadequate cultivation techniques by developing new, novel methods such as a diffusion chamber to isolate aerobic organoheterotrophs from intertidial marine sediments (39), extinction culturing with low-nutrient media from oligotrophic marine environments (16), extending incubation times to accommodate slow-growing bacteria (37, 57), encapsulation of cells in gel microdroplets (74), and novel cultivation strategies (25) combined with facile, high throughput methods for screening such as plate wash PCR (PWPCR) (66).

Not only is there a lack of cultivated representatives in such phyla as *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Cytophagales*, *Actinobacteria*, *Firmicutes*, and *Planctomycetes*, there is little to no information linking their abundance and distribution to edaphic properties. Oftentimes scientists are faced with the conundrum of trying to cultivate an organism having little to no information about their physiology. Information generated from soil survey(s) linking edaphic properties to the abundance and/or composition of these phyla provides a roadmap to bring about their successful isolation as well as insight into their physiology. This knowledge can be used

to develop strategies, such as media compositions and incubation conditions, to successfully isolate these poorly cultivated phyla. When members of these phyla have been successfully isolated, we can determine if the trends observed in the soil surveys are consistent with their physiology.

Members of the phylum *Acidobacteria* are a prime example of a dominant group of soil bacteria with few cultivated representatives and no information linking their abundance and/or composition to edaphic properties. The *Acidobacteria* is a phylum of soil bacteria that are known to be present in soil in high abundance in various types of environments including soils and sediments (3, 24), soil crusts of sand dunes (64), wastewater (18, 45), water distribution systems (48), peat bogs (23), acid mine drainage (41), hot springs (33), shallow submarine hydrothermal vents (63), and on the surface of Paleolithic cave paintings and catacombs (58-60, 76, 77). *Acidobacteria* is a large, monophyletic group of *Bacteria* historically harboring eight different subdivisions with few cultured representatives (21, 23, 29, 36-38, 56, 57, 66) with recent evidence of eighteen additional subdivisions, some unique to uranium contaminated subsurface sediments (2). Their common occurrence and high abundance suggests they play an ecologically significant role in the soil environment which remains unknown due to the lack of cultivated representatives. In addition to the lack of cultivated representatives, there is little to no information linking these eight monophyletic subdivisions of the *Acidobacteria* to various edaphic properties such as moisture, pH, carbon content, nitrogen content, or soil type, which could provide insight into new methods for their successful isolation as well as features unique to a particular subdivision.

The purpose of this study was three-fold: (i) determine the acidobacteria composition in the different treatments at the Kellogg Biological Station Long Term Ecological Research (KBS LTER) site; (ii) assess relationships between the acidobacteria composition and edaphic properties; and (iii) increase the recoverability of soil microorganisms, specifically acidobacteria, using recognized supplements for increased recovery such as low nutrients in the form of organic carbon (16, 50), extended incubation times (37, 57), inclusion of quorum sensing compounds (N-acyl homoserine lactones) (9, 12) as well as novel methods such as the catalase enzyme and anthraquinone disulfonate and incubations under atmospheric gas concentrations native to soil.

MATERIALS and METHODS

Molecular Survey

Soil collection Soil samples were collected August 2005 from the Michigan State University W.K. Kellogg Biological Station Long Term Ecological Research Site (KBS LTER, Hickory Corners, MI, USA). The KBS LTER is a 48-hectare plot designed to study ecological processes in agroecosystems, which prior to its establishment in 1989 was uniformly farmed for more than 50 years. A description of this site can be accessed at http://www.kbs.msu.edu. The soil type is defined as a Typic Hapludalf, consisting of sandy to silty clay loam with moderate fertility. Two series of soil cores (2.5 cm diameter) were collected from the upper 7 centimeters of five sampling stations in two replicate 1-ha plots from early succssional, mid succesional, and conventional agricultural soils (Table 2.1), as well as additional un-replicated samples from the upper 7 centimeters of the deciduous forest site and lower 13 to 20 centimeters of treatments 7

Table 2.1. Description of soil samples from the KBS LTER.

Treatment	Description	Crops	Tillage	Abbreviation
Treatment 1	Conventional agriculture	Corn/soybean/wheat	Conventional till	T1
Treatment 7	Early-successional community	Herbaceous plants	Historically tilled	T7
Treatment 8	Mid-successional community	Herbaceous plants	Never tilled	T8

and 8. These pooled soil cores from each plot were homogenized with a 2-mm sieve, and portions were used to determine soil pH and soil moisture (54) (Table 2.2); the remaining soil was frozen in liquid nitrogen and stored at -80°C.

DNA Extraction and PCR DNA was extracted using the UltraClean Fecal DNA MoBio DNA Extraction Kit (MoBio Lab. Inc., Carlsbad, CA) as per the protocol, further purified using the Promega Wizard DNA Clean-Up System (Promega, Madison, WI), and quantified with a Perkin Elmer UV/Vis Spectrometer Lambda 14. The 16S rDNAs were amplified using the polymerase chain reaction (PCR) with the *Acidobacteria* specific forward primer ACD31F (3) and general bacterial broadly inclusive reverse primer 1492R (43, 44). Each 25 µl PCR reaction contained 1x PCR Buffer, 1 mM magnesium chloride, 0.03 mM of each dNTP, 0.2 µM each primer, and 5U taq polymerase (Invitrogen, Carlsbad, CA). The PCR program consisted of the following steps: (1) 95°C for 3 minutes, (2) 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds (repeated for 30 cycles), (3) 72°C for 10 minutes, and (4) a hold at 4°C. Purified genomic DNA from *A. capsulatum* (ATCC 51196) was used as a positive control. PCR reactions were electrophoresed on a 1% agarose gel and stained with GelStar Nucleic Acid Stain (BioWhittaker Molecular Applications, Rockland, MA). Gel images were captured using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 fitted with an ethidium bromide (590 nm bandpass) filter. PCR products were cloned into the Invitrogen TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) as per the protocol. The PCR product was then re-amplified

Table 2.2. Soil pH, percent soil moisture, carbon concentration, and nitrogen concentration.

Treatment, replicate[†]	*pH*[a]	*% Soil Moisture*[b]	*Carbon conc.*[c]	*Nitrogen conc.*[c]
T1 0-7cm R1	6.06 ± 0.05	14.94 ± 0.15	0.73 ± 0.17	0.08 ± 0.03
T1 0-7cm R2	6.28 ± 0.04	15.49 ± 0.06	0.81 ± 0.24	0.10 ± 0.03
T7 0-7cm R1	6.66 ± 0.03	19.26 ± 0.23	0.77 ± 0.32	0.09 ± 0.04
T7 0-7cm R4	6.83 ± 0.01	17.37 ± 0.12	1.02 ± 0.31	0.11 ± 0.04
T8 0-7cm R1	6.06 ± 0.06	23.57 ± 0.15	1.02 ± 0.31	0.13 ± 0.04
T8 0-7cm R2	6.19 ± 0.04	23.84 ± 0.10	1.60 ± 0.51	0.14 ± 0.04

[†] Treatment abbreviations (Table 2.1), depth, and replicate number are listed.
[a,b] Indicates average ± standard deviation (n=2).
[c] Carbon and nitrogen concentrations ± standard deviation is averaged data collected at the KBS LTER website from 1989 to 2001.

using the modified-M13F and modified-M13R (40) primers with the PCR conditions as described above. PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) upon a 1:8 dilution of the enzyme, incubated at 37°C for 30 minutes and 80°C for 15 minutes, and submitted for sequencing with the 531R primer (5' TAC CGC GGC TGC TGG CAC 3'). Sequencing reactions were sent to the Michigan State University Research Technology Support Facility (RTSF) (http://genomics.msu.edu/index.html). Sequences were aligned using ARB Software (47). Other sequences were downloaded from Genbank and the Ribosomal Database Project (RDP) (15) for the generation of phylogenetic trees.

Data analysis These acidobacterial 16S rRNA gene clone libraries (50 clones per library) were analyzed using the ARB Software (47). Known representatives of the eight monophyletic subdivisions were used to determine the relative percentage of soil clones in each subdivision. The relationship between the relative percentages of the subdivision and various edaphic properties was assessed using regression analysis.

Cultivation Experiments

Soil Sampling for Cultivation Soil samples were collected at various times between August 2001 and October 2002 from the KBS LTER, primarily treatment 8 along with treatments 1 and 7, and the KBS deciduous forest site. Soil cores (2.5 cm diameter x 10 cm depth) were collected from replicate plots and stored at 4°C until extraction typically no more than 48 hours after sampling. Five soil cores were homogenized under microoxic (vol/vol: 2% oxygen, 5% carbon dioxide, 93% nitrogen)

conditions and approximately 30 grams of soil was added to 100 mL of a phosphate-buffered salts solution (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10 mM; KH_2PO_4, 2 mM), buffered to a pH of 7.0, and amended with 2.24 mM sodium pyrophosphate ($Na_4P_2O_7*10H_2O$) added as a dispersal agent and 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) added as a reducing agent. The microoxic atmosphere was maintained within a flexible vinyl chamber fitted with an oxygen sensor/controller (Coy Laboratory Products, Grass Lake, MI). The soil suspension was stirred with a magnetic stir bar for 30 minutes, large soil particles were allowed to settle for 30 minutes, and an aliquot was used for 10-fold decimal dilutions in the same buffer used for the initial soil suspension. Aliquots (50 µl) of selected dilutions were then spread onto media of various compositions (below) with at least four replicate plates per dilution.

Total direct counts to determine the number of cells/gram of soil (dry wt.) were made by using 5-(4,6-dichlorotriazine-2-yl) aminofluorescein (DTAF) (4) with an epifluorescence microscope (Axioskop, Carl Zeiss Inc., Oberkochen, Germany) at 1000X magnification under blue illumination (BP 450-490 exciter filter, FT 510 nm beam splitter, and LP 520 nm barrier filter) in a dark room. Soil moisture was determined as described above and was used with total direct counts to estimate the number of cells/gram (dry wt.) of soil.

Media Composition and Incubation Conditions The isolation medium consisted of vitamins (excluding thiamine), inorganic salts, and trace elements as described previously (66); hereafter, this medium will be referred to as VSB-# (VSB- "vitamins and salts base"; # indicates the respective pH of the basal solution). VSB was buffered to pH 7.0

with 10 mM HEPES (VSB-7) and amended with one or more of the following additives, prepared in distilled water unless otherwise noted: soil extract, 10 % (vol/vol); a mixture of one-carbon compounds (methanol, 5 mM; methylamine, 3 mM; dimethylamine, 1 mM); washed agar; the humic acid analog anthraquinone-2,6-disulfonic acid disodium salt (AQDS; final concentration 25mM) as a potential electron acceptor; a mixture of N-acylhomoserine lactones (N-(butyryl, heptanoyl, hexanoyl, β-ketocaproyl, octanoyl, and tetradecanoyl)-DL-homoserine lactones) (Sigma-Aldrich Co., St. Louis, MO) prepared in ethyl-acetate acidified with 0.1% (vol/vol) acetic acid at a final concentration in the media of 1 μM each as possible growth-promoting signals; catalase, added directly to plates at a concentration of 65 U/ml or to cooled, molten agar just prior to pouring plates at 130 U/ml to protect cells from peroxides; and a mixture of yeast extract, Bacto protease peptone #3, casamino acids, and dextrose at 0.05 g/l each (Table 2.3). Plates were incubated for 20 to 30 days at 12°C or 23°C under one of the following atmospheres: CO_2-enriched hypoxia (vol/vol: 2% O_2, 5% CO_2, and bal N_2); CO_2-enriched air (vol/vol: 95% air, 5% CO_2), or air. Plates were screened for the presence of acidobacteria using the Plate Wash PCR (PWPCR) procedure described below. A chi-square test was used to assess the significance of treatments that increased the frequency with which acidobacteria colonies were detected on the plates. ANOVA was used to assess the significance of treatments on the total number of colonies formed.

Plate Wash PCR Replicate agar plates were selected for screening with group-specific 16S rRNA gene primers using the Plate Wash PCR (PWPCR) (66) (Figure 2.1). Plates were flooded with 2 mL of Bead Solution from the Ultraclean Dry Soil DNA kit

Table 2.3. Medium additives used for cultivation experiments.

Medium Additive	Rationale
N-acyl homoserine lactones (aHSL)	Quorum sensing compounds
Anthraquinone disulfonate (AQDS)	Humic acid analogue
Low temperature (12°C)	Facultative psychrophiles
Catalase	Protection from peroxides
Low light	Mimic soil environment, prevent peroxide formation
Elevated carbon dioxide concentrations (5% CO_2/95% air)	Requirement for carbon dioxide for growth; CO_2 fixation
Microoxic Oxygen Concentrations (2% oxygen, 5% CO_2, bal. N_2)	Microaerophilic lifestyle
Extended incubation times	Slow growing microorganisms (low *rrn* copy number)
Low nutrients (organic carbon)	Mimic soil environment, mimic oligotrophic environment
C1 compounds	Methylotrophy
Soil extract	Provide nutrients, cofactors, additives necessary for growth
Washed agar	Remove excess nutrients, mimic oligotrophic environment

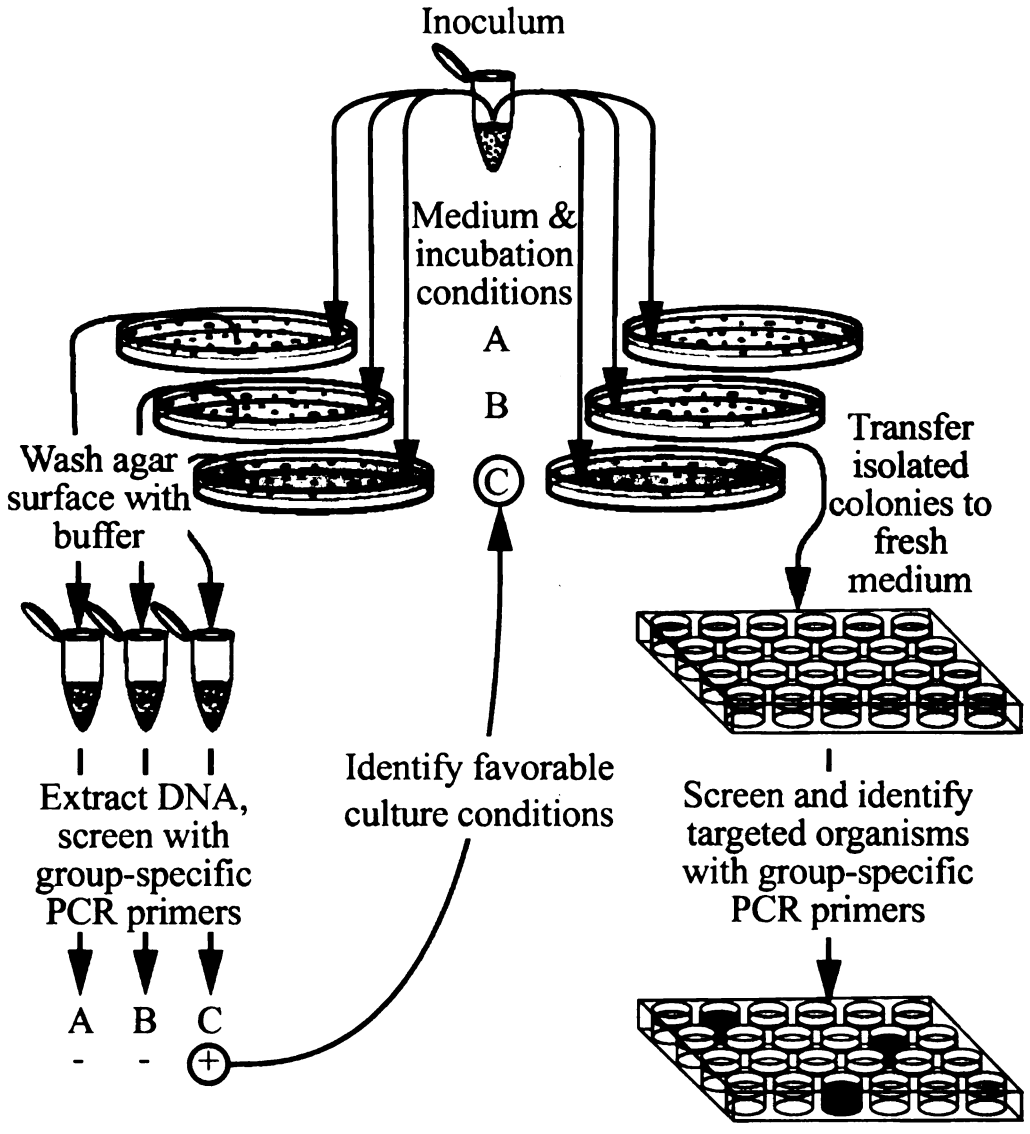


Figure 2.1. Plate Wash PCR method used to detect the presence of the target organism(s) on the three treatments (A, B, & C). Treatment C indicates a positive detection for the target organism (66).

for fecal samples (MoBio Laboratories, Carlsbad, CA) and a sterile spreader was used to scrape off colony material. The colony slurry was added to a dry bead tube from the soil DNA kit along with solutions S1 and IRS. To further aid in cell lysis, a 50 µl aliquot of lysozyme (50 mg/ml, Sigma-Aldrich, St. Louis, MO) was added to the resuspended colony material, and the tube was incubated for 45 minutes in a 56°C water bath. After this incubation step, cell lysis and DNA extraction were carried out per the manufacturer's protocol. The concentration and purity of each extracted DNA sample was estimated spectrophotometrically by determining the absorbance at wavelengths between 220 to 320 nm.

Isolation of novel acidobacteria from soil The plates were screened for the presence of acidobacteria using PWPCR (Figure 2.1) (66). Once media and atmospheres which supported the growth of acidobacteria were identified, all isolated colonies and patches of colonies were transferred to plates containing fresh medium and incubated under the same conditions. These isolated colonies or patches of cell growth from individual sectors were then grouped and screened for the presence of target organisms. Isolation plating ensued for patches of cell growth that were positive for target organisms.

RESULTS and DISCUSSION

***Acidobacteria* composition** The specific phylogenetic affiliations of partial sequences (ca. 500 nucleotides) of 100 cloned 16S rRNA genes (2 replicate libraries, consisting of 50 clones each) from 0 to 7 cm of treatments 1, 7, and 8 revealed the

presence of subdivision 1, 3, 4, 5, and 6 (Figure 2.2). Members of subdivisions 2, 7, and 8 were not detected because the 16S rRNA gene sequences of these subdivisions are not amplificable with the specific forward primer, ACD 31F (3).

When the relative percentage of each subdivision was represented as a pie chart, there was a clear indication that the subdivisions were responding to soil management (Figure 2.2) revealing that particular treatments selected for different subdivisions. The mid-successional treatment with no history of tillage (T8) contained members of subdivision 1 and 6 with their average percent composition of ca. 27% and 38%, respectively. The acidobacteria community from the mid-successional treatment with a history of tillage (T7) revealed that subdivision 1 became less dominant, while subdivision 4 and 6 became more dominant, ca. 35% and 43%, respectively. Finally, the conventional agricultural treatment (T1) was dominated by subdivision 4 with ca. 44% of the total acidobacterial community.

In summary, the mid-successional soil (T8) was dominated by members of subdivision(s) 1 and 6 whereas the conventional agriculture soil (T1) was dominated by members of subdivision 4 (Figure 2.3). The shift in the relative percentages of subdivision 4 from the mid-successional (T8) to the conventional agriculture (T1) soil were statistically significant ($p < 0.03$) with a Student's t-test; however the shifts in the composition of subdivision(s) 1 and 6 were not. These data taken together demonstrate that the management of agriculture resulted in a dramatic shift in the acidobacteria composition, with members of subdivision 4 becoming more dominant.

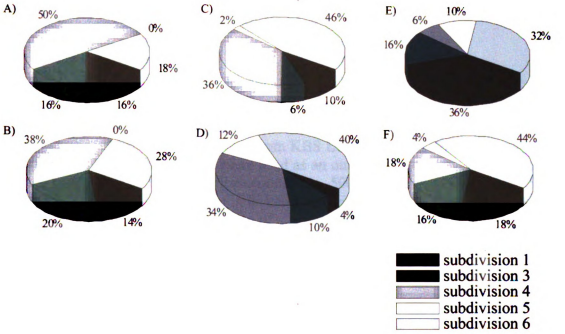
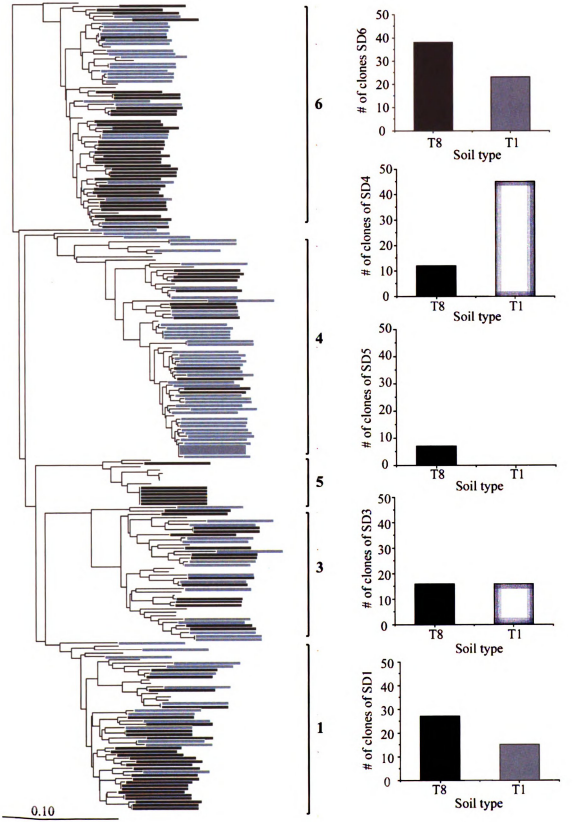


Figure 2.2. Distribution of *Acidobacteria* subdivisions among the different agricultural regimes: (A) T1 R1 0-7 cm; (B) T1 R2 0-7cm; (C) T7 R1 0-7cm; (D) T7 R4 0-7cm; (E) T8 R1 0-7cm; and (F) T8 R2 0-7cm.

Figure 2.3. Neighbor joining phylogenetic tree of the phylum *Acidobacteria* with 16S rRNA gene derived from DNA extracted from KBS LTER soil. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). All boxes in black and gray represent clones (partial sequence ca. 500 bp) from upper layers of T8 and T1, respectively. The subdivision is indicated to the right of the phylogenetic tree. Bar graphs are illustrated with the number of clones from each subdivision (SD#) with respect to the two treatments. Scale bar indicates 0.10 changes per nucleotide.


6
4
5
3
1
0.10
# of clones SD6
# of clones of SD4
# of clones of SD5
# of clones of SD3
# of clones of SD1
50
40
30
20
10
0
T8
T1
Soil type

Influence of soil properties The relative percentage of clones of each subdivision varied with depth and treatment, suggesting edaphic properties may influence the distribution of the various subdivisions. Members of subdivision 3 correlated positively to soil moisture ($p < 0.09$, $\alpha = 0.10$) (Figure 2.4) suggesting that wetter soils are preferred; 32% of the increase in relative percentage of subdivision 3 can be explained by soil moisture at the time of sampling. This suggests that members of subdivision 3 might be capable of growing under low or no oxygen conditions, given their prevalence in wetter soils that are typically oxygen depleted. Additionally, in situ higher soil moisture can influence the activity of the microbial community by enhancing the diffusion of nutrients (13) thus influencing the community structure (6, 7, 11) and activity (20, 65) of soil microbes.

The relative abundance of subdivision 1 clones in each treatment appeared to be related to soil pH, with the greatest abundance occurring in mildly acidic soils (Figure 2.5). This is consistent with recent publications regarding the physiology of members of subdivision 1. Members of subdivision 1 formed more colonies in media with pH between 5.5 and 7.0 (56) and recent characterization of subdivision 1 strains in the genus *Terriglobus* (25), indicated that the optimal pH range for this subdivision is between 5.0 and 6.0 (Chapter 3) (25). Taken together, these results suggest that members of subdivision 1 of the *Acidobacteria* have a preference for mildly acidic pH conditions.

Previous studies have implicated members of subdivision 1 with the capacity to oxidize one-carbon compounds such as methanol (51). There was a negative correlation between the net influx of methane into the soil and the relative percentage of subdivision 1 ($p < 0.07$, $\alpha = 0.10$) (Figure 2.6). A larger proportion of the acidobacterial community

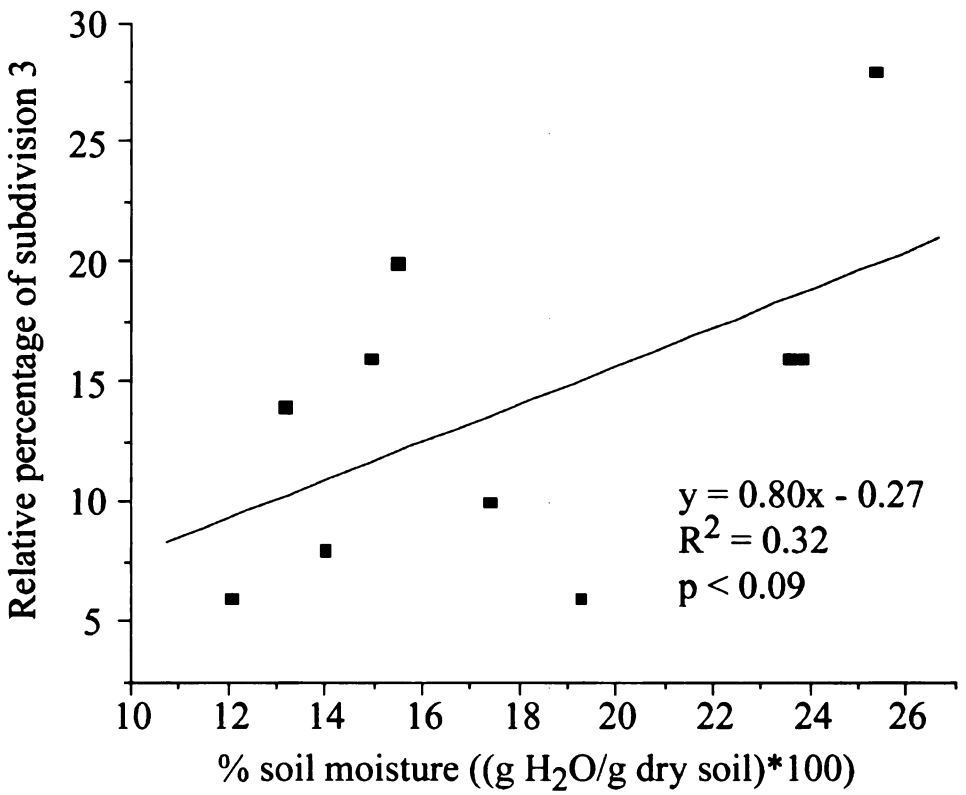


Figure 2.4. Relationship between relative percentage of acidobacteria subdivision 3 and % soil moisture.

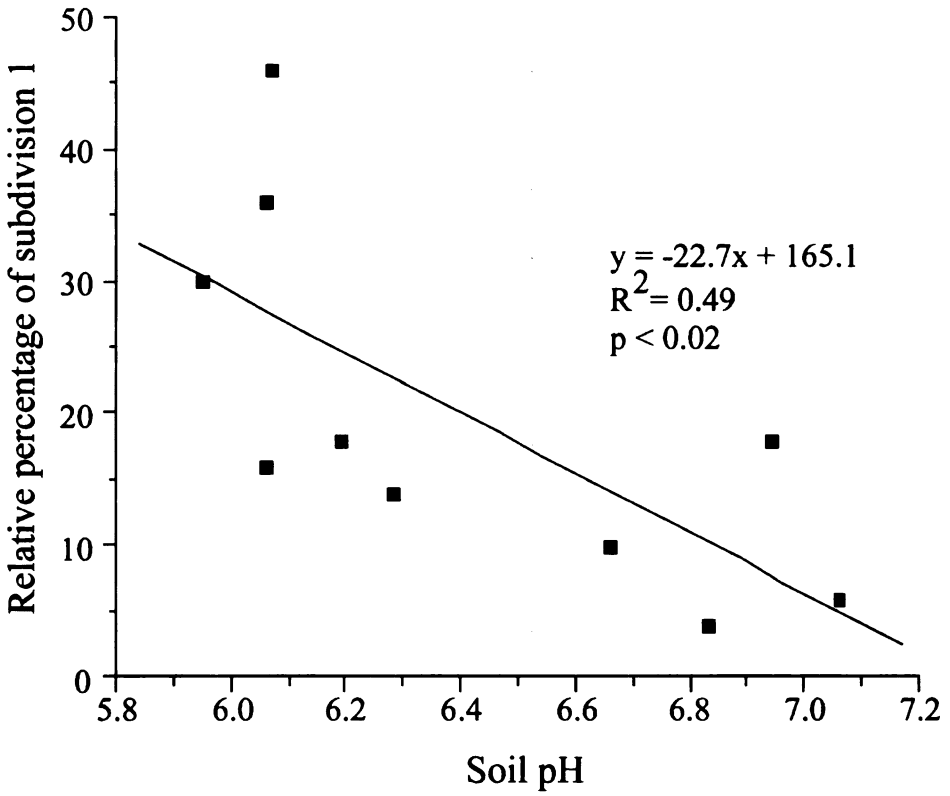


Figure 2.5. Relationship between relative percentage of acidobacteria subdivision 1 and soil pH.

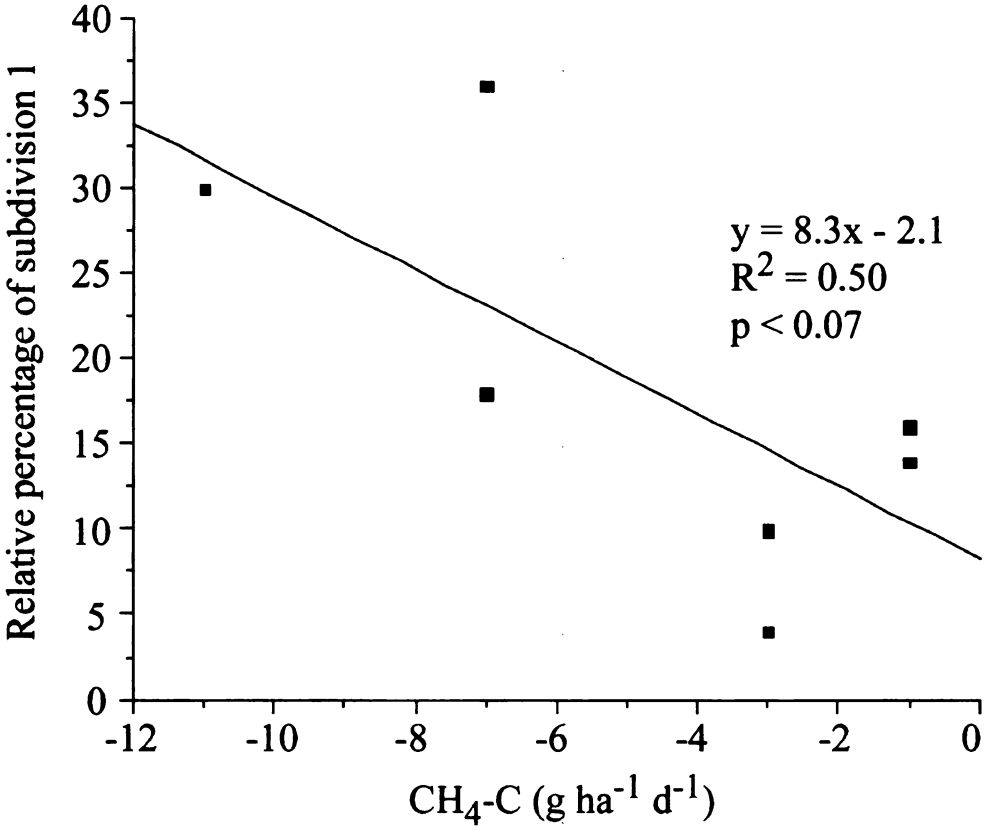


Figure 2.6. Relationship between the relative percentage of subdivision 1 and methane fluxes. Methane data was averaged for each treatment from historical data obtained from the KBS LTER website for methane flux data collected between 1992 and 2001; a detailed description of the sampling can be found in a previous study by Robertson and collegues (55).

contained members of subdivision 1 when there was approximately a 6-fold increase in the total methane flux into the soil. The affinities for known methane-oxidizing microorganisms and environmental concentrations of methane do not correlate suggesting that there could be an unexplored population of methanotrophs which is responsible for this discrepancy (17); perhaps members of subdivision 1 are a bacterial group responsible for this discrepancy or they could respond favorably to nearby methanotrophic communities.

A statistically significant negative correlation was observed between the relative percentage of acidobacteria subdivision 4 and the carbon concentration of each site ($p < 0.0008$) (Figure 2.7). Ninety-one percent of the increase in the relative abundance of subdivision 4 can be explained by the lower carbon concentrations suggesting that members of subdivision 4 might be oligotrophic, preferring lower carbon conditions for growth. Additionally, these data suggest that members of subdivision 4 are better competitors for carbon since they were found more prevalently in low carbon environments which coincided with environments with the higher nitrous oxide fluxes. Their percentages correlated positively to these fluxes ($p < 0.01$) (Figure 2.8) suggesting preliminarily that they either responded favorably to the nearby denitrifying community or are capable of denitrification since typically denitrifiers are good competitors for carbon, which is a major factor responsible for their distribution (71).

Cultivation experiments The average recovery of soil microorganisms was 3.5 to 5 x 10^7 CFU/gram of soil (dry wt), which based on total direct counts, is 2.5 to 6% of the total microbial community (Figure 2.9, panel A). These recoveries were similar to

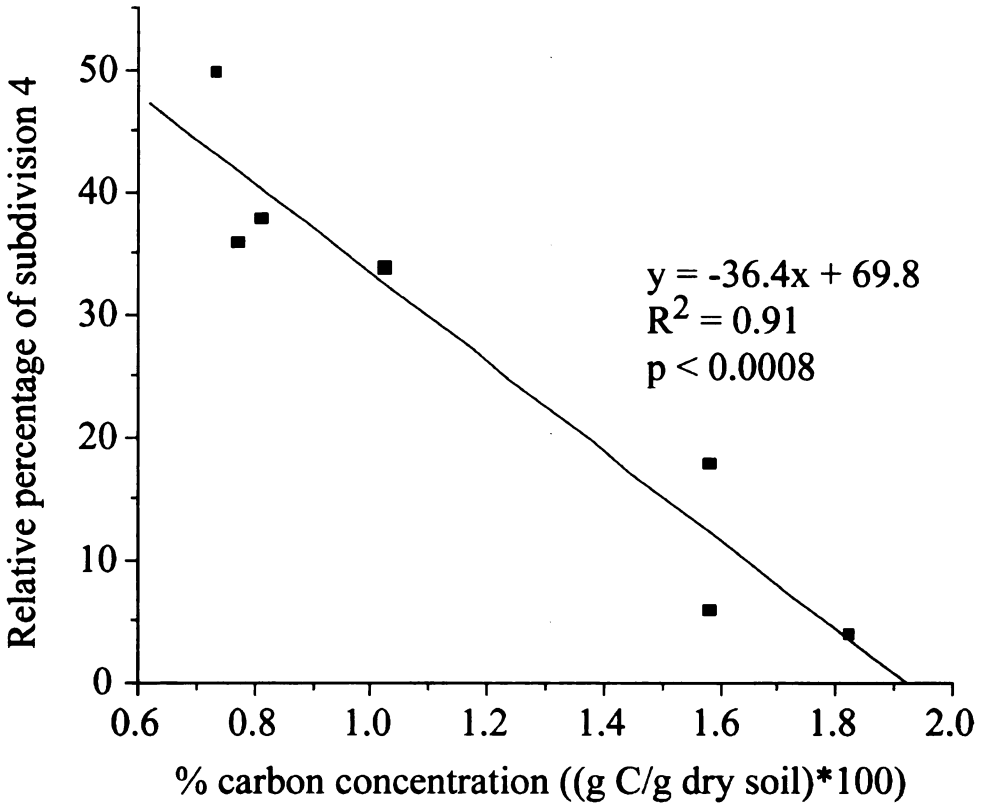


Figure 2.7. Relationship between relative percentage of acidobacteria subdivision 4 and carbon concentration. Carbon data was averaged for each treatment from historical data obtained from the KBS LTER website for carbon data collected between 1989 and 2001; a detailed description of the sampling can be found at the KBS LTER website (http://lter.kbs.msu.edu/).

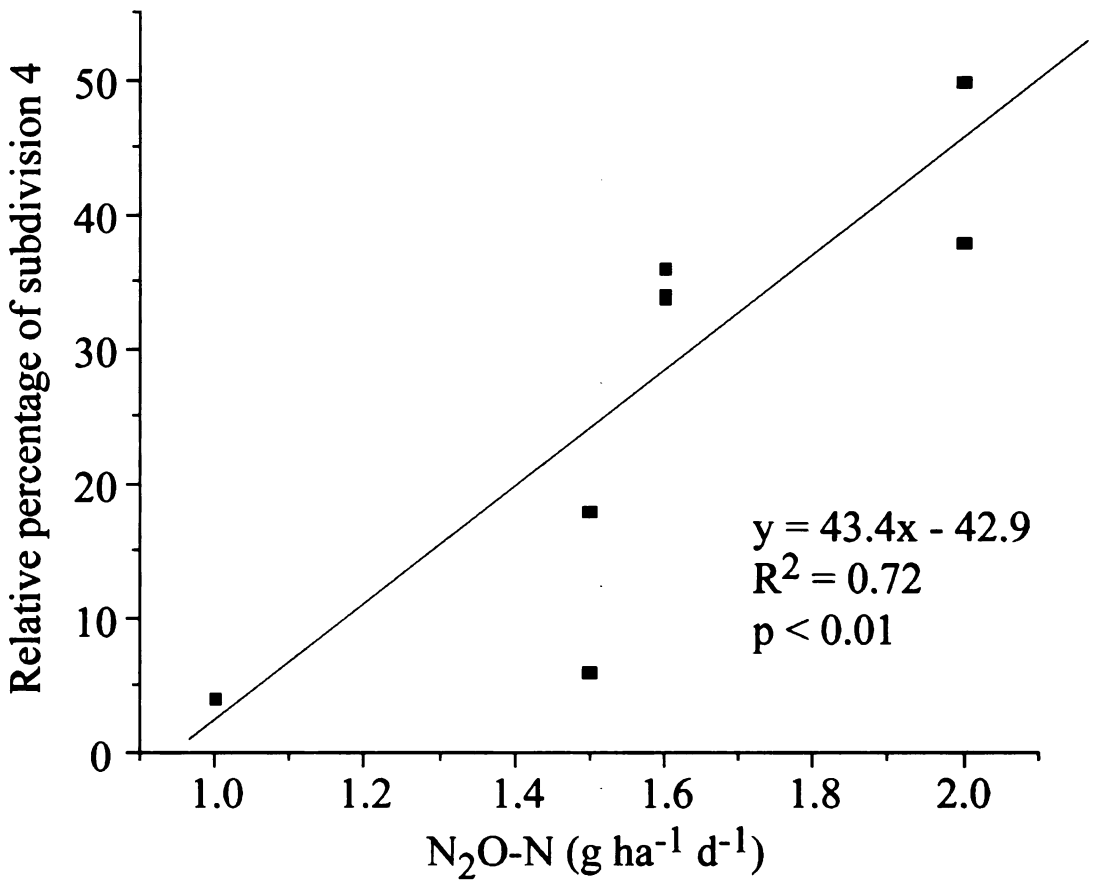
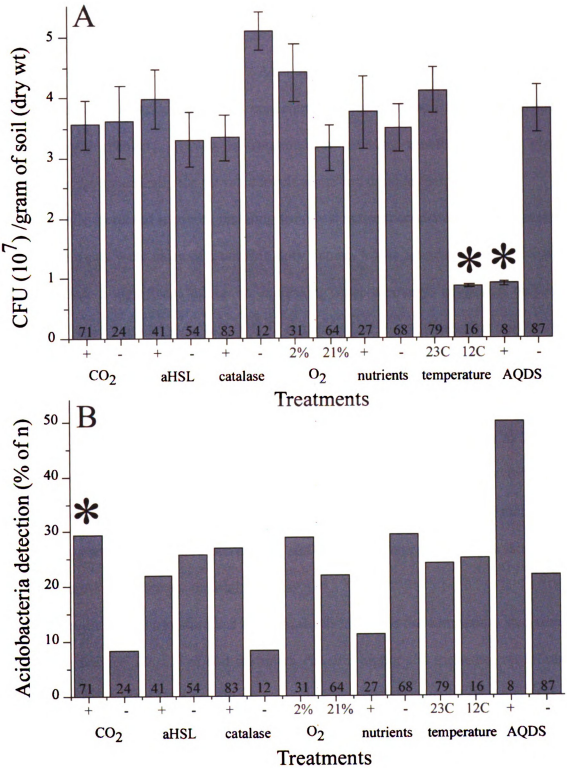


Figure 2.8. Relationship between the relative percentage of subdivision 4 and nitrous oxide fluxes. Nitrous oxide data was averaged for each treatment from historical data obtained from the KBS LTER website for nitrous oxide flux data collected between 1992 and 2001; a detailed description of the sampling can be found in a previous study by Robertson and collegues (55).

Figure 2.9. Summary of cultivation experiments for total recovery of colonies per gram of soil (dry weight) (Panel A) and acidobacteria detection (% of n) (Panel B). Panel A represents the average CFU recovery from soil +/- the standard error; the number of times a particular treatment was employed (n) is listed at the base of each bar. Panel B represents the percentage of treatments that yielded a positive test for acidobacteria using PWPCR from the plates of panel A. The asterisks "*"indicates treatments that yielded significant difference (panel A, ANOVA $\alpha = 0.05$; panel B, chi square goodness of fit, $\alpha = 0.10$).

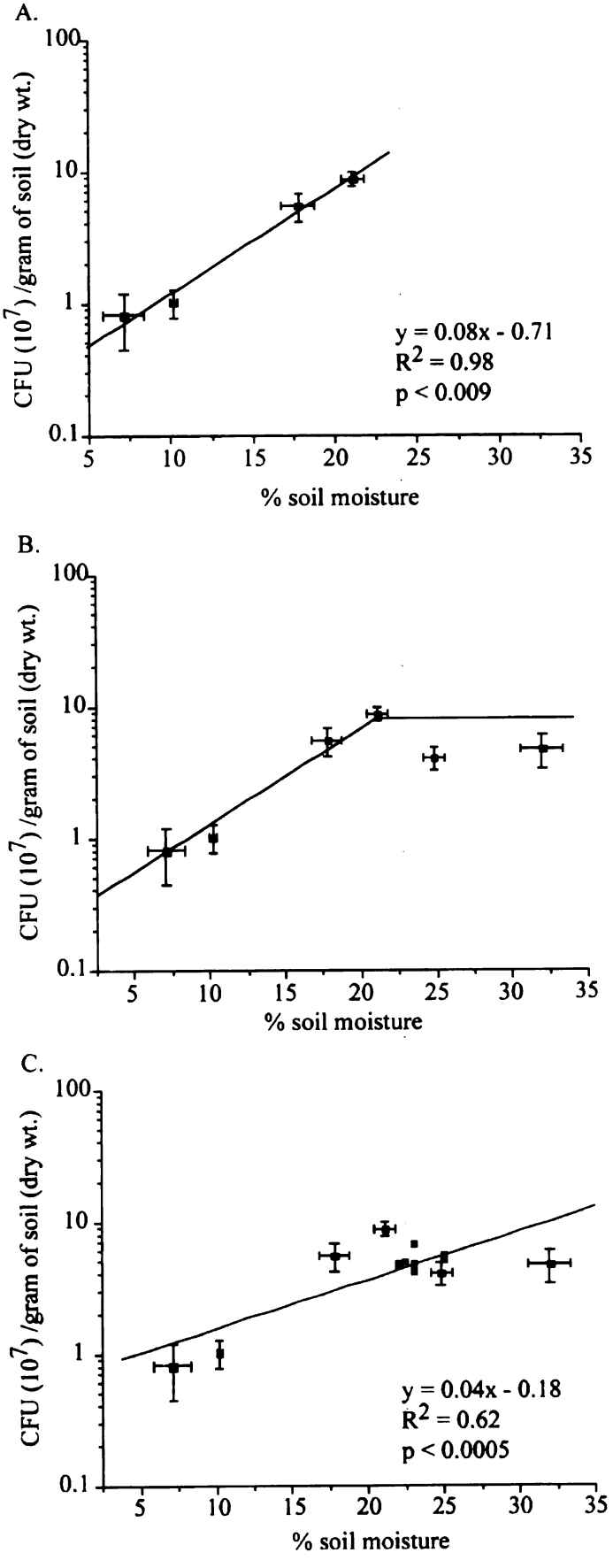

A
CFU (10^7)/gram of soil (dry wt)
0
1
2
3
4
5
71 24 41 54 83 12 31 64 27 68 79 16 8 87
+ - + - + - 2% 21% + - 23C 12C + -
CO_2 aHSL catalase O_2 nutrients temperature AQDS
Treatments
B
Acidobacteria detection (% of n)
0
10
20
30
40
50
71 24 41 54 83 12 31 64 27 68 79 16 8 87
+ - + - + - 2% 21% + - 23C 12C + -
CO_2 aHSL catalase O_2 nutrients temperature AQDS
Treatments

cultivation experiments with low nutrients and extended incubation times (16, 37). All treatments were identical in terms of recovery with the exception of the inclusion of AQDS or were incubated at 12°C. This suggests that the extended incubation time was responsible for the increased recovery. Although the use of our novel cultivation strategy of incubation under atmospheric gas concentrations native to soil did not significantly increase total recovery, it was instrumental in isolating members of the phylum *Acidobacteria*, specifically the elevated levels of carbon dioxide (below).

While increased recovery resulting from low nutrient concentrations and extended incubation times were more of a confirmatory nature, it was interesting to note that soil moisture had a significant effect on increasing total recovery, suggested only once previously in the mid-1930s (69). Soil moisture had a positive effect on the total recovery of aerobic, heterotrophic soil microorganisms after 20 to 25 days of growth from treatment 8 soil (Figure 2.10, panel A), similar to a previous study with garden soil (Figure 2.10, panel C, gray boxes). As soil moisture increased from 7% to 21%, the number of colony forming units significantly increased ca.10-fold ($p < 0.009$), but recovery reached an asymptote above a soil moisture of 21% (Figure 2.10, panel B). The asymptotic nature of the curve above 21% soil moisture suggests that there is a limit of soil moisture's effectiveness on total recovery.

Previously soil moisture has been shown to influence the community structure (6, 7, 11) and activity (20, 65) of soil microbes. In situ, higher soil moisture can influence the activity of the microbial community by enhancing the diffusion of nutrients (13) and decreasing oxygen tension (53, 62). Conversely, the low recoveries resulting from low

Figure 2.10. Relationship between total recovery of aerobic, heterotrophic soil bacteria from T8 soil and % soil moisture. Panel A depicts a significant increase in total recovery up to 21% soil moisture, whereas panel B illustrates the limit of soil moisture influence on total recovery. Panel C represents the effect of recovery on aerobic heterotrophs from KBS LTER treatment 8 soil (black boxes) and a garden plot (gray boxes) (69).


A.
100
10
1
0.1
CFU (10^7) /gram of soil (dry wt.)
y = 0.08x - 0.71
R^2 = 0.98
p < 0.009
5
10
15
20
25
30
35
% soil moisture
B.
100
10
1
0.1
CFU (10^7) /gram of soil (dry wt.)
5
10
15
20
25
30
35
% soil moisture
C.
100
10
1
0.1
CFU (10^7) /gram of soil (dry wt.)
y = 0.04x - 0.18
R^2 = 0.62
p < 0.0005
5
10
15
20
25
30
35
% soil moisture

moisture soil inoculum suggests that osmotic stress may have been detrimental to these cells. Drier soils typically have a low water potential, meaning it is harder for an organism to extract water from the environment, therefore water can become limiting. In order for organisms to maintain their cell integrity, they either produce compatible organic solutes or take up ions from surrounding environment (19) to create a high intercellular solute concentration to promote an inward diffusion of water. When taken from this low water potential environment and introduced to a high water potential environment, such as the creation of a soil suspension solution for plating, these cells could be more susceptible to cell lysis resulting from the rapid influx of water to their high intercellular solute concentration. This scenario could drastically reduce their culturability as compared to cells originating from a high water potential environment. Osmotic stress has been shown previously to be responsible for decreased culturability of pure cultures, especially in gram-negative bacteria (30). Our results suggest that soil moistures above 21% produce a sufficiently low water potential environment conducive with the creation of our soil suspension.

Although the treatments tested did not reveal significant increases in total recovery of aerobic heterotrophs from soil, the inclusion of N-acyl homoserine lactones (Figure 2.9, panel A, aHSL) and incubation under microoxic conditions (Figure 2.9, panel A, 2% O_2) did increase the total recovery. The inclusion of N-acyl homoserine lactones increases the recoverability of soil microorganisms ca. 1.2-fold, as seen previously (9, 12), perhaps due to gene activation for such activities as siderophore production (28), biofilm formation (22), starvation survival (70), or utilization (35, 73). The concentrations of oxygen were manipulated during incubation to better simulate

conditions in soil aggregates, and it increased the total recovery ca. 1.4-fold. Although upland soils like those at the KBS LTER are generally well-aerated, they include pockets of anoxia within soil aggregates, and hence also include transitions zones between oxic and anoxic conditions (34, 61).

Detection and isolation of acidobacteria with PWPCR The PWPCR method, in addition to isolating novel members of the acidobacteria (Figure 2.11), revealed insight into this phylum through the detection of acidobacteria over the multitude of treatments (Figure 2.9, panel B). Across treatments used for isolation, acidobacteria were detected more frequently under atmosphere(s) containing elevated levels of carbon dioxide ($0.05<p<0.10$), suggesting that they might be capable of using elevated levels of carbon dioxide perhaps through autotrophic pathways.

Additionally, acidobacteria were more frequently detected under low carbon concentrations and along with extended incubation times could have provided the ideal conditions to isolate potential oligotrophic acidobacteria (25). The use of low carbon concentrations has been shown previously to improve the recovery of bacteria from soils (31, 32, 67, 68), marine environments (16), heterotrophic planktonic bacteria from lake water (12), and more diverse communities of pseudomonas from soil (1). The inclusion of the catalase enzyme as well as incubation under microoxic conditions increased the frequency of acidobacteria detection by ca. 3.3- and 1.3-fold, respectively. Taken together, this result suggests that some acidobacteria are microaerophiles, preferring low levels of oxygen. Furthermore, the catalase enzyme decreased overall recoveries of soil microorganisms (Figure 2.9, panel A), but increased acidobacteria detection

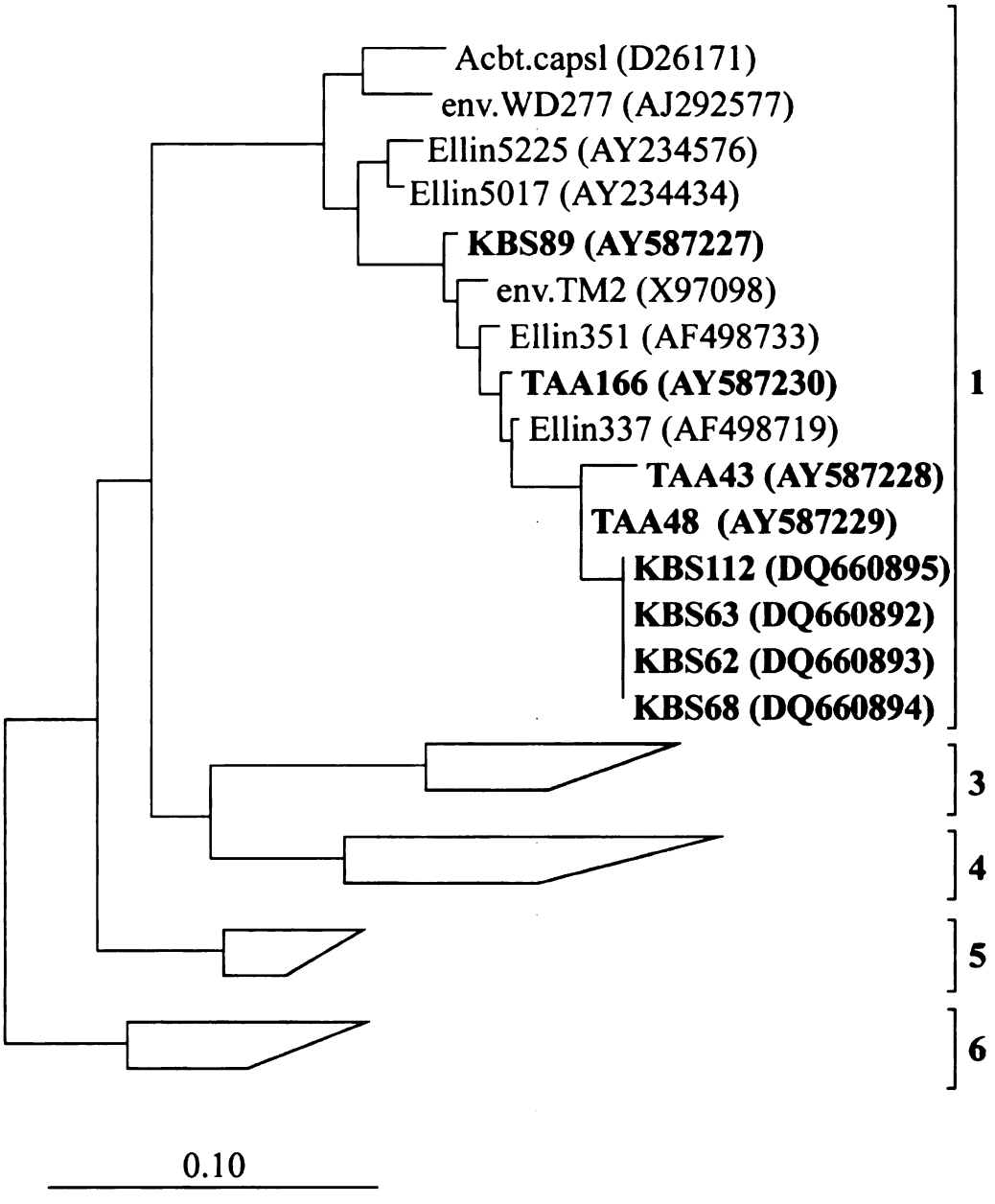


Figure 2.11. Maximum likelihood tree of the *Acidobacteria* subdivisions 1, 3, 4, 5, and 6 (indicated to the right of the group) based on the 16S rRNA gene using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). Strains from this study are in bold text. Each gray trapezoid represents a different subdivision. The scale bar indicates 0.10 changes per nucleotide.

(Figure 2.9, panel B), thereby potentially selecting for microaerophilic acidobacteria. Previous studies suggest that catalase-negative microorganisms are abundant in the soil environment (14, 27), and the inclusion of the catalase enzyme would provide protection for cells from dangerous peroxides resulting from the oxidation of the low concentrations of added carbon to the plates. Micro-zones of elevated carbon dioxide and/or lower levels of oxygen located in the soil matrix provide a great range of habitats and niches which are ideal for soil microorganisms.

SUMMARY

Soil is a heterogeneous environment containing over one million species of bacteria per gram (26), however, these microorganisms are poorly understood, since they are not well represented in culture, such as members of the phylum *Acidobacteria.* Before the start of these experiments, there was little to information available on this diverse phylum, except for the information on the historical strains, *A. capsulatum*, *H. foetida*, *G. fermentas* and generic soil surveys illustrating the phylum's ubiquity. The acidobacteria 16S rRNA gene survey in this study generated useful information pertaining to the composition of the acidobacteria community among the KBS LTER treatments along with an assessment of the impact certain edaphic properties have on the eight, monophyletic subdivisions.

Figure 2.12 represents a summary for the changes in the acidobacteria community composition in relation to edaphic properties observed at the KBS LTER. Subdivisions 1, 3, 4, 5, and 6 were present in the soils at the KBS LTER, with members of subdivision 4 being the dominant subdivision in the conventional agriculture treatment and

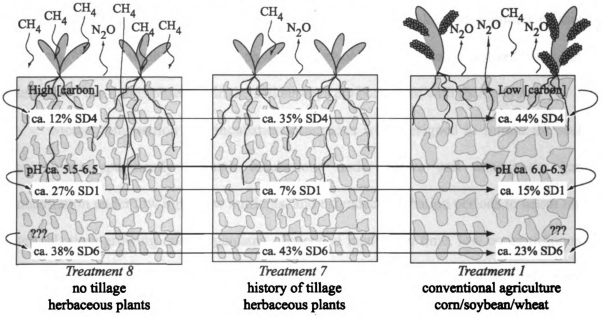


Figure 2.12. Summary of acidobacteria community composition and the edaphic properties that influence the composition of subdivisions (SD) 1, 4, and 6.

subdivisions 1 and 6 being dominant in the mid-sucessional community with no history of tillage. Additionally, the subdivisions responded to soil moisture (subdivision 3), carbon concentration (subdivision 4), soil pH (subdivision 1), methane fluxes (subdivision 1), and nitrous oxide fluxes (subdivision 4). These data, along with general knowledge about the soil environment, were instrumental in the development of cultivation methods used to isolate these strains as well as additional strains (Chapter 4). Moreover, these trends may help to bear insight into the physiology of members of this phylum. When members of this phylum have been successfully isolated, we can determine if the trends observed in the soil surveys are consistent with their physiology.

Media composition and incubation conditions were designed to mimic the soil environment, specifically through incubations under native soil atmosphere gas concentration under low light, low nutrients, and extended incubation periods to select for potential, slow-growing microorganisms. Extended incubation time, low concentrations of nutrients, and soil inoculum with high moisture (significantly) improved total recovery of aerobic heterotrophs from soil. A facile, high- throughput method called Plate Wash PCR (PWPCR) was developed to rapidly screen a multitude of plates for the target organism(s) using group-specific primers. These methods lead to the successful isolation of *Acidobacteria* as well as providing trends linking acidobacteria detection to our various amendments and treatments which helped to bear insight into their physiology.

In conclusion, the microbial community in the soil is an area we know very little about. The need to retrieve this not yet cultured majority (8, 52) is becoming more and more evident with the footprint of the soil community becoming more defined with molecular surveys. The retrieval of these recalcitrant microbes requires patience and an

understanding of environmental factors that affects their composition. These studies suggest that a combination of molecular and growth-based approaches such as those realized in this study are the strategies needed to isolate these "uncultivables."

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CHAPTER 3

ISOLATION AND CHARACTERIZATION OF SOIL BACTERIA THAT DEFINE *TERRIGLOBUS* GEN. NOV., IN THE PHYLUM *ACIDOBACTERIA*

These results have been published in the article: Eichorst, S.A., J.A. Breznak, and T.M Schmidt. 2007. Isolation and characterization of soil bacteria that define, *Terriglobus* gen. nov., in the phylum *Acidobacteria*. Appl. Environ. Microbiol. Vol. 73, No. 8, pgs. 2708-2717.

INTRODUCTION

Soils typically contain 10^9-10^{10} microorganisms per gram (dry weight) that may represent more than a million bacterial species (21). However, characterization of the small fraction of microbes that has been cultivated provides only a glimpse of their physiological capacity and potential impact on soil ecosystems. The absence of pure cultures or genome sequences makes it difficult to ascertain the roles of specific microbes in soil environments: this is particularly true for bacteria in the phylum *Acidobacteria,* which are broadly distributed in soils but poorly represented in culture.

The phylum *Acidobacteria* is defined by a large collection of 16S rRNA gene sequences (>1,500 in the Ribosome Database Project) (10) retrieved from diverse environments including: soils and sediments (3, 17), soil crusts of sand dunes (69), wastewater (13, 41), water distribution systems (49), peat bogs (15), acid mine drainage (33), hot springs (26), shallow submarine hydrothermal vents (67), and on the surface of

Paleolithic cave paintings and catacombs (62-64, 75, 76). I*n situ* hybridization with acidobacteria-specific probes has also confirmed the presence of intact acidobacteria in many environments, and revealed multiple cellular morphotypes, including cocci, short rods, and thin filaments (46).

Acidobacterium capsulatum was the first described member of the phylum *Acidobacteria.* It was among eight strains isolated from acidic mine drainage and acidic muds in the early 1990's (33, 34). This was the first report of the isolation of acidophilic, heterotrophic bacteria from an acidic mineral environment other than *Acidiphilium* (33). By the mid-1990's, the growing collection of ribosomal RNA gene sequences from molecular surveys of diverse environments resulted in the recognition of *A. capsulatum* as a member of a large, deeply branching, monophyletic lineage within the *Bacteria* (25). The phylum *Acidobacteria* was named after the only described species at the time (46).

Shortly after the characterization of *A. capsulatum*, *Holophaga foetida* was isolated and described as a novel, homoacetogenic bacterium capable of degrading methoxylated aromatic compounds (43). Based in part on the 81.6% divergence in the 16S rRNA gene sequences of these two cultivars, the phylum was sometimes referred to as *Holophaga/Acidobacterium.* G*eothrix fermentans,* a strain capable of Fe(III) reduction, was later placed in the *Holophaga/Acidobacterium* phylum based on the similarity of its 16S rRNA gene sequence to that of *H. foetida* (ca. 94%) (9).

The phylum *Acidobacteria* is now officially recognized in the Bergey's Manual of Systematic Bacteriology (22) and includes three genera with cultured representatives: *Acidobacterium* (33), *Geothrix (9)*, and *Holophaga (43).* The genus *Solibacter* was recently proposed as the fourth genus in this phylum (www.jgi.doe.gov). There are

currently eight recognized monophyletic subdivisions within this phylum (28) that encompasses the molecular diversity first recognized as *Acidobacteria* (38), and additional unnamed and mostly uncharacterized cultivars in subdivisions 1, 2, 3, and 4 (14, 29-31, 60, 61, 70). A recent survey of 23S rRNA genes in microbial communities associated with Paleolithic paintings uncovered additional novel acidobacteria, expanding the number of subdivisions to as many as eleven (76).

The breadth of divergence of 16S rRNA gene sequences in the phylum *Acidobacteria* (ca. 77% based on >1000 nearly full length sequences, this study) is similar to that within the metabolically diverse phylum *Proteobacteria* (28), suggesting the capacity for extensive metabolic diversity. Acidobacteria are oftentimes the most abundant bacteria represented in molecular surveys of soil environments: as many as half of all clones from Arizona soil samples clustered in the phylum *Acidobacteria* (16), as did more than 40% of PCR-amplified and cloned rRNA-encoding genes in soils of alpine ecosystems (44). In a comprehensive review of acidobacterial abundance in soil communities (29), acidobacteria averaged ca. 20% of the total bacterial community. Although the metabolic potential of acidobacteria is poorly described, their abundance suggests a major impact on nutrient cycling in soil environments.

To learn more about the metabolic properties and potential ecological roles of members of this poorly explored phylum, we sought to cultivate and characterize new strains from terrestrial habitats. Using incubation conditions and media designed to mimic their natural environment, eight strains of the phylum *Acidobacteria* were isolated from soil as well as from the hindguts of soil-dwelling termites. These strains were characterized, with emphasis on properties that may bear on their ecological roles in soil.

Results from the physiological and phylogenetic characterization warrant creation of a new genus, *Terriglobus*.

MATERIALS and METHODS

Phylogenetic survey and isolation of strains Soil samples were collected between August 2001 and August 2005 from the Michigan State University W.K. Kellogg Biological Station Long Term Ecological Research (KBS LTER) site. The KBS LTER is a 48-hectare research site established in 1989 to study ecological processes in agroecosystems. The dominant soil types are of the Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs) series. A detailed description of this site can be accessed at http://www.kbs.msu.edu.

For the survey of acidobacterial diversity, soil cores (2.5cm diameter x 20 cm depth, divided into 0-7cm and 13-20cm) were collected from four different treatments at the KBS LTER: treatment 1 (T1) is managed as a conventional agriculture site with a rotation of corn/soybean/wheat; treatment 7 (T7) is a successional plant community on historically tilled soil – tillage was abandoned after spring plowing in 1989; treatment 8 (T8) is a successional plant community on never-tilled soil; and the fourth treatment is native deciduous forest. Five soil cores per plot were pooled and homogenized using a 2-mm sieve, portions of the sieved soil were used to determine soil pH and soil moisture (58), and the remaining soil was frozen immediately in liquid nitrogen, and stored at -80 ° C until use. DNA was extracted from soil samples using the UltraClean Fecal DNA MoBio DNA Extraction Kit (MoBio, Carlsbad, CA). The 16S rRNA genes were amplified from the DNA by using the PCR with an acidobacterial-specific forward

primer (ACD31F: 5' GAT CCT GGC TCA GAA TC 3') (3) and a broadly inclusive bacterial reverse primer (1492R: 5' GGT TAC CTT GTT ACG ACT T 3') (39, 40). Each 25µl PCR reaction contained 1x PCR buffer, 1mM $MgCl_2$, 0.03mM of each dNTP, 0.2µM each primer, and 5U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Thermal cycling consisted of the following steps: (1) 95°C for 3 minutes; (2) 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds (repeated 30X); (3) 72°C for 10 minutes. Genomic DNA purified from *A. capsulatum* (ATCC Number 51196) was used as a positive control. PCR products were electrophoresed through a 1% agarose gel in 0.5x tris-borate-EDTA (TBE) and visualized with GelStar Nucleic Acid Stain (BioWhittaker Molecular Applications, Rockland, MA).

PCR products were cloned with the Invitrogen TOPO TA Cloning Kit for Sequencing (Invitrogen Carlsbad, California) and the inserts were re-amplified using the modified-M13F (F2) (5' CAG TCA CGA CGT TGT AAA ACG ACG GC 3') and modifed-M13R (F4) (5' CAG GAA ACA GCT ATG ACC ATG 3') (32) with the PCR conditions described above. The PCR products for sequencing were treated with ExoSAP-IT (USB, Cleveland, OH) using a modification of the manufacturer's protocol (ExoSAP enzyme was diluted 1:8 and the reaction was incubated at 37°C for 30 minutes, followed by an 80°C incubation for 15 minutes) and submitted for sequencing with the 531R (5' TAC CGC GGC TGC TGG CAC 3') primer. Sequencing was performed at the Michigan State University Research Technology Support Facility (genomics.msu.edu/index.html). Sequences were aligned with other sequences downloaded from GenBank using an integrated aligner (ARB Software) (47) as well as manual corrections based on secondary structure models of the 16S rRNA gene.

For isolation attempts, five cores (2.5 cm diameter x 10 cm depth) from the never tilled, successional treatment (T8) were collected and homogenized with a sterile spatula in a 500mL beaker under a hypoxic atmosphere (vol/vol: 2% O_2, 5% CO_2, 93% N_2) within a flexible vinyl chamber fitted with an oxygen sensor/controller (Coy Laboratory Products, Grass Lake, MI). Portions of the homogenized soil were used to determine soil moisture (58). Approximately 30 grams of soil were added to 100 mL of a phosphate-buffered salts solution (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4 and 2 mM KH_2PO_4); pH was adjusted to 7.0, and supplemented with 2.24 mM $Na_4P_2O_7 \cdot 10H_2O$ as a dispersal agent, and dithiothreitol (1mM) as a reducing agent (73). The soil suspension was stirred vigorously with a magnetic stir bar for 30 minutes. Denser soil aggregates were then allowed to settle for 30 minutes, after which an aliquot of the supernatant fraction was used for total direct cell counts after staining with 5-(4,6-dichlorotriazine-2-yl) aminofluorescein (DTAF) (4). Another aliquot was used to prepare 10-fold decimal dilutions in the same buffer for subsequent inoculation of the isolation medium consisting of vitamins (excluding thiamine), inorganic salts, and trace elements as described previously (70); hereafter, this composition will be referred to as VSB-# (VSB- "vitamins and salts base"; # indicates the respective pH of the basal solution). VSB was buffered to a pH 7.0 with 10mM HEPES (VSB-7) and amended with one or more of the following additives, prepared in distilled water unless otherwise noted: the humic acid analog anthraquinone-2,6-disulfonic acid disodium salt (AQDS; final concentration 25mM) as a potential electron acceptor; a mixture of N-acylhomoserine lactones prepared in ethyl-acetate acidified with 0.1% (vol/vol) acetic acid at a final concentration in the media of 1 uM each N-(butyryl, heptanoyl, hexanoyl, β-ketocaproyl, octanoyl, and tetradecanoyl)-

DL-homoserine lactones (Sigma-Aldrich Co.) as possible growth-promoting signals; catalase, added directly to plates at a concentration of 65 U/ml or to cooled, molten agar just prior to pouring plates at 130 U/ml to protect cells from peroxides; and a mixture of yeast extract, Bacto protease peptone #3, casamino acids, and dextrose at 0.05 g/l each. Cultivation conditions for samples from the hindguts of soil-dwelling termites, *Reticulitermes flavipes*, have been described previously (70).

Plates were incubated for 20-30 days at 12°C or 23°C under one of the following atmospheres: CO_2-enriched hypoxia (vol/vol: 2% O_2, 5% CO_2, and bal N_2); CO_2-enriched air (vol/vol: 95% air, 5% CO_2), or air. Plates were screened for the presence of acidobacteria using Plate Wash PCR (PWPCR) (70). A chi-square test was used to assess the significance of treatments that increased the frequency with which acidobacteria colonies were detected on the plates. ANOVA was used to assess the significance of any treatments on the total number of colonies formed.

Optimization of Growth Rate VSB was amended with a mixture of organic compounds (yeast extract, protease peptone, casamino acids, and glucose - 0.15 g/l each) to assess capacity for growth under a range of conditions. This complex medium (designated VSM-#, where # = pH) was used to determine the effect of pH on growth rate. Strains were grown at room temperature (23°C) between pH 2.0 and 7.5 at intervals of 0.5 pH units: MES was used to buffer at pH ranges between 5.0-6.0, 4-morpholinepropanesulfonic acid (MOPS) was used at pH ranges between 6.5-7.5, and citric acid was used as a buffer below pH 5.0, all at a final concentration of 10mM. Culture tubes (Bellco™ cat. no. 2048-18150) containing 5-10 mL of medium, were

stoppered under an air atmosphere and held on a reciprocating shaker operating at ca. 190 strokes/min. to disrupt cellular flocs. Optical density at 600nm was monitored periodically with a Thermo™ Spectronic model 20D+ spectrophotometer. Inocula consisted of a 1:100 dilution of cultures in mid/late log phase growth (ca. $1x10^8$ cells/mL) in R2B - a complex medium containing yeast extract, amino acids, peptone, carbohydrates, pyruvate, and inorganic salts (56). After identifying the optimal pH for growth, the range of growth-permissive temperatures was determined by assessing growth rate at 4°C, 12°C, 23°C, or 37°C in VSM-6 for all strains except KBS 89 which was grown in VSM-5.

To determine if growth rate was influenced by the elevated concentration of carbon dioxide used during isolation, strains were grown on VSM-6 in triplicate culture tubes and were incubated with either 0.05% CO_2 (vol/vol, balance air) or 5% CO_2 (vol/vol, balance air). Sodium bicarbonate (7 mM) was used as an additional buffer under elevated levels of carbon dioxide. To control for the possible influence of increased concentration of sodium when sodium bicarbonate was added, growth was also monitored in a medium containing 7 mM sodium chloride without a CO_2-enriched atmosphere. Analysis of variance (ANOVA) was used to assess if culture conditions resulted in significant differences in growth rate (SAS System version 8e software; SAS Institute Inc., Cary, NC).

Colony and cell morphology Bacterial colonies on plates of R2A medium were examined under a Nikon SMZ-2T dissecting microscope at 10-15X for size, pigmentation, form, elevation, and margin (68). Gram stain reactions were performed as

described previously (65). Gross cell morphology and motility were assessed by using phase contrast microscopy with a Zeiss Axioskop Microscope (Carl Zeiss Inc., Thornwood, NY). Cell shape, length, and width were measured with the CMEIAS image analysis software (45). Transmission and scanning and electron microscopy (using the polylysine procedure), including whole cell negative staining (using 2% uranyl acetate in water), were completed at the Michigan State University Center for Advanced Microscopy (www.ceo.msu.edu).

Carbon source utilization The ability of strains to use various organic compounds for aerobic growth was tested in replicate 25mL Erlenmeyer flasks containing 5 mL of VSB-6 for all strains except KBS 89 (VSB-5) amended with the following carbon sources at a final concentration of 10mM (unless indicated otherwise): D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose, L-arabinose, D-mannitol, D-sorbitol, sucrose, D-maltose, D-raffinose, D-cellobiose, methyl cellulose (0.1% v/v, final conc.), carboxymethyl cellulose (0.1% v/v, final conc.), sodium acetate, sodium pyruvate, sodium formate, sodium succinate, a mixture of organic acids (sodium citrate, sodium pyruvate, sodium fumarate, sodium D-L-lactate, and malic acid; 0.02% each, final conc.), a mixture of purines and pyrimidines (adenine, guanine, thymine, cytosine, and uracil; 10μg/mL each, final conc.), D-glucuronate, D-galacturonate, D-gluconic acid, trimethoxybenzoate, syringate, ferulate, vanillate, sodium benzoate, resorcinol, and tannic acid (0.1% v/v, final conc.). Flasks were incubated at room temperature (ca. 23°C) and shaken on an orbital shaker (ca. 190 rotations/min). A positive result was defined as visible turbidity after incubation for ca. 14 days.

Strains were also tested for their ability to grow anaerobically by fermentation of glucose, lysine, or ornithine (BBL™ Enterotube II, Sparks, MD), or by anaerobic respiration with nitrate (10mM), iron citrate (10mM), or AQDS (8mM) as the electron acceptor with glucose (10mM) as the sole carbon and energy source in VSB-6 for all strains except KBS 89 (VSB-5) with the addition of 7 mM bicarbonate. The headspace gas for these tests was 85% nitrogen, 10% hydrogen, and 5% carbon dioxide, except for samples with nitrate, which had a headspace of helium.

Carotenoid characterization Replicate 50 mL cultures of the pigmented strains in 250 mL side-arm flasks were grown in R2B to late-log phase under elevated carbon dioxide (5% carbon dioxide) and either 20% oxygen or 2% oxygen in the dark. Cultures were normalized to the same optical density at 600 nm and then cells were harvested by centrifugation at 5,000 x g for 25 minutes. The cell pellet was resuspended in a mixture of acetone:methanol (7:2 vol/vol), incubated overnight at 4°C in the dark (18), centrifuged to remove cell debris, and the absorption spectra of the extracts were determined between 220 and 800 nm with a Perkin Elmer model Lambda 14 scanning UV/Vis Spectrometer. Carotenoids were redissolved in chloroform for comparison of their spectra with previously characterized carotenoids (7).

16S rRNA gene phylogeny Nearly full length sequence of the 16S rRNA gene (ca. 1500 nt) was generated. Briefly, the 16S rRNA gene was amplified using the acidobacteria-specific forward primer, ACD31F and the broadly inclusive bacterial reverse primer 1492R using the conditions described above. PCR products were cloned

and prepared for sequencing as described above. Ten sequencing primers were used to obtain at an average coverage ranging from 4 to 8x of the 16S rRNA gene are as follows: ACD31F, 1492R, 338F (5' ACT CCT ACG GGA GGC AGC 3'), 338R (5' GCT GCC TCC CGT AGG AGT 3'), 531R, 810R (5' GGC GTG GAC TTC CAG GGT ATC T 3'), 776F (5' AGC AAA CAG GAT TAG ATA CCC TGG 3'), 1087F (5' GGT TAA GTC CCG CAA CGA 3'), modified-M13F, and the modified-M13R. Each primer was used in duplicate sequencing reactions. Sequences were assembled using the DNA Star LaserGene Software (Madison, WI) and aligned using the ARB Software (47), and compared to acidobacterial 16S rRNA gene sequences downloaded from GenBank. The phylogeny algorithms in ARB were used for the generation of the phylogenetic trees; PAUP* Version 4.0b10 was used for bootstrapping analysis (72). The 16S rRNA gene sequences of the *Acidobacteria* strains described in this study have been deposited in GenBank with accession numbers AY587227 through AY587230 and DQ660892 through DQ660895.

Characterization of genomic DNA The mole percent G + C content of genomic DNA from strains KBS 63, KBS 89, TAA 43, and TAA 166 was determined as described previously (51). Briefly, genomic DNA was extracted using a Qiagen Genomic DNA Extraction Kit (Qiagen, Valencia, CA), and approximately 5 µg of DNA was digested with P1 nuclease and alkaline phosphatase. The nucleosides were separated and quantified using a Shimadzu™ High Pressure Liquid Chromatograph fitted with a UV detector and a VP Series Alltima C18 column (250 x 4.6mm; particle size 5µm) (Alltech

Associates, Inc., Deerfield, IL). Genomic DNA purified from *A. capsulatum* (ATCC Number 51196) was used as a positive control.

The number of 16S rRNA-encoding genes was determined by non-radioactive Southern hybridization after restriction endonuclease digestion of genomic DNA, as described previously (35, 36) (http://rrndb.cme.msu.edu/rrndb/servlet/controller). A DIG-labeled, acidobacteria-specific 16S rRNA gene probe was made targeting regions between 31 and 531 (*E.coli* numbering). Genomic DNA purified from *A. capsulatum* was used as a positive control.

Other physiological tests Catalase and oxidase tests were performed using standard methods (65, 68). *Escherichia coli* strain REL 607, a derivative of *E.coli* B/r (42), was used as a positive control for the catalase test and a negative control for the oxidase test. *Pseudomonas aeruginosa* ATCC 10145 was used as a positive control for the oxidase test. Fatty acid profiles were generated and analyzed by Microbial ID (Newark, DE; www.microbialid.com) after all strains, including *A. capsulatum,* were grown on glucose yeast extract (GYE) medium adjusted to within their optimal pH range.

RESULTS and DISCUSSION

Occurrence and isolation of acidobacteria Acidobacteria are abundant members of the microbial community in soils at the KBS LTER; their rRNAs account for ca. 1 to 6% of the total bacterial rRNA (8). The specific phylogenetic affiliations of these acidobacteria were determined from samples collected during August 2005 from different management treatments and soil depths at the KBS LTER. Partial sequences (ca. 500

nucleotides) of 50 cloned 16S rRNA genes from each depth and treatment (total number of clones was 550) revealed the presence of acidobacteria from subdivisions 1, 3, 4, 5, and 6. The relative abundance of clones of each subdivision varied with depth and treatment, suggesting that physical and chemical properties of the soil may influence the distribution of the various subdivisions. In particular, the percentage of acidobacteria in subdivision 1 was correlated with soil pH, generally increasing in mildly acidic soils (Figure 3.1, panel A, gray boxes). To determine if the relationship between soil pH and relative abundance of subdivision 1 acidobacteria was significant for other terrestrial environments as well, previously published sequences from a variety of soil environments (15, 23, 38, 44, 50, 53, 54, 59, 69) were analyzed. The combined data sets reveal a significant correlation ($R^2 = 0.38$, $p < 0.004$; Figure 3.1, panel A) between pH and the percentage of acidobacteria associated with subdivision 1. This result is consistent with a previously determined relationship indicating that as soil pH decreased, there was an increased abundance of subdivision 1 acidobacteria in relation to the total bacterial community (60).

The strains characterized in this study were isolated from soils under a never-tilled, successional community with herbaceous plants (T8) with an average soil pH of 6.1 and a high percentage of subdivision 1 acidobacteria (27%; Figure 3.1, panel B). Media and incubation conditions were designed to mimic the microenvironments thought to be experienced by microbes in these soils. In particular, relatively low concentrations of organic carbon in conjunction with extended incubation periods were used to accommodate oligotrophic, slow-growing acidobacteria (35). The concentrations of

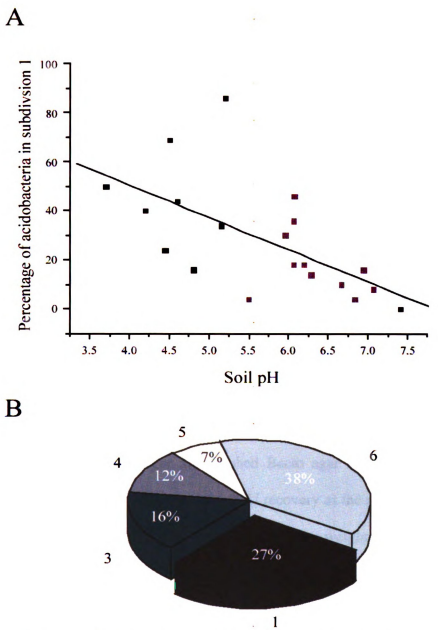
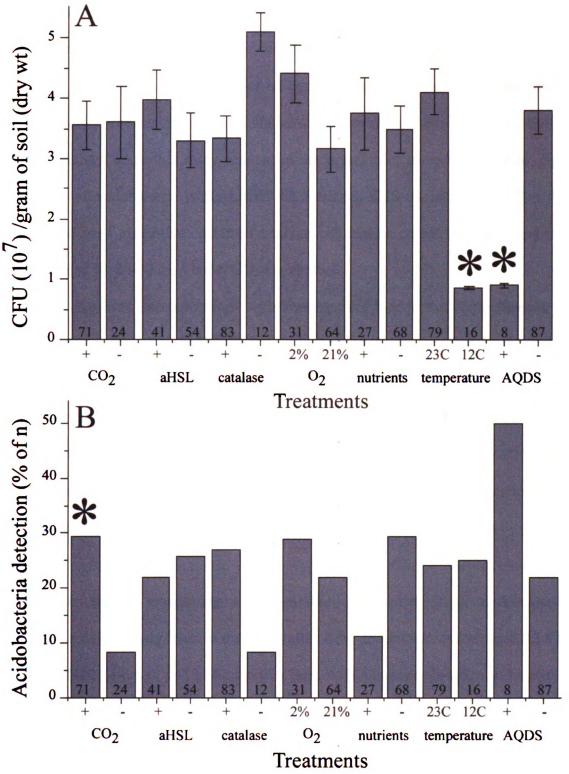


Figure 3.1. Summary of acidobacterial 16S rRNA gene clone libraries survey. Panel A depicts the percent abundance of acidobacteria subdivision 1 out of the total acidobacterial community at various soil pHs. Data from the KBS LTER are depicted as gray boxes (■), whereas data from published soil surveys are depicted as black boxes (■). If a range in pH was given in these studies, the average of that range was used for this analysis. The equation of the line is $y = -13.1x + 103$ with an R^2 value = 0.38 and p-value < 0.004. Panel B depicts the average percent abundance of acidobacteria subdivisions 1, 3, 4, 5, & 6 (subdivisions are indicated next to each pie wedge) from replicate plots of the never-tilled, successional community with herbaceous plants at the KBS LTER, depth of 0-7cm.

oxygen and carbon dioxide during incubation were also manipulated to better simulate conditions in soil aggregates. Although upland soils like those at the KBS LTER are generally well-aerated, they include pockets of anoxia within soil aggregates, and hence also include transitions zones between oxic and anoxic conditions (27, 66). Moreover, metabolism of heterotrophic microbes in soil leads to increased concentrations of carbon dioxide (20), which has the potential to influence the pH of microenvironments. Accordingly, hypoxic and CO_2- enriched incubation atmospheres were included, as was the incorporation of catalase in the media to minimize the damaging effects of hydrogen peroxide produced during aerobic respiration.

With the exception of decreased recoveries at lower temperature and in the presence of AQDS, the recovery of soil microorganisms under the various conditions tested was between 3.5 to 5 x 10^7 CFU/gram of soil (dry wt), which was 2.5 to 6% of the direct cell count (Figure 3.2, panel A). Previous experiments done on three different types of solidifying agents (Bacto Agar and washed Bacto agar in VSB-7 and agarose in distilled water) indicated a decrease in total CFU recovery as the purity of the solidifying agent increased: agar, ca. 5.2 x 10^7 CFU/gram of soil dry weight washed agar, ca. 4.4 x 10^7 CFU/gram of soil dry weight; and agarose, ca. 0.3 x 10^7 CFU/gram of soil dry weight when grown under oxic conditions. Agar was chosen as a solidifying agent to increase our recovery of soil microorganisms, thereby presumably increasing our chances of isolating member of the phylum *Acidobacteria*. Across treatments used for isolation, acidobacteria were detected more frequently under atmosphere(s) containing elevated levels of carbon dioxide ($0.05<p<0.10$) (Figure 3.2, panel B). This was apparently due to

Figure 3.2. Summary of cultivation experiments for total recovery of colonies per gram of soil (dry weight) (Panel A) and acidobacteria detection (% of n) (Panel B). Panel A represents the average CFU recovery from soil +/- the standard error; the number of times a particular treatment was employed (n) is listed at the base of each bar. Panel B represents the percentage of treatments that yielded a positive test for acidobacteria using PWPCR from the plates of panel A. The asterisks "*"indicates treatments that yielded significant difference (panel A, ANOVA $\alpha = 0.05$; panel B, chi square goodness of fit, $\alpha = 0.10$).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0
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CO_2 aHSL catalase O_2 nutrients temperature AQDS
Treatments
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71 24 41 54 83 12 31 64 27 68 79 16 8 87
+ - + - + - 2% 21% + - 23C 12C + -
CO_2 aHSL catalase O_2 nutrients temperature AQDS
Treatments

a mild acidification of the medium under 5% carbon dioxide (plates incubated under 5% CO_2 had a pH ca. 6.5), which favored growth of these acidobacteria, rather than to a requirement by cells for exogenous carbon dioxide per se (see below).

Although overall recoveries of bacteria here were similar to other studies that used low nutrient concentrations and extended incubation times (12, 14), the specific conditions used in this study resulted in the isolation of several novel, pink-pigmented strains of acidobacteria (strains, KBS 62, KBS 63, KBS 68, and KBS 112). All were isolated from plates that contained a mixture of organic carbon substrates and that were incubated for 20-30 days under CO_2-enriched air.

Previous isolation efforts with homogenized hindguts of *Reticulitermes flavipes* soil-dwelling termites under similar cultivation conditions resulted in the isolation of three acidobacteria strains (strains TAA 43, TAA 48, and TAA 166), which appeared to be allocthonous contaminants from nearby soil (J.T. Wertz, B.S. Stevenson, and J.A. Breznak, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. 2003, abstr. N-223, 2003) and strain KBS 89 from soils at the KBS LTER. These strains are also characterized further in the current study.

Colonial and cell morphology and pigmentation Colonies of all acidobacteria were small, approximately 1mm in diameter (after 7 day incubation, except for KBS 89 which took about 14 days), and had a circular form with a convex elevation and entire margin when grown on VSM-6, -5(KBS89) or R2A. With the exception of KBS 89, all isolates took approximately 3-5 days before visible, smooth, butyrous colonies were seen on the surface of the plate of the VSM-6, -5(KBS 89) while KBS 89 took approximately 14-16

days to form visible colonies. All strains achieved maximal growth rates in complex medium, such as R2B, at room temperature (ca. 23°C) with doubling time between 10 and 15 hours.

Cells of all strains are short, plump, Gram negative, nonmotile rods measuring 1 μm x 0.6 μm when grown on R2A (Figure 3.3). Cells possessed exaggerated convoluted outer membranes when viewed by TEM as is typical of a Gram-negative type cell wall. All strains produced an extracellular matrix of as yet unknown chemical composition, but which apparently caused cells to stick together tightly (Figure 3.3, panel f) in colonies and form visible flocs in liquid culture, qualitatively to the same extent under microoxic and oxic conditions. The formation of such extracellular material in soils may serve as a form of protection from predation, or a web to trap water or nutrients (57), and may be involved in the formation of soil aggregates.

Four strains (KBS 62, 63, 68, and 112) were pink, owing to the presence of a pigment(s), whose solvent extractability and absorption spectrum were typical of carotenoids. Interestingly, the pigmentation was more pronounced in cells grown under 20% O_2 than under 2% O_2 (Figure 3.4, panel A). The ratio of the absorbance maxima of the two dominant peaks in acetone:methanol (A_{498} and A_{528}) were nearly identical, 1.2 ± 0.0 and 1.3 ± 0.1, respectively, suggesting the same carotenoid(s) was produced under each oxygen regime (Figure 3.4, panel B). The visible absorption spectrum of strain KBS 63 pigment(s) in chloroform had maxima at 473, 505, and 539 nm, which are most similar to those of spirilloxanthin (475, 505, and 543 nm) (7).

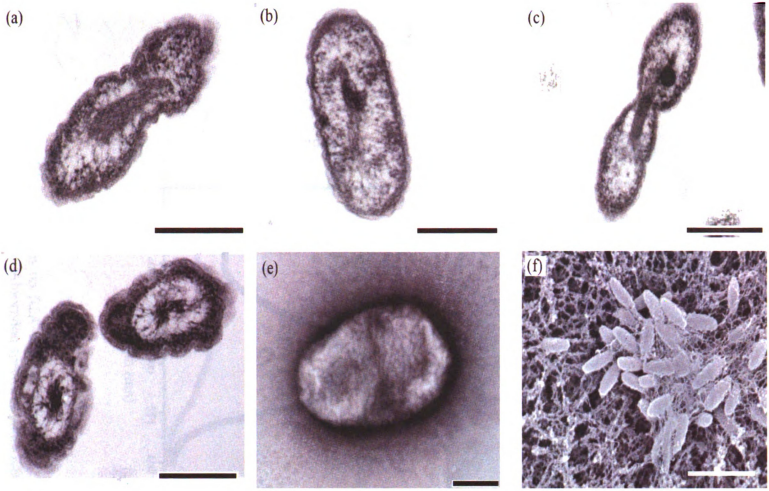

Figure 3.3. Electron micrographs of thin sections of strains TAA 43 (a), TAA 48 (b), TAA 166 (c), and KBS 63 (d) grown in R2A. Negatively stained cell of strain KBS 63 (e) and a scanning electron micrograph of strain KBS 89 (f). Bars: (a-e) 500 nm, and (f) 2 μm.

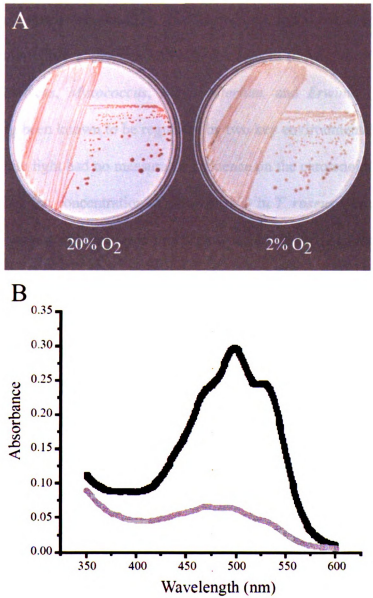

Figure 3.4. Strain KBS 63 grown on R2A under 20% and 2% oxygen (vol/vol) of nitrogen & 5% CO_2 mix) (panel A). Absorption spectra of strain KBS 63 cells extracted in acetone:methanol (7:2) after growth in 20% (■) and 2% (■) oxygen on R2B (panel B).

Carotenoids are naturally occurring accessory pigments that typically absorb visible light maximally between 450 and 550 nm (48), and are known to play roles in light-harvesting for photosynthesis and protection from photooxidative damage (6), as well as quenching singlet oxygen (11). Carotenoids are found not only in phototrophic bacteria, but in numerous, phylogenetically diverse genera of heterotrophic soil bacteria, including *Streptomyces*, *Myxococcus*, *Flavobacterium,* and *Erwinia* (2). Carotenoid synthesis has long been known to be regulated by two key environmental factors, oxygen and light (2). While light had no measurable influence on the carotenoid(s) content of the *Terriglobus* strains, the concentration of carotenoid(s) in *T. roseus* increased in response to oxygen. We suggest that increased production of carotenoid(s) under 20% oxygen as compared to 2% oxygen concentration represents a response to oxidative stress. The potential antioxidant property of carotenoids is well known: β-carotene is effective in quenching singlet oxygen (11) and the addition of carotenoid genes to *E.coli* inhibits peroxidation (1). Specifically in heterotrophs, carotenoids can influence growth rate and survival (52) presumably through the inactivation of reactive oxygen species generated during respiration. Differential expression of carotenoids might also be advantageous in the soil environment, where oxygen gradients change in response to soil moisture and depths, with higher concentrations of oxygen occurring in upper layers of soil that are also subject to more intense solar radiation where carotenoids would also provide protection against the damaging effects of ultraviolet radiation.

The frequency of acidobacteria detection was notably higher in the presence of catalase (Figure 3.2, panel B), suggesting that acidobacteria are sensitive to damage by reactive oxygen species. All strains, except for KBS 89, tested positive for the presence

Table 3.1. Phenotypic characteristics of *Terriglobus, Acidobacteriaceae,* and *Acidobacterium* strains.

Characteristics	*Terriglobus* Strains		*Acidobacteriaceae* Strains		*Acidobacterium*
	KBS 63[†]	**TAA 43**[‡]	**KBS 89**	**TAA 166**	***A.capsulatum***[a]
Origin	soil	termite hindgut	soil	termite hindgut	acidic mine drainage
Cell Shape[b]	Rod	Rod	Rod	Rod	Rod
Length (μm)	1.1 ± 0.2	1.2 ± 0.2	ca. 1	1.1 ± 0.3	1.1 - 2.3
Width (μm)	0.6 ± 0.1	0.6 ± 0.1	ca. 0.5	0.6 ± 0.0	0.3 - 0.8
Pigment	pink	white	white	white	orange
rrs copy number	2	1	1	1	1
G + C content (mol %)[‖]	59.8 ± 0.5	58.1 ± 0.04	59.7 ± 1.8	54.7 ± 0.7	60.8 (62.7 ± 0.1 this study)
Catalase	+	+	-	+	+
Oxidase	-	-	-	-	-
Motility	-	-	-	-	+
pH range (optimum)	5 – 7 (6)	5 – 6.5 (5)	ND	5 – 7 (6)	3 – 6
Growth at 4°C	-	-	-	-	ND
Growth at 12°C	+	+	+	+	ND
Growth at 25°C	+	+	+	+	ND
Growth at 37°C	-	-	-	-	+
Differences Carbon Utilization:					
D-Ribose	-	-	+	-	ND
D-Sorbitol	-	-	+	-	ND
Purine & Derivative Mix[c]	-	+	-	-	ND

[†] Data are identical for strains KBS 62, KBS 63, KBS 68, and KBS 112.
[‡] Data are identical for strains TAA 43 and TAA 48.
[‖] The mole percent G+C content was only done for strains KBS 63, KBS 89, TAA 43, and TAA 166.
[a] Reference: characteristics described from a previous study (33). ND "not determined"
[b] Cell length and width was determined from a culture in mid-log phase on R2B medium.
[c] The mix of adenine, guanine, thymine, cytosine, and uracil had a final concentration of 10 μg/ml (total concentration of sugar derivative was 50μg/ml).

of catalase (Table 3.1), and some strains produced a carotenoid(s) which was preferentially expressed under higher oxygen concentrations (Figure 3.4). It would be beneficial for a soil microorganism to have mechanisms to deal with the damaging effects of reactive oxygen species, since there are oxygen gradients in the three-dimensional soil structure that change with properties such as soil moisture and nearby microbial communities.

Physiological properties of strains Physiological properties of all strains are tabulated in Table 3.1. The growth rates of all strains grown in VSM- 5 (KBS 89) or VSM-6 (all others) were highest (avg. $\mu \approx 0.15$) at room temperature under air. Growth was observed over a pH range of 4.5 to 7.0. It is not surprising that the pH optima of the strains were similar to that of their native environment. Most soils are slightly acidic due to the presence of humic substances, which make up 70-80% of the soil organic matter (24). Sait et al. recently suggested that there is an effect with pH on the isolation and distribution of subdivision 1 acidobacteria. Specifically, representatives of subdivision 1 formed more colonies in media having a pH 5.5 as compared to a pH of 7.0 after a 4 month incubation (60). Furthermore, the results of our molecular-based survey reveals that subdivision 1 acidobacteria are found in a higher abundance in mildly-acidic to acidic soil environments (Figure 3.1, panel A). Taken together, these results suggest that members of subdivision 1 of the *Acidobacteria* have a preference for mildly acidic pH conditions, which is consistent with their ubiquitous distribution in soil.

We examined the effect of CO_2 on the growth of isolated strains to explore the greater frequency at which acidobacteria formed colonies on plates incubated under 5%

CO_2 (Figure 3.2, panel B). There was no significant difference in growth rate of strains KBS 63, TAA 43, and TAA 166 incubated under an atmosphere of air (ca. 0.05% CO_2 vol/vol) or 5% CO_2 (balance air) (p-value = 0.83, 0.98 and 1.0, respectively). Therefore, the increased frequency of appearance of acidobacteria on plates incubated under 5% CO_2 appears to be a result of mild acidification of the isolation medium. The degree of acidification that can occur on plates of media without added bicarbonate and incubated under elevated levels of carbon dioxide (pH ca. 6.5) is consistent with the optimal pH of the strains (Table 3.1).

The relatively slow growth rate of the acidobacteria strains is consistent with the presence of one (strains TAA 43, 48, 166, and KBS 89) or two (strains KBS 62, 63, 68, and 112) genes coding for the 16S rRNA gene (Figure 3.5). Previous studies have indicated that the number of rRNA operons correlates with the rate at which bacteria respond to resource availability and how efficiently those resources are utilized (35, 71).

The metabolism and growth of bacteria in soil environment are frequently limited by carbon availability, but subject to periodic fluctuations in resource resulting from events including rain or spring snow melt (74). Populations of *Acidobacteria* are more abundant in soil environments where resource availability is low (19), and can be described as oligotrophic bacteria because of this distribution in soil, their relatively slow growth rate, and possession of either one or two copies of the 16S rRNA encoding gene – a genomic marker of oligotrophic bacteria (35). Although the acidobacteria characterized in this study do not fit the definition of an obligate oligotroph because they are capable of growth on rich media, they do fit into the broader notion of an oligotroph as being an organism adapted to low-nutrient environments (37).

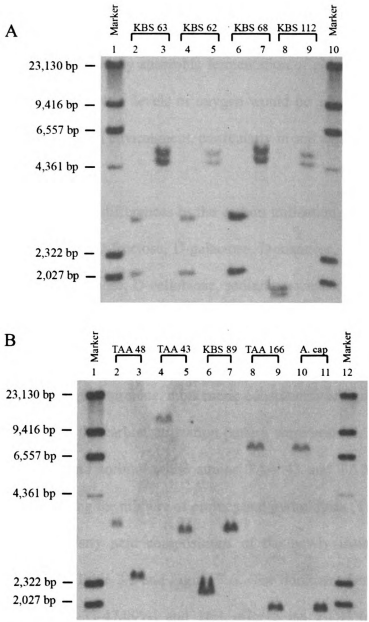

Figure 3.5. Southern hybridization of digested genomic DNA from strains KBS 62, KBS 63, KBS 68, KBS89, KBS 112, TAA 43, TAA 48, & TAA 166. In panel A, isolates were cut with *EcoRV* (lanes 2, 4, & 6), *Sma* I (lane 3, 5, 7, & 9), and *Rsa* I (lane 8). In panel B, isolates were cut with *Sma* I (lanes 2, 4, 6, 8, & 10) and *Apa* I (lanes 3, 5, 7, 9, & 11). The lambda DNA *Hind* III marker (lane 1 & 10 (panel A) and lanes 1 & 12 (panel 12)) provided size estimates.

All strains were able to grow in a 2% oxygen atmosphere as well as atmospheric concentrations of oxygen, with or without elevated levels of carbon dioxide, but they could not grow anaerobically with nitrate, iron (Fe(III)), or AQDS as external electron acceptor, nor could they grow by anaerobic fermentation of glucose, lysine, or ornithine (Table 3.1). Growth under low levels of oxygen would be advantageous in soil, since oxygen is depleted in the soil environment, particularly in soil aggregates as soil moisture increases (5, 55, 74).

There were nominal differences in the carbon utilization profile; all strains were able to oxidize D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, D-arabinose, sucrose, D-maltose, D-raffinose, D-cellobiose, sodium succinate, D-glucuronate, and D-gluconic acid; however they were not able to oxidize D-mannitol, methyl cellulose, carboxymethyl cellulose, sodium acetate, sodium pyruvate, sodium formate, maleic acid, the organic acid mix, D-galacturonate, monomeric constituents of lignins, or humic acids. Only two differences in the carbon utilization pattern were noticed, strain KBS 89 was able to oxidize ribose and sorbitol while strains TAA 43 and TAA 48 were the only strains capable of oxidizing the mixture of purines and pyrimidines (Table 3.1).

The whole-cell fatty acid compositions of the newly isolated strains and *A. capsulatum* are listed in Table 3.2 and Figure 3.6. The dominant fatty acids for the new strains are 15:0 iso (36.33-47.09%) and 16:1 ω7*c*/15 iso 2-OH (summed feature 3) (26.18-30.24%). These results were compared to the Microbial ID database, but did not correspond to any known species. *A. capsulatum* was sent as a source of comparison, and its dominant fatty acids were 15:0 iso (40.28%) and 18:1 ω9*c* (21.21%).

Table 3.2. Whole-cell fatty acid composition (%) of *Terriglobus, Acidobacteriaceae,* and *Acidobacterium* strains grown in GYE medium. The most abundant fatty acids that distinguish the *Terriglobus* strains from *A. capsulatum* are presented in bold font.

Fatty acid	*Terriglobus* Strains		*Acidobacteriaceae* Strains		*Acidobacterium*
	KBS 63	TAA 43	KBS 89	TAA 166	*A. capsulatum*
Saturated					
14:0	3.42	4.25	1.16	3.68	1.58
15:0	0.64	0.74	0.00	0.60	0.57
16:0	7.77	9.14	7.33	8.71	4.29
16:0 N alcohol	0.52	3.54	0.50	5.01	4.61
17:0	0.70	0.00	0.93	0.00	1.85
18:0	0.38	0.68	0.75	0.59	2.34
20:0	0.76	0.00	4.07	0.00	0.00
Unsaturated					
14:1 ω5c	1.07	0.67	1.00	0.57	0.00
15:1 ω6c	0.83	0.92	0.43	0.71	0.00
16:1 ω5c	0.43	0.00	0.00	0.56	0.00
17:1 ω8c	0.00	0.00	0.00	0.00	3.97
18:1 ω9c	**0.00**	**0.00**	**0.00**	**0.00**	**21.21**
18:1 ω7c	0.00	0.84	0.00	1.27	0.45
18:1 ω5c	1.05	0.00	0.00	0.00	2.24
19:0 cyclo ω8c	0.00	0.00	0.00	1.10	0.00
20:2 ω6,9c	0.67	0.00	0.00	0.00	0.00
Methyl-branched					
13:0 iso	4.02	0.00	4.12	0.00	0.00
15:0 iso	43.88	36.33	47.09	37.06	40.28
15:0 anteiso	0.48	0.70	0.00	1.03	0.00
16:0 iso	0.00	0.00	0.00	0.58	0.00
iso 17:1 ω9c	0.00	0.00	1.25	0.00	0.90
iso 17:1 ω5c	4.24	7.27	2.33	0.00	7.40
17:0 iso	0.53	0.79	1.60	0.78	2.15
17:0 anteiso	0.27	0.60	0.00	0.74	0.49
19:0 anteiso	0.00	1.82	0.00	0.00	0.00
Hydroxy					
15:0 iso 3-OH	0.00	0.00	0.58	0.00	0.00
17:0 iso 3-OH	0.00	0.00	0.00	0.00	0.77
Summed Feature					
15:1 iso H/13:0 3-OH	0.59	0.00	0.36	0.00	0.32
16:1 ω7c/15 iso 2-OH	**27.08**	**30.24**	**26.18**	**26.99**	**4.30**
17:1 iso i/anteiso b	0.00	0.00	0.00	8.07	0.00
18:2 ω6,9c/18:0 anteiso	0.64	1.47	0.31	1.96	0.00
Unknown					
Unknown 11.799	0.00	0.00	0.00	0.00	0.28

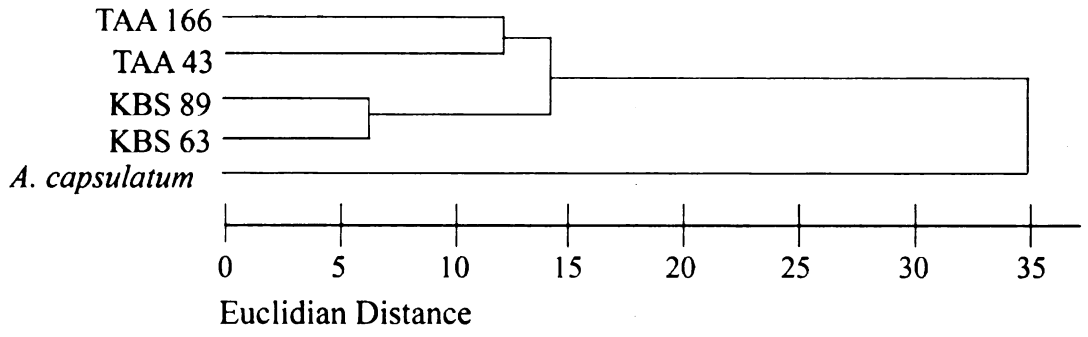

Figure 3.6. Euclidian distance dendrogram for *Terriglobus*, *Acidobacteriaceae*, and *Acidobacterium* strains generated from the fatty acid profiles.

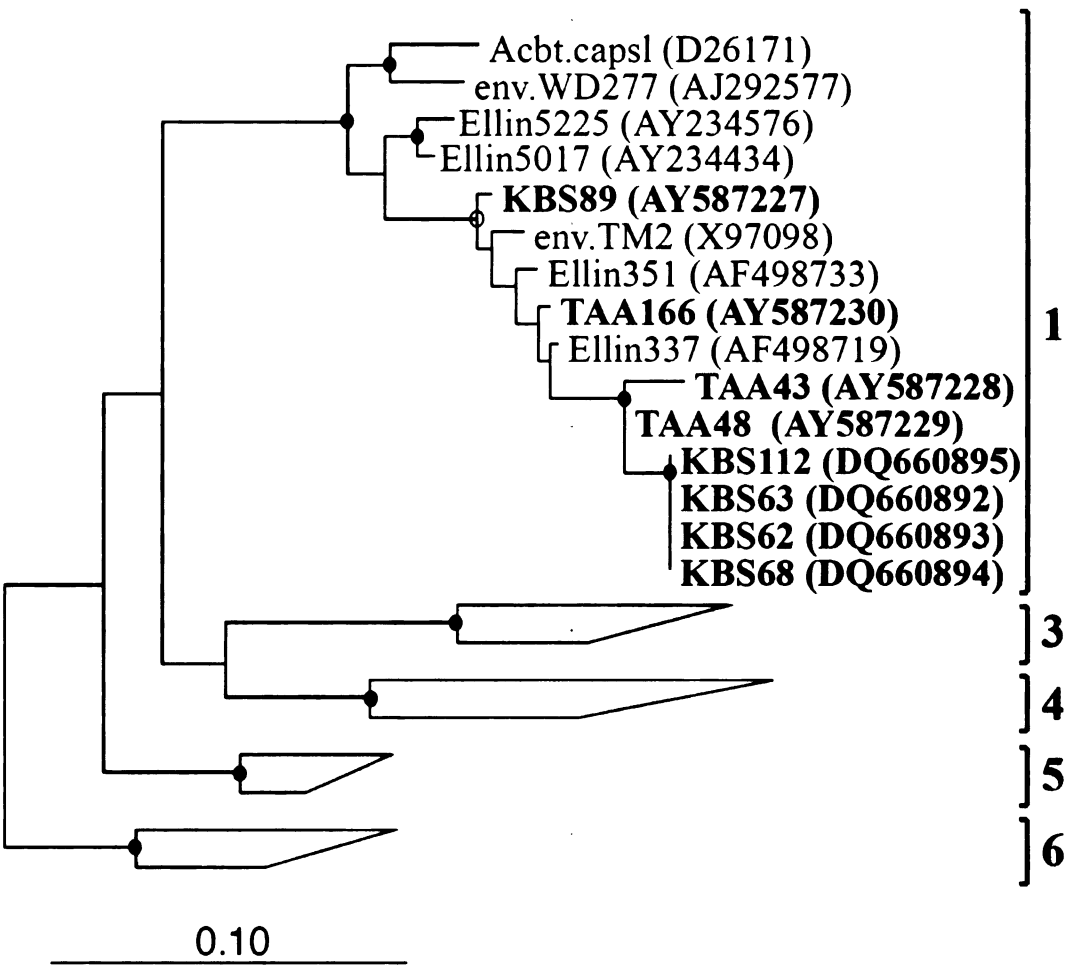

Figure 3.7. Maximum-likelihood tree of the *Acidobacteria* subdivisions 1, 3, 4, 5, and 6 (indicated to the right of the group) based on 16S rRNA gene using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). Strains from this study are in bold font. Each gray trapezoid represents a different subdivision with approximately 8 representative sequences. Internal nodes supported by a bootstrap value of >95% are indicated with a filled circle (•) and of >75 with an open circle (o). The scale bar indicates 0.10 changes per nucleotide.

Six of the strains (KBS 62, KBS 63, KBS 68, KBS 112, TAA 43, and TAA 48) are sufficiently similar to one another, but distinct from previously isolated acidobacteria (Figure 3.7), to warrant creation of a new genus. The similarity of the 16S rRNA gene sequences within these six strains is 96% and, on average, only 92% similar to *A.capsulatum*. Moreover, unlike *A. capsulatum* these new strains do not require extremely low pH conditions (pH 3.0-6.0), they cannot grow at 37°C, and they contain 16:1 ω7*c*/15:0 iso 2-OH as a dominant fatty acid and not 18:1 ω9*c*, which is dominant in *A capsulatum* (Table 3.2, Figure 3.6). Hence, we propose that these six strains be regarded as members of a new genus, *Terriglobus*, with KBS 63 as the type strain of the new species, *roseus*, which also includes the other pink-pigmented strains (KBS 62, KBS 68, and KBS 112). The nomenclature and description of the genus and species are given below. We are postponing application of specific epithets to strains TAA 43 and TAA 48 until further work is done to determine their relatedness to strains comprising the species, *T. roseus*. Although the characteristics of strains KBS 89 and TAA 166 are consistent with the description of *Terriglobus*, there is insufficient metabolic information currently available about the previously isolated and closely related strains Ellin 351 and Ellin 337 (61) to include all four strains in the genus *Terriglobus* at this time.

Description of *Terriglobus* gen. nov. *Terriglobus* gen. nov. (Ter.ri.glo' bus. L. fem n. *terra*, earth; L. masc. n. *globus* ball, clump; N.L. masc. n. *Terriglobus* clump of earth) literally translated as "clump of earth" in the phylum *Acidobacteria*. Gram negative, short, plump, rods (ca. 1μm x 0.6 μm) when grown in R2B medium. Moderate acidophiles (pH range for growth is 5.0 - 7.0, optimal for KBS 63 is 6.0 and TAA 43 is

5.0). Produce an extracellular matrix which apparently facilitates cell clumps in liquid media incubated under an atmosphere containing either 2% or 20% molecular oxygen. G+C content ca. 59 mol% (Table 3.1); 15:0 iso and 16:1 ω7*c*/15:0 iso 2-OH as dominant fatty acids (Table 3.2). Members of the phylum *Acidobacteria*, subdivision 1. The type species is *Terriglobus roseus*.

Description of *Terriglobus roseus* sp. nov. *Terriglobus roseus* sp. nov. (ro' se.us. L. masc. adj. *roseus* rose-colored, pink), "a pink *Terriglobus*" (type strain KBS 63). Pink-pigmented colonies due to the presence of carotenoids; catalase positive; oxidase negative; and two copies of the *rrs*. Isolated from soils in a never-tilled, successional plant community at the KBS LTER. The type strain KBS 63 was deposited into the USDA Agricultural Research Service Culture Collection (NRRL B-41598^T) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM 18391).

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CHAPTER 4

DESCRIPTION OF NOVEL MEMBERS OF SUBDIVISION 1 AND 3 IN THE PHYLUM *ACIDOBACTERIA* FROM AGRICULTURAL SOIL

INTRODUCTION

Members of the phylum *Acidobacteria* are abundant and diverse members of the soil environment. They have been retrieved from various environments including: soils and sediments (5, 15), soil crusts of sand dunes (51), wastewater (10, 29), water distribution systems (32), peat bogs (13), acid mine drainage (24), hot springs (18), shallow submarine hydrothermal vents (49), and on the surface of Paleolithic cave paintings and catacombs (45-47, 62, 63). It is a diverse phylum, historically harboring eight, monophyletic subdivisions with recent evidence of eighteen additional subdivisions, some unique to uranium contaminated subsurface sediments (4). Their common occurrence and high abundance suggests they play an ecologically significant role(s) in the soil environment.

Recently, scientists have made successful attempts to cultivate members of subdivision 1, 2, 3, and 4 (11, 13, 14, 16, 20-22, 42-44, 52) from soil and sediment environments. To date, cultivation efforts have been most successful with members of subdivision 1, with more than forty isolates obtained from this subdivision; however, there are only a few cultivated representatives from subdivisions 2, 3, and 4. In order to cultivate new strains in these poorly represented subdivisions, the information generated from the acidobacteria soil survey (Chapter 2) was used to develop additional cultivation

strategies such as the influence of soil pH (subdivision 1), soil moisture (subdivision 3), and low carbon concentrations (subdivision 4). Soil from the conventional agriculture site at the KBS LTER (treatment 1) was used as the source of inoculum in an attempt to isolate members of subdivision 4, given their high prevalence in this soil (Chapter 2).

These experiments were successful at isolating additional members of subdivision 1 as well as a novel strain in subdivision 3 using medium with plant polymers as the carbon source at low pHs. As such, the characterization of newly isolated strains had particular emphasis on determining the ability of these strains to use xylan and more recalcitrant carbon sources such as benzoate, syringate, cellulose, and resorcinol as well as their ability to use nitrate since the inoculum was from a conventional agricultural soil, which has high inputs of nitrates. Microorganisms that harbor active enzymes to degrade recalcitrant carbon sources are essential for maintaining an active carbon flow in the soil, since typically these compounds are slow to decompose. Nitrate as well as nitrite, under anoxic conditions, can be used as an electron acceptor for anaerobic respiration and be reduced to gaseous forms of nitrogen such as nitric oxide, nitrous oxide, and dinitrogen gas, a process commonly referred to as denitrification. This process results in a loss of fixed nitrogen, ca. 20 to 30% of the added nitrogenous fertilizers to agricultural fields (56), as well as producing the dangerous greenhouse gas, nitrous oxide. Results from the physiological and phenotypic characterization warrant the creation of a new genus in subdivision 1 and in subdivision 3.

MATERIALS and METHODS

Cultivation experiments Soil samples were collected from the conventional agriculture plot (treatment 1) and never tilled successional plot (treatment 8) in July 2006 from the Michigan State University W.K. Kellogg Biological Station Long Term Ecological Research (KBS LTER) site. The KBS LTER is a 48-hectare research site established in 1989 to study ecological processes in agroecosystems. The dominant soil types are of the Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs) series. A detailed description of this site can be accessed at http://www.kbs.msu.edu.

For isolation attempts, five cores (2.5 cm diameter x 10 cm depth) from the conventional agricultural plot (T1) were collected and homogenized with a 2 mm sieve on the bench top. Portions of the homogenized soil were used to determine soil moisture (40). Approximately 30 grams of soil were added to 100 mL of a phosphate-buffered salts solution (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4 and 2 mM KH_2PO_4); pH was adjusted to 7.0, and supplemented with 2.24 mM $Na_4P_2O_7 \cdot 10H_2O$ as a dispersal agent, and dithiothreitol (1mM) as a reducing agent (57). The soil suspension was stirred vigorously with a magnetic stir bar for 30 minutes on the bench top. Denser soil aggregates were then allowed to settle for 30 minutes, after which an aliquot was used to prepare 10-fold decimal dilutions in the same buffer for subsequent inoculation of the isolation medium consisting of VL55 medium (22) with either R2B carbon (VL55-R2B), a mixture of plant polymers (VL55-PP), or xylan from birchwood (VL55-xylan); hereafter, this composition will be referred to as VL55-(R2B, PP, or xylan) - # "VL55 medium with either carbon as R2B, plant polymer mix, or xylan"; # indicates the respective pH of the basal solution. The plant polymer mix contained 0.05 grams/liter

of the following components: xylan (derived from oat spelts), xanthan, pectin, xylan (derived from birchwood), methyl cellulose, and xylan (derived from larch) (Sigma-Aldrich Co. St. Louis, MO). The R2B carbon mixture contained 0.05 grams/liter of the following components: yeast extract, protease peptone #3, casamino acids, dextrose, and soluble starch. The xylan mixture contained 0.05 gram/liter of xylan from Birchwood (Sigma-Aldrich Co.). The final pH of the plates was buffered to either 5.5 or 6.5 with 10 mM of MES, and they were incubated for 30 days at 23°C under one of the following atmospheres: CO_2-enriched hypoxia (vol/vol: 2% O_2, 5% CO_2, and bal N_2); CO_2-enriched anoxia (vol/vol: 85% nitrogen, 10% hydrogen, 5% CO_2), or air. Plates were screened for the presence of acidobacterial colonies after ca. 30 days of growth using PWPCR (52).

Isolation and standard growth medium During the isolation of these strains, an alternative medium was sought that would provide better growth of the strains, KBS 83 and KBS 96: this provided to be modified hyphomicrobium medium "337" (36) (MHM). MHM contained (per liter): NaCl 1.0 g; $MgCl_2 \cdot 6H_2O$ 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; NH_4Cl, 0.25 g; KH_2PO_4, 0.2 g; KCl, 0.5 g; Na_2SO_4, 0.28 g; KNO_3, 5 g; KPO_4, 0.15 mM, 20 mM respective buffer; 10 mM designated carbon source (for KBS 83) or a mixture of organic compounds (below) (for KBS 96); along with 1 mL SL-10 trace element solution; 1 mL vitamin B12 (50mg/mL); and 1 mL vitamin mix as described previously (16). All other strains were successfully isolated and routinely grown on R2B - a complex medium containing yeast extract, amino acids, peptone, carbohydrates, pyruvate, and inorganic salts (38).

Optimization of growth rate MHM was amended with 10 mM glucose (KBS 83) or a mixture of organic compounds (yeast extract, protease peptone, casamino acids, and glucose – 0.15g/l each) (KBS 96) to assess capacity for growth under a range of conditions. This complex medium (designated MHM-#, where # indicates pH) was used to determine the effect of pH on growth rate. Strains were grown at room temperature (23°C) between pH 3.0 and 7.0 at intervals of 0.5 pH units: MES was used to buffer at pH ranges between 5.0 to 6.0, 4-morpholinepropanesulfonic acid (MOPS) was used at pH ranges between 6.5 to 7.0, and citric acid was used as a buffer below pH 5.0, all at a final concentration of 20 mM. Culture tubes (Bellco™ cat. no. 2048-18150) containing 5 mL of medium, were stoppered under an air atmosphere and held on a reciprocating shaker, horizontally, operating at ca. 50 strokes/min. to disrupt cellular flocs. Optical density at 600nm was monitored periodically with a Thermo™ Spectronic model 20D+ spectrophotometer. Inocula consisted of a 1:100 dilution of cultures in mid/late log phase growth (ca. $1x10^8$ cells/mL) in MHM-5 with 10 mM glucose (KBS 83) or MHM-5 with 0.3x R2B (KBS 96).

Strain KBS 83 was tested for its ability to grow under hypoxic conditions with the following concentrations of oxygen in the headspace (vol/bal. He): 2%, 4%, 8%, 16%, and 21% on MHM-5.5. A final concentration of 2.5 mM glucose was used to ensure an excess of oxygen in the headspace of the 750 mL sealed, side-arm flasks. Similar experiments were performed for strain KBS 96, however this strain was grown on MHM-5 with a mixture of organic compounds (yeast extract, protease peptone, casamino acids, and glucose - 0.05 g/l each) due to some unknown auxotrophic requirement for better growth. The headspace was purged every third day of growth and oxygen re-added at the

respective concentration. Strains were grown at room temperature, on an orbital shaker at 150 RPM, and growth was monitored as described above. Inocula consisted of a 1:100 dilution of cultures in late log/early stationary phase growth (ca. $1x10^8$ cells/mL) in MHM-5 with respective carbon source, presumed to be depleted in oxygen.

Carbon utilization Strains KBS 83 & KBS 96 were tested for their ability to oxidize various carbon sources. Strains were grown under oxic conditions on the following carbon sources for ca. 30 days, with a 10 mM final concentration on MHM-5.5: D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose, L-arabinose, D-mannitol, D-sorbitol, sucrose, D-maltose, D-raffinose, succinate, acetate, formate, pyruvate, benzoate, ferulate, resorcinol, syringate, and trimethoxybenzoate. Methyl cellulose, pectin, cellulose, and a xylan mix, containing xylan from oat spelts, birchwood, and larch were used with MHM-5.5 at a final concentration of 0.1% (wt/vol). Culture vessels and incubation were identical to the pH conditions (above). Due to an unknown, auxotrophic requirement of strain KBS 96 for faster growth, a small amount of yeast extract (5 μg/mL) was added to the medium for growth; growth for each respective carbon source was determined after the amount of growth on 5 μg/mL yeast extract alone was subtracted.

Capacity to utilize nitrate Strains KBS 83 & KBS 96 were tested for their ability to grow anaerobically and reduce nitrate by determining the concentration of ammonium, nitrite, and nitrous oxide while simultaneously monitoring the quantity of nitrate at the beginning and end of growth. Strains were grown in MHM-5.5 with 10 mM glucose in a

headspace of helium. Culture vessels and incubation were identical to the pH conditions (above). Nitrate, nitrite, and ammonium were measured at the Michigan State University Soil Testing Laboratory using the Lachat QuickChem automated flow injection ion analyzer. Nitrous oxide, if present, and carbon dioxide were measured using a Shimadzu™ thermal conductivity gas chromatograph series GC-2014 with the following settings: flow rate, 60 mL/min; column temperature, 55°C; injector temperature, 100°C; detector temperature, 100°C; and a thermal conductivity of 175 mA. Glucose concentrations were determined using the Glucose Assay Kit (Sigma Aldrich Co., St. Louis, MO).

16S rRNA gene phylogeny Nearly full length sequence of the 16S rRNA gene (ca. 1,500 nt) was generated by amplifying it with the broadly inclusive bacterial forward primer 8F and bacterial reverse primer 1492R (28). Each 25µl PCR reaction contained 1x PCR buffer, 1mM $MgCl_2$, 0.03mM of each dNTP, 0.2µM each primer, and 5U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Thermal cycling consisted of the following steps: (1) 95°C for 3 minutes; (2) 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds (repeated 30X); (3) 72°C for 10 minutes. Genomic DNA purified from *A. capsulatum* (ATCC Number 51196) was used as a positive control. PCR products were electrophoresed through a 1% agarose gel in 0.5x tris-borate-EDTA (TBE) and visualized with GelStar Nucleic Acid Stain (BioWhittaker Molecular Applications, Rockland, MA). PCR products were cloned with the Invitrogen TOPO TA Cloning Kit for Sequencing (Invitrogen Carlsbad, California) and the inserts were re-amplified using the modified-M13F (F2) (5' CAG TCA CGA CGT TGT AAA ACG ACG GC 3') and modifed-M13R (F4) (5' CAG GAA ACA GCT ATG ACC ATG 3') (23) with the PCR

conditions described above. The PCR products for sequencing were treated with ExoSAP-IT (USB, Cleveland, OH) using a modification of the manufacturer's protocol (ExoSAP enzyme was diluted 1:8 and the reaction was incubated at 37°C for 30 minutes, followed by an 80°C incubation for 15 minutes) and submitted for sequencing with ACD31F, 1492R, 338F (5' ACT CCT ACG GGA GGC AGC 3'), 338R (5' GCT GCC TCC CGT AGG AGT 3'), 531R, 810R (5' GGC GTG GAC TTC CAG GGT ATC T 3'), 776F (5' AGC AAA CAG GAT TAG ATA CCC TGG 3'), 1087F (5' GGT TAA GTC CCG CAA CGA 3'), modified-M13F, and the modified-M13R. Each primer was used in duplicate sequencing reactions. Sequencing was performed at the Michigan State University Research Technology Support Facility (genomics.msu.edu/index.html). Sequences were assembled using the DNA Star LaserGene Software (Madison, WI) and aligned using the ARB Software (31), and compared to acidobacterial 16S rRNA gene sequences downloaded from GenBank. The phylogeny algorithms in ARB were used for the generation of the phylogenetic trees; PAUP* Version 4.0b10 was used for bootstrapping analysis (54).

Colony and cell morphology Bacterial colonies on plates of MHM medium were examined under a Nikon SMZ-2T dissecting microscope at 10-15X for size, pigmentation, form, elevation, and margin (50). Gram stain reactions were performed as described previously (48) and cells were stained with India ink to determine if they were capsulated. Gross cell morphology and motility were assessed by using phase contrast microscopy with a Zeiss Axioskop Microscope (Carl Zeiss Inc., Thornwood, NY).

Transmission electron microscopy was completed at the Michigan State University Center for Advanced Microscopy (www.ceo.msu.edu).

Characterization of genomic DNA The mole percent G + C content of genomic DNA from strains KBS 83 and KBS 96 was determined as described previously (33). Briefly, genomic DNA was extracted using a Qiagen Genomic DNA Extraction Kit (Qiagen, Valencia, CA), and approximately 2 μg of DNA was digested with P1 nuclease and alkaline phosphatase. The nucleosides were separated and quantified using a Shimadzu™ High Pressure Liquid Chromatograph fitted with a UV detector and a VP Series Alltima C18 column (250 x 4.6mm; particle size 5μm) (Alltech Associates, Inc., Deerfield, IL). Genomic DNA purified from *A. capsulatum* (ATCC Number 51196) was used as a positive control.

The number of 16S rRNA-encoding genes was determined by non-radioactive Southern hybridization after restriction endonuclease digestion of genomic DNA, as described previously (25, 26) (http://rrndb.cme.msu.edu/rrndb/servlet/controller). A DIG-labeled, acidobacteria-specific 16S rRNA gene probe was made targeting regions between 31 and 531 (*E.coli* numbering). Genomic DNA purified from *A. capsulatum* was used as a positive control.

RESULTS and DISCUSSION

Isolation of new members of subdivision 1 & 3 The recovery of soil microorganisms using this new medium base, VL55, along with more native carbon sources such as plant polymer mixture and xylan was between 0.6 to 1.3 x 10^7 CFU/gram

of soil (dry wt) (Figure 4.1), which was lower then previous recoveries which ranged between 3.5 to 5 x 10^7 CFU/gram of soil (dry wt) (16). The major difference in these cultivation experiments from previous ones was the use of Gellan Gum, a bacterial exopolysaccharide, as the solidifying agent which was shown previously to be superior to agar for isolating novel members of the acidobacteria (21, 44). Regardless of the media additive or pH, the recovery under hypoxic conditions was similar, however under oxic conditions there was a ca. 1.3-fold higher recovery of microorganisms when xylan and the plant polymer mixture were used as the source of carbon with the conventional agriculture soil (treatment 1) as the source of inoculum as compared to the mid-successional soil (treatment 8) at pH 5.5 (Figure 4.1).

The inclusion of these compounds appears to have led to the successful cultivation of the new subdivision 1 strain KBS 83 which was isolated from VL55-PP at a pH of 5.5 from the conventional agriculture plot (treatment 1) at the KBS LTER after incubation at room temperature for ca. 30 days in an oxic atmosphere. Perhaps the use of these more recalcitrant carbon sources prevented substrate-accelerated death (53) for strain KBS 83 since these polymers must be hydrolysed before used as a source of carbon (7, 30), as suggested to be responsible for the increased viable counts of soil bacteria from Australian soil (44). The new strain in subdivision 3, KBS 96, was isolated on VL55-R2B under the same conditions described for KBS 83.

Genotypic, physiological, and morphological properties of KBS 83 and KBS 96 will primarily be described in this chapter. Additional strains isolated during these experiments were strains KBS 5 and 13, isolated on VL55-R2B at pH 5.5 and 6.5, respectively, and KBS 3 was isolated on VL55-PP, pH 5.5 from the never-tilled,

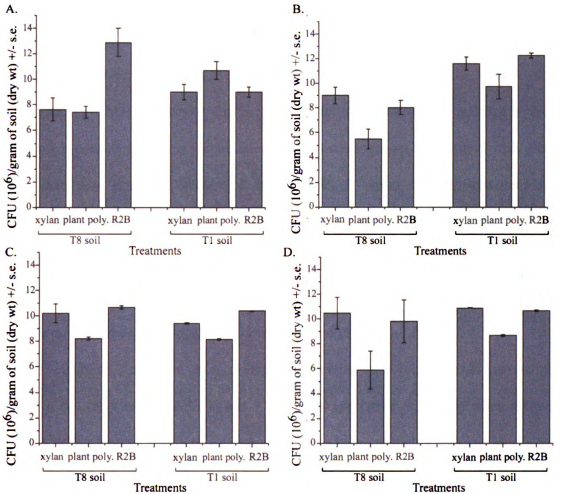

Figure 4.1. Summary of cultivation experiments. Panels A & B depict total recovery ± standard error after 30 days of growth from plates incubated under oxic conditions with the media at a pH of 5.5 and 6.5, respectively. Panels C & D depict total recovery after 30 days for growth from plates incubated under hypoxic conditions with media at a pH of 5.5 and 6.5, respectively.

successional community soil (treatment 8) at the KBS LTER after incubation at room temperature for ca. 30 days in an oxic or hypoxic (KBS 13) atmosphere. Strain KBS 146 was isolated previously from the conventional agricultural soil (treatment 1) on VL55-R2B at pH 6.0 under a hypoxic atmosphere.

Colony and cellular morphology Colonies of all acidobacteria were small, approximately 1 mm in diameter (after ca. 14-21 days of incubation), and had a circular form with a convex elevation and undulate margin when grown on MHM-5 with glucose under oxic conditions. Colonies of strains KBS 83 were smooth, glutinous white colonies taking approximately two to three weeks to form visible colonies, whereas the colonies of strain KBS 96 had smooth, butyrous pale yellow pigmented colonies taking approximately three weeks to form a visible colony. Neither strain KBS 83 nor KBS 96 were pigmented like strains of the *Terriglobus* genus (16).

Cells of all strains are short, plump, Gram negative, nonmotile rods measuring ca. 0.5 μm x 0.4 μm (strain KBS 83) and 1 μm x 0.4 μm (strain KBS 96) when grown on MHM-5 with glucose. Cells possessed exaggerated convoluted outer membranes when viewed by TEM as is typical of a Gram-negative type cell wall (Figure 4.2, panels A&B (strain KBS 83), C&D (strain KBS 96)). Both strains produced an extracellular matrix of as yet unknown chemical composition, but which apparently caused cells to stick together tightly in colonies and form visible clumps in liquid culture similar to the previously isolated strains in the *Terriglobus* genus (16). Strain KBS 83 typically appears in clumps of four cells as seen under phase contrast and transmission electron

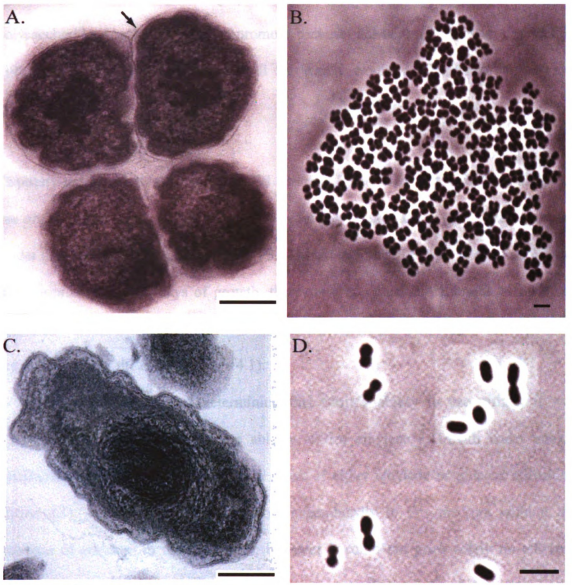

Figure 4.2. Phase contrast and transmission electron micrograph of KBS 83 (panels A & B) and KBS 96 (panels C & D). Arrow in panel A indicates the capsular material layer produced by KBS 83. Scale bar indicates 1 μm (panels B & D) and 200 nm (panels A & C).

microscopy (Figure 4.2, panels A&B), and are held together in this tetrad by capsular material visible in electron micrograph (Figure 4.2, panel A). Capsules or extracellular polysaccharides has been shown to promote bacterial adhesion (9), prevent desiccation (39), and soil aggregation (1, 3), all of which would be beneficial for a soil microorganism.

Capacity to utilize nitrate Strains KBS 83 and KBS 96 were tested for their ability to use nitrate either via denitrification, nitrate reduction to ammonium, or nitrate reduction. Under the conditions tested, strain KBS 83 does not appear to be capable of using nitrate. After approximately 25 days of growth, there were no detectable amounts of nitrite or higher concentrations of ammonium in the spent medium, and nitrate concentrations were not different from the control (Table 4.1).

During the course of determining this strain's ability to use nitrate, it was discovered that strain KBS 83 was able to utilize ethylene. Growth under anoxic conditions preliminarily appears to be a result of either ethylene or glucose utilization (Table 4.1). This strain could not grow anaerobically in this medium without the presence of ethylene in the headspace; however it could not grow solely on ethylene under anaerobic conditions. It was interesting to note that growth of this strain under these conditions was inconsistent, reaching a maximum optical density ranging from 0.02 to 0.102 at 600 nm; perhaps the inconsistent maximum optical density noted during growth resulted from possible impurities of the ethylene gas. Ethylene is a plant hormone used to promote fruit ripening of higher plants. This highly unusual substrate has been seen previously for growth in *Nitrosomonas europaea* (19) as well as other soil

Table 4.1. Summary of growth and end products of KBS 83 & KBS 96 under anoxic conditions with nitrate.

		Strain KBS 83		Strain KBS 96	
Concentrations (mM)		Blank	T25[†]	Blank	T13[†]
Carbon source					
	Glucose	9.7 ± 0.2	9.0 ± 0.3	9.7 ± 0.2	7.9 ± 0.8
	Ethylene	5.3 ± 0.3	2.6 ± 0.4	5.3 ± 0.3	4.2 ± 0.2
End products					
	NO_3^-	ca. 49.3	49.0 ± 0.9	ca. 49.3	47.8 ± 1.1
	NO_2^-	ca. 0	0 ± 0	ca. 0	1.1 ± 0.1
	NH_4^+	ca. 5.93	6.0 ± 0.3	ca. 5.93	5.5 ± 0.2
	N_2O	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	CO_2	0 ± 0	0.17 ± 0.9	0 ± 0	0.46 ± 0.04

[†] Indicates the number of days it took for culture to reach stationary phase.

microorganisms (12), furthermore certain plants and microbes such as *Pseudomonas syringae* (59) and *Penicillium cyclopium* (8) can produce ethylene at sufficiently high concentrations for growth of other organisms.

Alternatively strain KBS 96 exhibited a substantially different pattern of growth under these conditions where growth commenced after ca. 3 days instead of ca. 17 days as seen with strain KBS 83. After approximately 13 days of growth, ca. 3% of the nitrate was converted into nitrite and ca. 20% of the glucose was utilized (Table 4.1). The ammonium concentrations were not substantially higher from the initial concentrations and no nitrous oxide was detected in the headspace. Like strain KBS 83, it can also use ethylene given the ca. 1.2-fold reduction of ethylene in the headspace; there was no growth without the presence of ethylene in the headspace.

These results preliminarily suggest that nitrate was being reduced to nitrite while glucose and/or ethylene were either being oxidized through a respiratory pathway or glucose being oxidized by fermentation. Preliminary there was ca. 2-fold higher growth rate with ethylene and glucose in the presence of nitrate as compared to the absence of nitrate. Although very little of the nitrate was reduced to nitrite, it appears that there was enough energy produced for growth from the partial reduction of nitrate to nitrite for anaerobic respiration (for both glucose and ethylene) based on free energy (ΔG) calculations even with scenarios where high free energy products were produced. Since nitrate was in excess, the low optical density readings of the strain were presumed to be of result a build-up of an inhibitory compound(s) resulting from growth or limiting concentration of a cofactor such as molybdenum which is necessary for the metalloprotein, nitrate reductase.

Strain KBS 96 preliminarily appears to be capable of reducing nitrate to nitrite under anoxic conditions, but not is able to denitrify. This trait is fairly ubiquitous and is the most widely distributed variant of using nitrogenous oxides for respiratory purposes (64). Perhaps this ability could provide a selective advantage allowing them to survive during rainfall events, creating anoxic pockets in the soil especially in fertilized fields high in nitrate. Additionally, this physiology could be used to develop novel cultivation methods to isolate additional strains in this poorly represented subdivision. Readily available strains of subdivision 1, *Terriglobus roseus* strain KBS 63, *Terriglobus sp.* strain TAA 43 (16), and *Acidobacteriaceae* strain KBS 146 were not able to grow under anoxic conditions with nitrate and glucose as the carbon source. Strain KBS 96 was initially cultivated under aerobic conditions, however it was further isolated in pure culture on the MHM-5 with 10 mM glucose under anaerobic conditions, illustrating its capacity to form a colony on a plate under these nitrate reducing conditions, typically taking about three weeks.

Other physiological properties The characteristics of the new strains are summarized in Table 4.2. Similar to the *Terriglobus* strains (16), KBS 83 and KBS 96 preferred slightly acidic pH conditions. KBS 83 grew at pHs between 4.5 and 6, growing optimally at 5.0, whereas KBS 96 grew between pHs 4.0 and 6, optimally growing at 6.0 which is consistent with the pH of their native environment. As suggested previously, members of subdivision 1 have a preference for mildly acidic pH conditions (16, 43). Preliminary these data suggests that members of subdivision 3 might also follow this trend given the growth of strain KBS 96 under mildly acidic conditions.

Table 4.2. Phenotypic characteristics of *Terriglobus*, *Acidobacteriaceae*, and *Acidobacterium* strains.

Characteristics	*Acidobacteriaceae* KBS 96	*Acidobacteriaceae* KBS 83	*Terriglobus* KBS 63[a]	*Acidobacteriaceae* KBS 89[a]	*Acidobacterium* *A.capsulatum*[b]
Subdivision	3	1	1	1	1
Origin	agricultural soil	agricultural soil	successional community soil	successional community soil	acidic mine drainage
Gram reaction	negative	negative	negative	negative	negative
Cell Shape	rod	rod	rod	rod	rod
Length (µm)	ca. 1	ca. 0.5	1.1 ± 0.2	ca. 1	1.1 - 2.3
Width (µm)	ca. 0.4	ca. 0.4	0.6 ± 0.1	ca. 0.5	0.3 - 0.8
Capsule	-	+	-	-	+
Pigment	pale yellow	white	pink	white	orange
rrs copy number	2	1	2	1	1
G + C content (mol %)[‖]	60.6 ± 0.41	62.2 ± 0.07	59.8 ± 0.5	59.7 ± 1.8	62.7 ± 0.1
Catalase	+	+	+	-	+
Oxidase	+	-	-	-	-
Motility	-	-	-	-	+
pH range (optimum)	4.0 – 6(6)	4.5 – 6 (5)	5 – 7 (6)	ND	3 – 6
Capacity to reduce NO_3	+	-	-	-	ND
Capacity to denitrify	-	-	-	-	ND
Capacity of DNRA	-	-	-	-	ND
Growth at 4°C	-	-	-	-	ND
Growth at 12°C	+	+	+	+	ND
Growth at 25°C	+	+	+	+	ND
Growth at 37°C	+	+	-	-	
Differences in carbon utilization					
Monosaccharides					
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	ND
D-Galactose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Ribose	+	-	-	+	ND
D-Xylose	+	+	+	+	+

Table 4.1 contd.					
L-Arabinose	+	+	+	+	+
D-Mannitol	+	-	-	-	ND
D-Sorbitol	+	+	-	+	ND
Disaccharides					
Sucrose	+	+	+	+	ND
D-Maltose	+	+	+	+	+
Trisaccharides					
D-Raffinose	+	+	+	+	ND
Organic Acids					
Sodium acetate	-	-	-	-	ND
Sodium pyruvate	-	-	-	-	ND
Sodium formate	-	-	-	-	ND
Sodium succinate	-	+	+	+	ND
Other carbon cmpnds					
Xylan mix	+	+	+	-	ND
Benzoate	-	-	-	-	ND
Syringate	+	+	-	-	ND
Pectin	+	+	-	-	ND
Trimethoxybenzoate	-	+	-	-	ND
Methyl cellulose	+	+	-	-	ND
Ferulate	+	+	-	-	ND
Resorcinol	-	-	-	-	ND
Cellulose	+	+	ND	ND	ND

[a] Reference: characteristics described from a previous study (16). ND "not determined"
[b] Reference: characteristics described from a previous study (24). ND "not determined"

Strain KBS 83 grew significantly faster at oxygen concentrations between 4% to 16% (avg. p-value = 0.01) as compared to atmospheric concentrations of oxygen, growing optimally at 8% oxygen (μ = 0.014 hr^{-1}) (Figure 4.3), suggesting that it was microaerophilic. Microaerophiles are defined as organisms not capable of growing or growing poorly under atmospheric concentrations of oxygen presumably due to an increased sensitivity to toxic forms of oxygen such as H_2O_2, O_2^-, and OH·(27). Although strain KBS 83 was catalase positive, suggesting that cells have the capacity to deal with peroxides, this enzyme's effectiveness might be low, similar to certain *Campylobacter* species classically described as microaerophiles (41). The preference of this strain for low levels of oxygen is consistent with their native environment; oxygen can be depleted in the soil environment, particularly in soil aggregates when soil moisture increases (6, 35, 58). Strain KBS 96 qualitatively grew better under atmospheric concentrations of oxygen; this strain's growth requirements demanded the use of a undefined medium which made optimal growth testing under different oxygen concentrations difficult since the concentration of oxygen was not in excess relative to the unknown amount of carbon. Nevertheless, it was clear the strain KBS 96 preferred more oxygen rich environments.

Characterization data from recently isolated strains of the *Acidobacteria* (16) along with data obtained from cultivation experiments using PWPCR suggest that some acidobacteria are microaerophiles. The inclusion of the catalase enzyme as well as incubation under microoxic conditions increased with frequency of acidobacteria detection by ca. 3.3 and 1.3-fold, respectively on initial cultivation experiments (Chapter 2). The newly isolated strain KBS 83 grows optimally at low concentrations of oxygen.

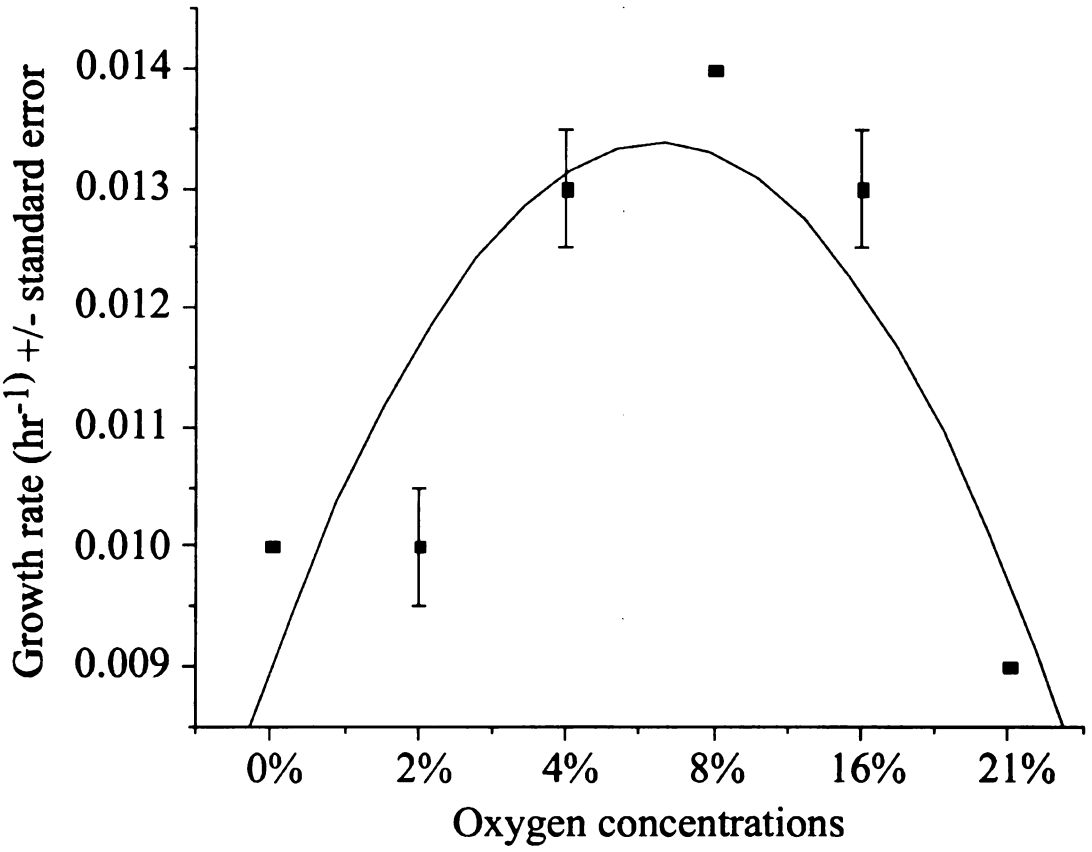

Figure 4.3. Summary of growth rates for KBS 83 under different concentrations of oxygen. Note growth rate for an oxygen concentration of 0% was obtained from studies assessing the capacity to use nitrate.

Although not following the classic definition of microaerophily, members of the recently described genus *Terriglobus* produce a carotenoid(s) whose quantity per cell increased in response to oxygen, suggestive of an oxidative stress response (16). These data taken together suggest that oxygen and its damaging effects is a major concern to the lifestyle of acidobacteria, and strains harbor traits to respond accordingly.

Relatively slow growth rate in acidobacteria strains is becoming a trend with recent cultivars which is consistent with their low number of genes coding for the 16S rRNA gene. These new strains contain either one (KBS 83) or two (KBS 96) copies of the *rrs* gene (Figure 4.4). Additionally, recently sequenced genomes of *Acidobacterium* str. Ellin 345 and *Solibacter usitatus* strain Ellin 6076 reveal that both harbor one copy of the *rrs* gene (www.jgi.doe.gov), and in summary no strain in the phylum *Acidobacteria* to date contains an *rrs* copy number greater than 2. This is additional support for previous claims suggesting that members of the phylum *Acidobacteria* can be described as oligotrophic given their high abundance in soil environments where nutrients are low (17), relatively slow growth rate, and possession of either one or two copies of the 16S rRNA encoding gene – a genomic marker of oligotrophic bacteria (25).

There were major differences in the carbon utilization profiles of these new strains as compared to previously isolated strains in *Terriglobus* and *Acidobacteriaceae* (16). Strain KBS 83 and KBS 96 had the capacity to oxidize more native soil carbon such as xylan, cellulose, carboxymethyl cellulose, syringate, pectin, trimethoxybenzoate, and ferulate (Table 4.2), which are typically subject to slow turnover in the soil environment (6). I propose that the use of these more recalcitrant carbon compounds was responsible for its successful isolation of strain KBS 83, presumably because the use of

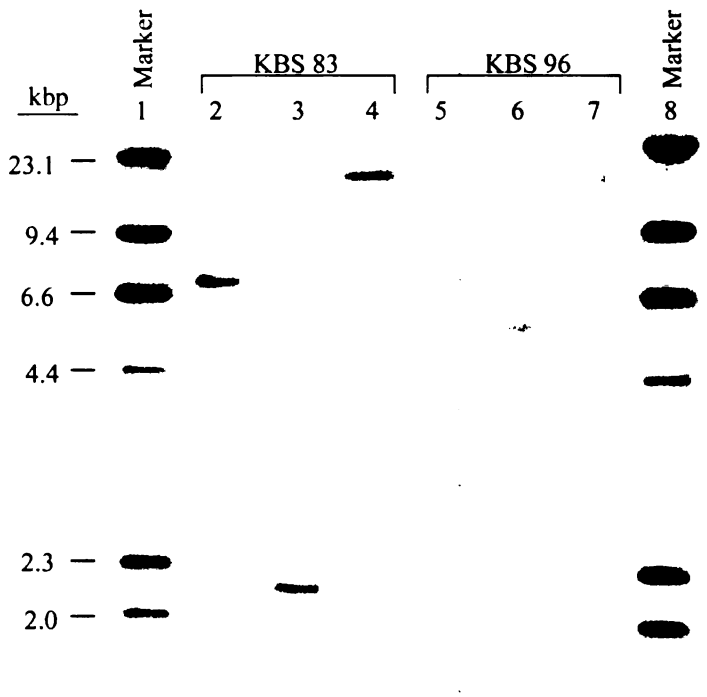

Figure 4.4. Southern hybridization of digested genomic DNA from strains KBS 83 & KBS 96. Strains were cut with *EcoRI* (lanes 2 & 5), *EcoRV*I (lane 3 & 6), and *HindIII* (lane 4 & 7). The lambda DNA *Hind* III marker (lane 1 & 8) provided size estimates.

these compounds prevented substrate-accelerated death (37, 53) since these polymers must be hydrolysed before used as a source of carbon (7, 30) as suggested to be responsible for the increased viable counts of soil bacteria from Australian soil previously (44).

Plants in terrestrial ecosystems contain 560 Pg of the total carbon content on the planet (61). Plant tissues are the dominant source of soil polysaccharides, specifically cellulose, hemicellulose, and lignin (55). Depending on the type of plant, xylans can comprise from 7% to 35% (dry weight of the plant) (60), and they are typically oxidized by nearby microbial communities. Although the soil environment is referred to as carbon limiting, plant polymers and products of the degradation are major sources of carbon for indigenous populations of microorganisms. Microorganisms that harbor active enzymes to degrade these plant polymers are essential for maintaining an active carbon flow in the soil.

The ability of these acidobacteria to oxidize more recalcitrant carbon compounds is a practical trait to have in the soil environment. One could predict that strains capable of oxidizing plant polymers might be prevalent in environments where carbon pools are low such as in the conventional agriculture soil (treatment 1) where carbon concentrations primarily consisting of degradation of plant material from corn, soybean, and wheat that is plowed into the soil annually and are ca. 2 to 3-fold lower as compared to the successional treatment (treatment 8). In order to test this prediction, acidobacteria 16S rRNA gene sequences from treatment 8 and 1 soils were analyzed, specifically assessing if there was a higher prevalence of sequences from treatment 1 in the clade

harboring the acidobacteria strain capable of oxidizing these more recalcitrant carbon sources based on the assumption that phylogeny follows function. Figure 4.5 depicts a phylogenetic tree of this clade along with the number of acidobacteria sequences from treatments 8 and 1. Approximately half of the clones in this clade belonged to treatment 1, suggesting that this trait is not unique to acidobacteria from low carbon environments and is fairly ubiquitous in both conventional agriculture and mid-successional community soils.

16S rRNA gene phylogeny Figure 4.6 depicts a phylogenetic tree of nearly full length sequences for the new strains. Strains KBS 13, 5, and 3 are ca. 97% similar to *T. roseus*, strain KBS 63, therefore preliminary appears to be members of the *Terriglobus* genus (16). The closest cultivar to strain KBS 146 is strain TAA 166 with a sequence similarity of ca. 99%.

Strain KBS 83 is more closely related to *A. capsulatum* (ca. 96% sequence similarity) than to members of *Terriglobus* and is most similar (ca. 99%) to an environmental clone from a polychlorinated biphenyl-polluted soil (env. WD277, AJ292577) (34). The strain belonging to subdivision 3, KBS 96 is ca. 94% similar to an environmental clone from a reaction system treating monochlorobenzene contaminated groundwater (env. GOUT8, AY050595) (2), and is only ca. 93% similar to *Solibacter usitatus* strain Ellin 6076.

Strain KBS 83 and KBS 96 are distinct from previously isolated acidobacteria to call for the creation of a new genus and species in subdivisions 1 and 3, respectively. Strain KBS 83 is a member of subdivision 1 and only 96% similar to *A. capsulatum*, and

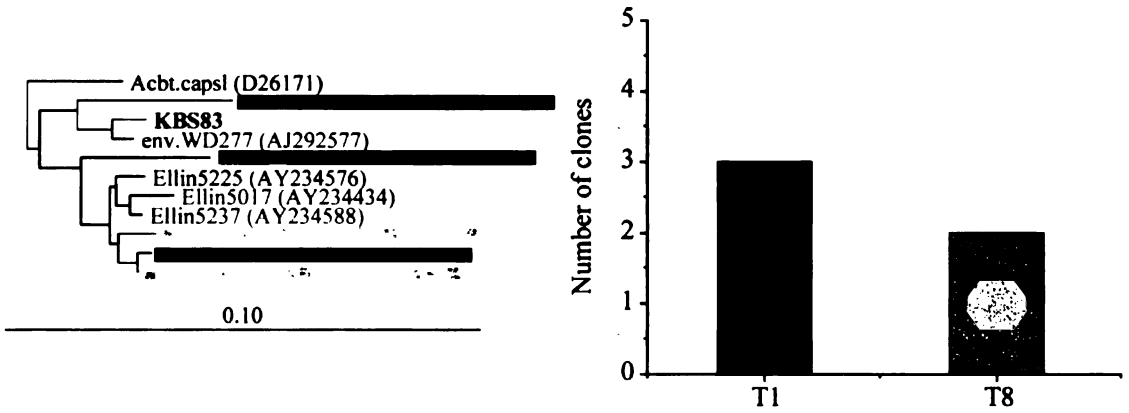

Figure 4.5. Neighbor-joining phylogenetic tree depicting the number of 16S rRNA gene clone sequences generated from treatments 1 and 8 (black and gray boxes, respectively) at the KBS LTER in the clade harboring strain KBS 83.

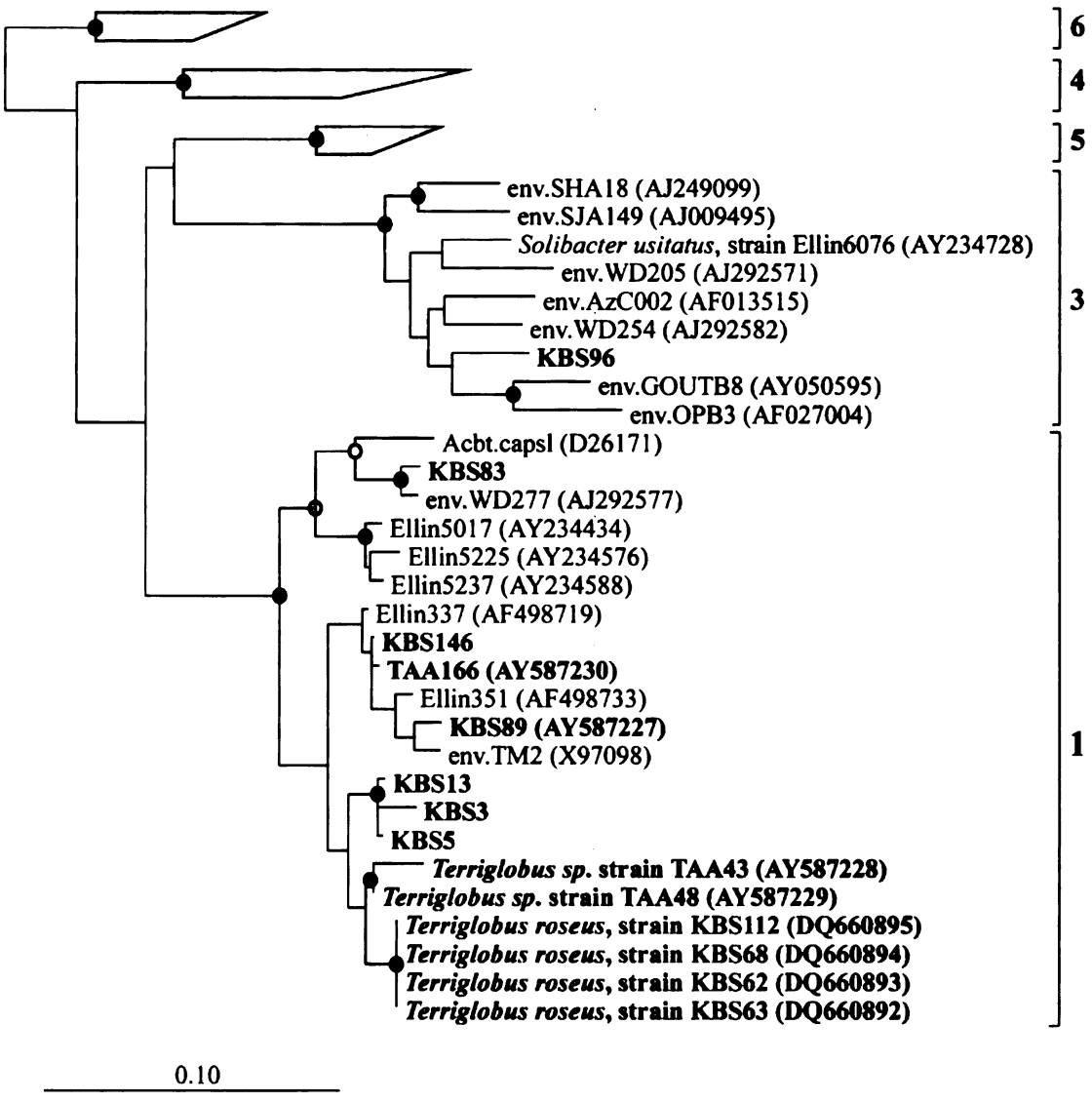

Figure 4.6. Maximum-likelihood tree of the *Acidobacteria* subdivisions 1, 3, 4, 5, and 6 (indicated to the right of the group) based on 16S rRNA gene using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). Strains from this study are in bold font. Each gray trapezoid represents a different subdivision with approximately 5 representative sequences. Internal nodes supported by a bootstrap value of >95% are indicated with a filled circle (•) and of >70 with an open circle (o). The scale bar indicates 0.10 changes per nucleotide.

94% similar to the *Terriglobus* strains. Unlike *A. capsulatum,* this strain cannot grow at low pH (pH 3.0-6.0) conditions, it is not motile, and it was isolated from soil, not an acidic mine drainage. Given the low sequence similarity, it does not appear to be members of *Terriglobus*. Strain KBS 96 is a member of subdivision 3 and only ca. 93% similar to previously isolated strain *Solibacter usitatus,* strain Ellin 6076. The specific epithets to strains KBS 83 and KBS 96 could not be determined for my dissertation, but will be given in the publication of this work.

Description of new genus and species in subdivisions 1 Gram negative, nonmotile capsulated short rods characteristically in groups of four (ca. 0.5 x 0.4 μm) when grown on MHM-5 with 10 mM glucose. Moderate acidophile with an optimal pH of 5. Colonies are non-pigmented, taking approximately two to three weeks to form a colony. Catalase positive; oxidase negative; one copy of the *rrs*; isolated from a conventional agriculture treatment at the KBS LTER; able to grow at 12°C, 25°C and 37°C, but not 4°C; G+C content ca. 62 mol%; microaerophilic growing optimally at oxygen concentrations between 4 to 16%; and able to oxidize more recalcitrant carbon sources such as xylan, syringate, pectin, methyl cellulose, ferulate, and cellulose, in addition to various mono-, di- and trisaccharides. Type strain KBS 83.

Description of new genus and species in subdivisions 3 Gram negative, nonmotile short rods (ca. 1.0 x 0.5 μm) when grown on MHM-5 with 10 mM glucose. Moderate acidophile with an optimal pH of 6. Colonies are pale yellow, taking approximately three weeks to form a colony. Catalase positive; oxidase positive; two

copies of the *rrs*; isolated from a conventional agriculture treatment at the KBS LTER; able to grow at 12°C, 25°C, and 37°C, but not 4°C; G+C content ca. 60 mol%; reduces nitrate to nitrite; can growth at low concentrations of oxygen, but prefers atmospheric concentrations; and able to oxidize more recalcitrant carbon sources such as xylan, syringate, pectin, trimethylbenzoate, methyl cellulose, ferulate, and cellulose in addition to various mono-, di- and trisaccharides. Type strain KBS 96.

These strains will be deposited into the USDA Agricultural Research Service Culture Collection and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

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CHAPTER 5

THE EXPLORATION OF METHANOTROPHY AND METHYLOTROPHY AMONG MEMBERS OF THE PHYLUM *ACIDOBACTERIA*

INTRODUCTION

Methane is a plentiful gas in the soil environment. Forests, grasslands, and other unsaturated soils are major sinks for atmospheric methane (11). Microorganisms are responsible for methane oxidization in these environments, consuming 20 to 60 Tg of methane per year (11). Unfortunately, these communities are largely unexplored as evident by the discrepancy between the rates of known methane oxidizers and soils' consumption of methane (8, 24).

Microorganisms that are capable of growth on one-carbon compounds such as methanol, methylated amines, halomethanes, and sulfur-containing methylated compounds are referred to as methylotrophs, while organisms capable of growing on methane are designated as methanotrophs. These compounds serve as the microorganism's sole source of carbon and energy (11) and this unique group of microbes are found in soils and sediments throughout the globe (12, 13, 24, 25, 34). Notably, this unique physiology is not limited to bacteria; it has also been observed in yeast as well as filamentous fungi (11).

Oxidization of these one-carbon compounds is a simple, yet elegant pathway converting single carbon compounds into complex, multi-carbon cellular material. Methane is oxidized to methanol by the methane monooxygenase enzyme and is

performed by two phylogenetically distinct groups of methane oxidizers, Type I and II, which differ by their assimilation pathway and the type of methane monooxygenase enzyme. Type I methanotrophs are members of the γ-*Proteobacteria* phylum, oxidizing methane with a membrane-bound or particulate methane monooxygenase (pMMO), and assimilating carbon into cellular material with the RuMP pathway. Type II methanotrophs are members of the α-*Proteobacteria* phylum oxidizing methane with a cytoplasmic soluble methane monooxygenase (sMMO) and assimilating carbon using the serine pathway (11). Methanol, resulting from the oxidization of methane or exogenous sources such as pectin or lignin degradation, is further oxidized to formaldehyde with the highly conserved periplasmic enzyme, methanol dehydrogenase (MDH) (11, 23). Remarkably, the presence of methanol promotes the oxidization of methane in pure cultures and soil enrichments (2). Formaldehyde is then shuttled to one of three assimilation pathways, ribulose monophosphate (RuMP), serine, or ribulose-1,5-bisphosphate carboxylase (RuBP), to create multi-carbon compounds for the generation of cellular material (Figure 5.1) (11).

Previous studies have implicated members of the phylum *Acidobacteria* with the capacity to oxidize one-carbon compounds. Radajewski and colleagues (26), after incubating oak forest soil samples with ^{13}C-methanol for a period of 40 days, revealed that acidobacterial DNA was present in the heavy fraction of a cesium chloride gradient (Figure 5.2) (26-28). Heavy-labeled acidobacterial DNA suggests these acidobacteria used the methanol as a substrate for growth thus incorporating the ^{13}C into their DNA. The ubiquity of acidobacteria in the soil along with the scarcity of knowledge correlating

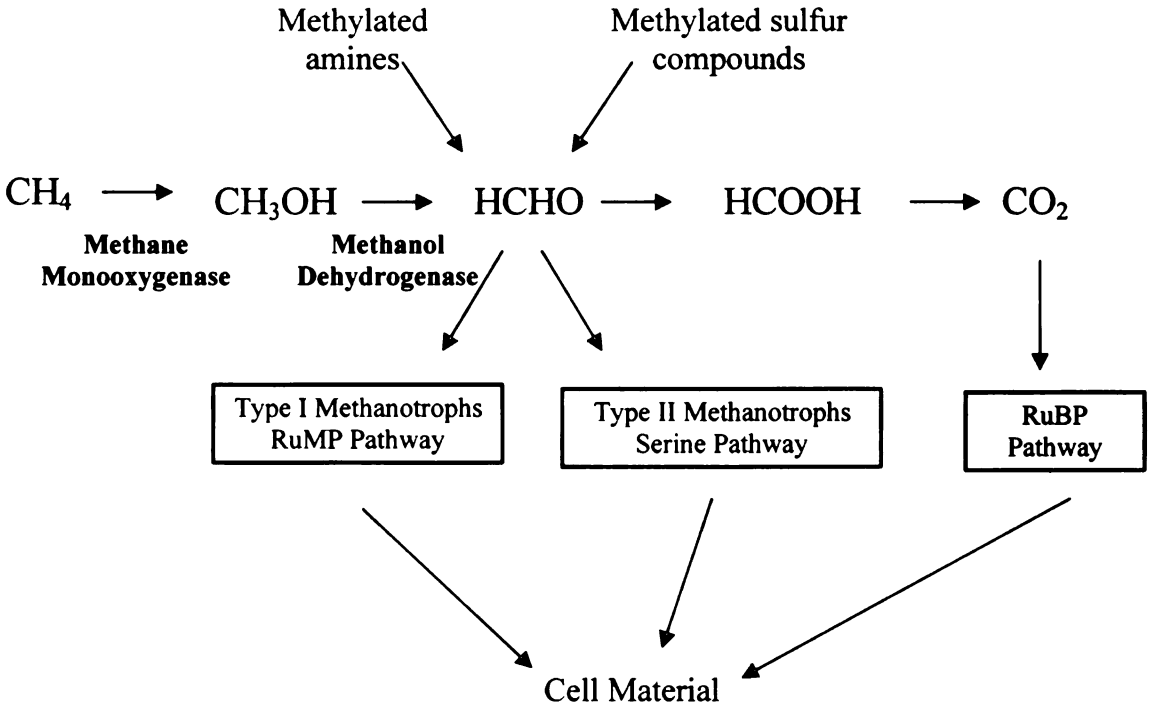


Figure 5.1. Simplified overview of the pathway for the oxidation and assimilation of one-carbon compounds into cellular material using the RuMP, RuBP, or serine pathways.

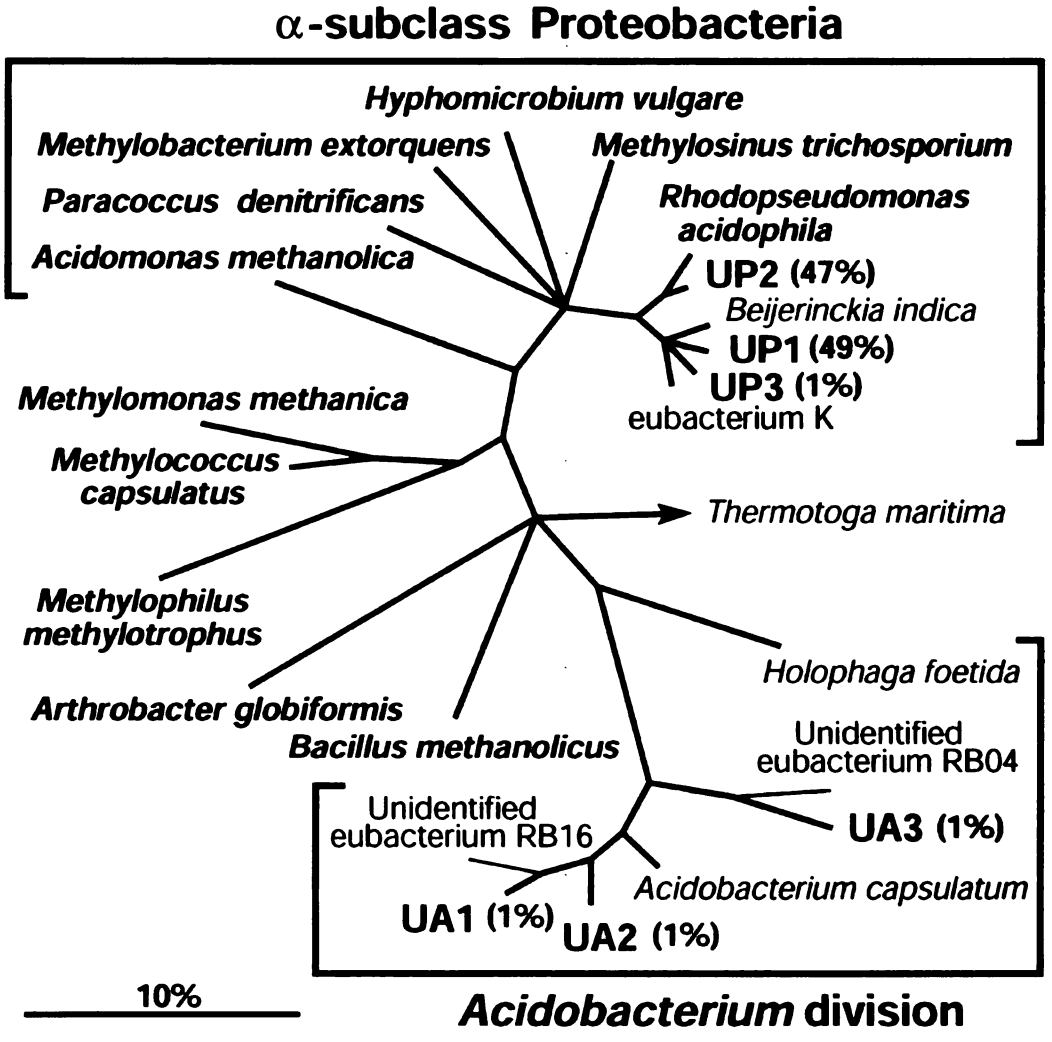


Figure 5.2. Acidobacteria clones obtained after incubation of acidic oak forest soil samples with 350 mM methanol (26).

upland soils' methane fluxes to known, cultivated microorganisms could be two pieces of the complex soil puzzle, which if fit together, can greatly improve our understanding of these bacteria in the soil. The goal of this study is to assess the metabolic potential of acidobacteria to oxide one-carbon compound(s) using growth and molecular-based approaches.

MATERIALS and METHODS

Pure culture approach

Media composition and growth conditions Strains were tested for the capacity to oxidize one-carbon compounds on VSB-7 as described previously (Chapter 3) as well as on a nitrate mineral salts medium (pH 7). Nitrate mineral salts, Whittenbury medium (NMS) contained the following components (per liter): $MgSO_4$, 0.49g; KNO_3, 0.1g; $CaCl_2 \cdot H_2O$, 0.2g; Whittenbury trace element solution (1mL/L); Na_2MoO_4, 0.0005g; FeEDTA, 0.0038g; $CuSO_4$, 0.8mg; phosphate stock solution (10mL); and vitamin stock solution (10mL). The Whittenbury trace elements solution contained the following (per liter): $FeSO_4 \cdot 7H_2O$, 0.5g; $ZnSO_4 \cdot 7H_2O$, 0.4g; $MnCl_2 \cdot 4H_2O$, 0.02g; $CoCl_2 \cdot 6H_2O$, 0.05g; $NiCl_2 \cdot 6H_2O$, 0.01g; H_3BO_3, 0.015g; and NaEDTA, 0.25g. The phosphate stock solution contained the following (per liter): KH_2PO_4, 26g, and Na_2HPO_4, 33g. The vitamin stock solution contains the following (per liter): biotin, 2mg; folic acid, 2mg; thiamin-HCl, 5mg; calcium pantothenate, 5mg; vitamin B12, 0.1mg; riboflavin, 5mg; and nicotinamide, 5mg. For solid medium, agar was added to a final concentration of 1.5% (g/vol).

Carbon Utilization Testing All strains were grown on replicate agar plates of VSB-7 and NMS with 10 mM of one of the following one-carbon compounds: methanol; formate; methylated amines containing dimethylamine (2.8 mM), trimethylamine (1.8 mM), and methylamine (5.4 mM); and dimethylsulfoxide. Plates were incubated at room temperature (ca. 23°C), under low light for a period of 40-60 days, under one of the following atmospheres: CO_2-enriched hypoxia (vol/vol: 2% O_2, 5% CO_2, and bal N_2); CO_2-enriched air (vol/vol: 95% air, 5% CO_2), or air. Liquid cultures of the NMS medium were grown with an oxic headspace supplemented with methane (30% vol/vol).

Strains KBS 63, TAA 43, and *A. capsulatum* were also grown on the VSB-6 with 50 mM and 100 mM methanol in 750 ml sealed, side-arm flasks. Some of these cultures were supplemented with a combination of 2.5 mM glucose and 1 mM formaldehyde. Optical density at 600 nm was monitored every 5 to 10 hours with a Thermo Spectronic model 20D+ spectrophotometer. The headspace was monitored for a period of 30 days using an Aerograph flame ionization gas chromatograph series 2440 (Varian Instrument Division, Palo Alto, California) with a 5 foot long, $1/8^{th}$ inch stainless column with Porapak Q packing material.

PCR amplification and cloning DNA was extracted from the pure cultures using the MoBio Fecal DNA Extraction Kit (MoBio Labs. Inc., Carlsbad, California). Genomic DNA was screened for the presence of the methanol dehydrogenase gene (*mxaf*) using primers *mxa* f1003 and *mxa* r1561 (23), and the different forms of methane monoxygenase using A189/A682 (active site for pMMO) and ammonium monooxygenase) (14), A189/mb661 (active subunit for pMMO) (4), mmox536f/898r (9)

and mmox206f/886r (15) (active subunit for sMMO), mmox206F/886R (active subunit for sMMO) (15). Purified genomic DNA from *M. capsulatum* (ATCC Number 19069-D) for was used as a positive control. Each 25µl PCR reaction contained 1x PCR Buffer, 1mM magnesium choloride, 0.02mM of each dNTP, 0.2µM each primer, and 5U *Taq* polymerase (Invitrogen, Carlsbad, California). The PCR program consisted of the following steps: (1) 95°C for 3 minutes, (2) 95°C for 30 seconds, specific annealing temperature for 30 seconds, 72°C for 45 seconds (step 2, repeated for 30 cycles), (3) 72°C for 10 minutes, and (4) hold at 4°C. In addition to these reactions, a PCR amplification of the 16S rRNA gene was performed to ensure the genomic DNA from the strains was amplifiable. Purified genomic DNA from *A. capsulatum* (ATCC Number 51196) was used as a positive control. PCR reactions were electrophoresed on a 1% agarose gel and stained with GelStar Nucleic Acid Stain (BioWhittaker Molecular Applications, Rockland, MA). If reactions yielded a positive amplification of the correct size, PCR products were cloned into the Invitrogen TOPO TA Cloning Kit for Sequencing (Invitrogen Carlsbad, California) as per the protocol instructed. The PCR product was then re-amplified using the following primers: modified-M13F and modified-M13R (19) with the PCR conditions as described above. PCR products were purified for sequencing using a 1:8 dilution of ExoSAP-IT (USB, Cleveland, Ohio) following incubation at 37°C for 30 minutes and 80°C for 15 minutes, and reactions were sent to the Michigan State University Genomics Technology Support Facility (RTSF) (http://genomics.msu.edu/index.html).

Environmental molecular-based approach

Soil microcosms Soil samples were collected July 10th, 2001 from the Michigan State University W.K. Kellogg Biological Station Long Term Ecological Research Site (KBS LTER). The KBS site is a 48-hectare plot designed to study ecological processes in agroecosystems, which prior to its establishment in 1989 was uniformly farmed for more than 50 years. A description of this site can be accessed at http://www.kbs.msu.edu. Soil cores (2.5 cm diameter x 10 cm depth) were collected from a mid-successional community with no history of tillage (treatment 8), and stored at 4°C for approximately two weeks prior to establishing the microcosms. Treatment 8 soil was used for this microcosm because preliminary clone analysis of acidobacterial 16S rRNA genes revealed a high sequence similarity to Radjewski's and colleagues (26) clone sequences.

The soil was homogenized in a sterile 500 mL beaker by gently mixing with a sterile spatula. Replicate, sealed microcosms consisted of approximately 10 grams in a 125 mL crimp-top vial supplemented with 336 μmoles of methanol. The remaining homogenized soil was frozen at -20°C and later used to extract genomic soil DNA for time zero in the clone library analysis (below). The water holding capacity was adjusted to ca. 35% with sterile distilled water (20). The microcosms were incubated at room temperature (ca. 23°C) under low light conditions.

The headspace was monitored routinely for methanol using the Aerograph flame ionization gas chromatograph series 2440 (Varian Instrument Division, Palo Alto, Calif.). The headspace was flushed every other week to maintain an oxic atmosphere. Once the initial addition of methanol was consumed, methanol was re-added at the same initial concentration.

Enrichment plating Upon the consumption of the second addition of methanol, a portion of the soil was used for DNA extraction (see below) and the remaining soil was used as a source of inoculum in an attempt to cultivate potential methylotrophic acidobacteria using a water agar based medium with 25 mM, 50 mM, and 490 mM of methanol. Plates were incubated at room temperature (ca. 23°C) under CO_2-enriched air for ca. 30 days. Plates were screened for the presences of acidobacteria using PWPCR (33).

Methanol microcosm clone libraries DNA was extracted using the MoBio Fecal DNA Extraction Kit (MoBio Labs. Inc., Carlsbad, California) from soil before methanol addition (T0) and after the consumption of the second addition of methanol (T90). T0 and T90 soil DNA was used to amplify the acidobacteria 16S rRNA gene using the phylum-specific *Acidobacteria* forward primer, ACD31F (1), and the broadly inclusive bacterial primer, 1492R (21). Amplicons were cloned into the Invitrogen TOPO TA vector and prepared for sequencing with conditions described above, and submitted with the 531R (5' TAC CGC GGC TGC TGG CAC 3') primer. Sequences were aligned using ARB Software (22) along with other sequences were downloaded from Genbank and the Ribosomal Database Project (RDP) (3) for the generation of the phylogenetic tree. ∫-LIBSHUFF was used to determine the similarities of the libraries before and after methanol additions (31, 32).

RESULTS

Pure culture approach

Strain similarity to acidic forest soil clones Clone sequences of the 16S rRNA genes obtained from acidic oak forest soil microcosms incubated with methanol (UA1, UA2, and UA3) (26) had a high sequence similarity to acidobacteria clones obtained from KBS LTER soil. KBS Acido Clone 29 was 98.8% similar to UA3; KBS Acido Clone 25 was 96.4% similar to UA1; and KBS Acido Clone 31 was 95% similar to UA2 (Figure 5.3). All clone sequences were associated with subdivision 1 of the phylum *Acidobacteria.*

Carbon ultization testing Strains, KBS 62, KBS 63, KBS 68, KBS 112, KBS 89, TAA 43, TAA 48, and TAA 166 were tested for their ability to oxidize different one-carbon compounds. These newly isolated strains had a sequence similarity to the putative methanol-oxidizing clones (26) of ca. 91% ranging from 88 to 94%. No growth was detected on the plates containing neither one-carbon compounds nor liquid cultures with 30% (vol/vol) methane in the headspace under these conditions. Positive controls of VSB-7 and NMS with glucose and glucose with one-carbon compound(s) resulted in growth indicating that the conditions were ample for growth of the strains. Neither growth nor depletion of methanol in the headspace was detected for KBS 63, TAA 43, or *A. capsulatum* in liquid cultures with 50 mM or 100 mM methanol with and without supplementation of 1 mM formaldehyde. Previous studies indicated that the presence of formaldehyde acts as an important trigger for the expression of one-carbon compound genes (6); therefore these cultures were supplemented with 1 mM formaldehyde. Controls containing glucose and methanol (

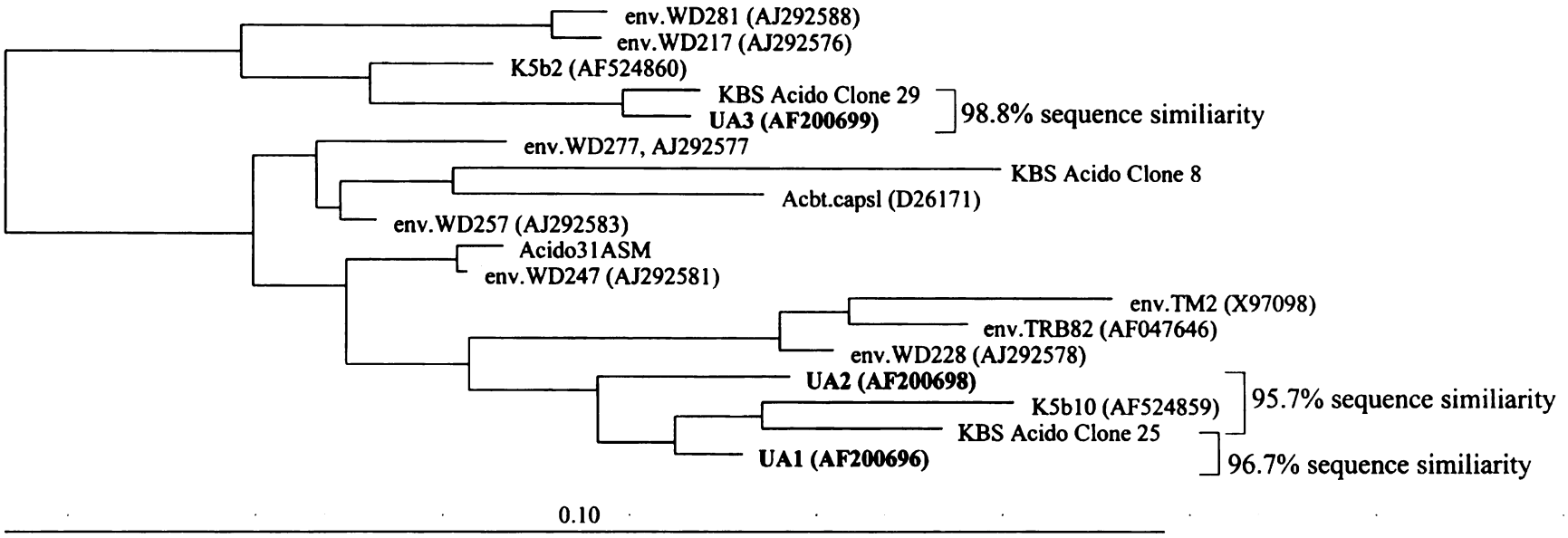


Figure 5.3. Maximum likelihood tree of the phylum *Acidobacteria*, subdivision 1. Sequences were obtained from Genbank and accession number are listed parenthetically. Sequences obtained from Radajewski and colleagues (26) are in bolded text. The scale bar indicates a 0.10 change per nucleotide.

with and without 1 mM formaldehyde) exhibited no difference in normal growth patterns, suggesting that methanol was not inhibitory to the any of the strains. Under all conditions tested, there was no evidence for these strains harboring the capacity to oxidize these one-carbon compounds.

PCR amplifaction of *mxaf* and *mmo* Purified genomic DNA of these acidobacteria stains did not yield positive amplification for the methanol dehydrogenase nor methane monooxygenase gene with readily available primer sets, even though positive controls yielded amplicons of the correct size. This result was not surprising since these primers were designed from the *Proteobacteria;* members of the phylum *Acidobacteria* could have a methane monooxygenase and/or methanol dehydrogenase gene, but these primer sets may not have amplified their genes, most likely due to the proteobacteria-specific primers not allowing any degeneracy for other diverse phyla.

Environmental molecular-based approach

Soil microcosm Since the KBS acidobacteria clones were similar in sequence to the putative methylotrophic Radajewski and colleagues' clones (26), methanol was used as an enrichment with soil from the KBS LTER. A steady decrease in methanol was observed for 90 days (Figure 5.4). Abiotic controls consisting of sterile soil supplemented with 338 μmoles of methanol had significantly higher ($p < 0.006$) concentrations of methanol compared to experimental replicates after 90 days, demonstrating that the depletion of methanol was due to a biological component, rather than abiotic degradation. Upon depletion of the second addition of methanol, the soil was

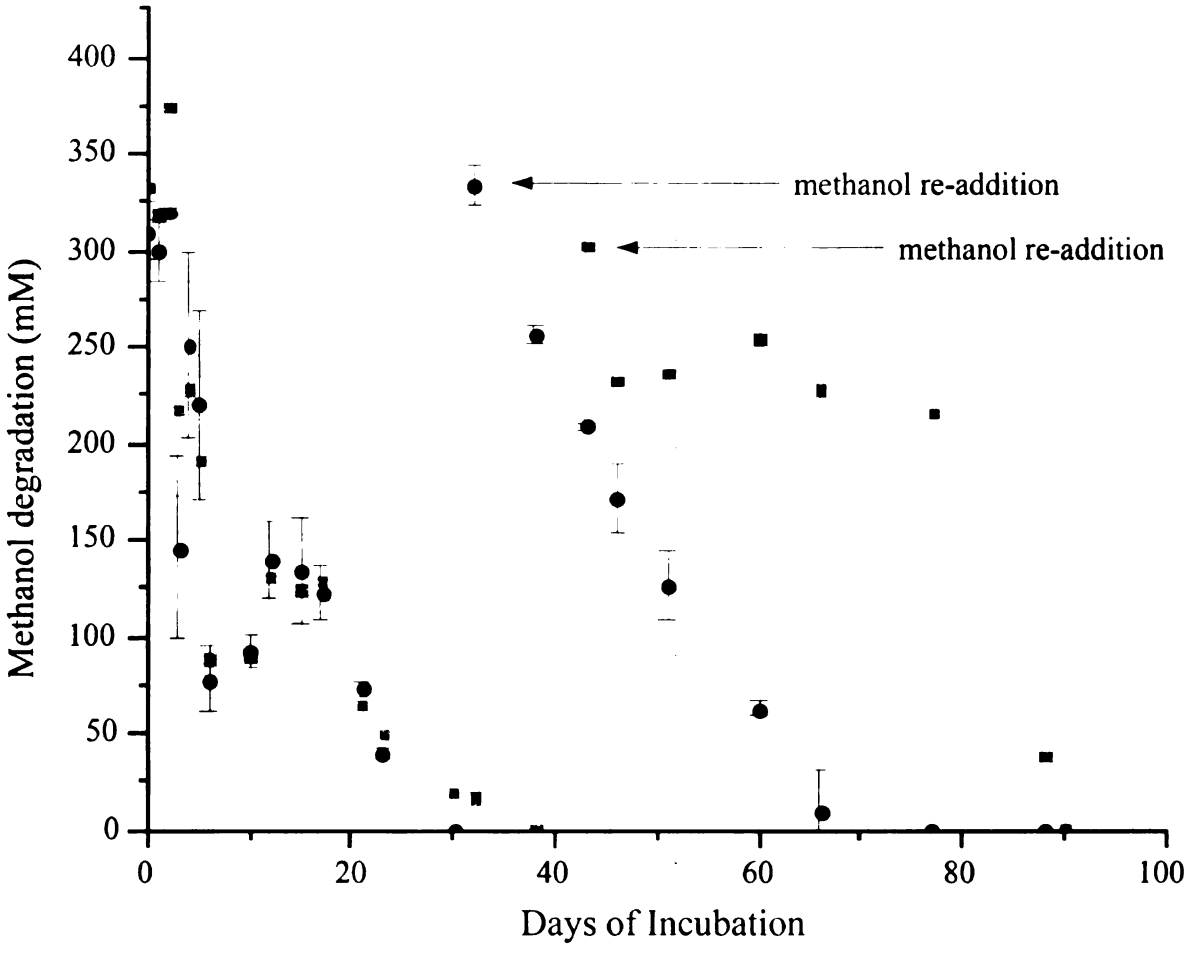


Figure 5.4. Methanol consumption by KBS LTER replicate soil microcosms, replicate 1 (gray boxes) and replicate 2 (black boxes). Each point represents the mean ± standard deviation from triplicate measurements of methanol in the headspace as measured by GC-FID.

used as a source of inoculum to isolate potential, methylotrophic acidobacteria. After thirty days of growth, the water agar treatment supplemented with 25 mM methanol yielded a positive amplification for acidobacteria (Figure 5.5, Sequence ID JAB 971) using PWPCR (33). Attempts to isolate acidobacteria from replicate plates were unsuccessful.

Phylogenetic analysis (∫ - LIBSHUFF) Acidobacteria clone libraries were made from microcosm soil before (T0) and after methanol addition (T90) to determine if acidobacteria responded to methanol additions. The composition of acidobacteria clone libraries following methanol incubation significantly differed from acidobacteria clone libraries before methanol incubation ($p < 0.001$). Furthermore, methanol enriched for a phylogenetically cohesive group of *Acidobacteria* in subdivision 1 (Figure 5.6). It was this subdivision where the clones from the Radajewski and colleagues' study (26) reside.

DISCUSSION and FUTURE DIRECTION

The goal of this study is to assess the capacity of acidobacteria to oxidize one-carbon compounds using growth and molecular-based approaches. At this time, there is no evidence to suggest that these newly isolated strains have the capacity to oxidize one-carbon compounds under the conditions tested. Nevertheless it is interesting to note that during the soil microcosm experiment there was a shift in the acidobacterial community after methanol consumption. Methanol clearly had an affect on the acidobacteria

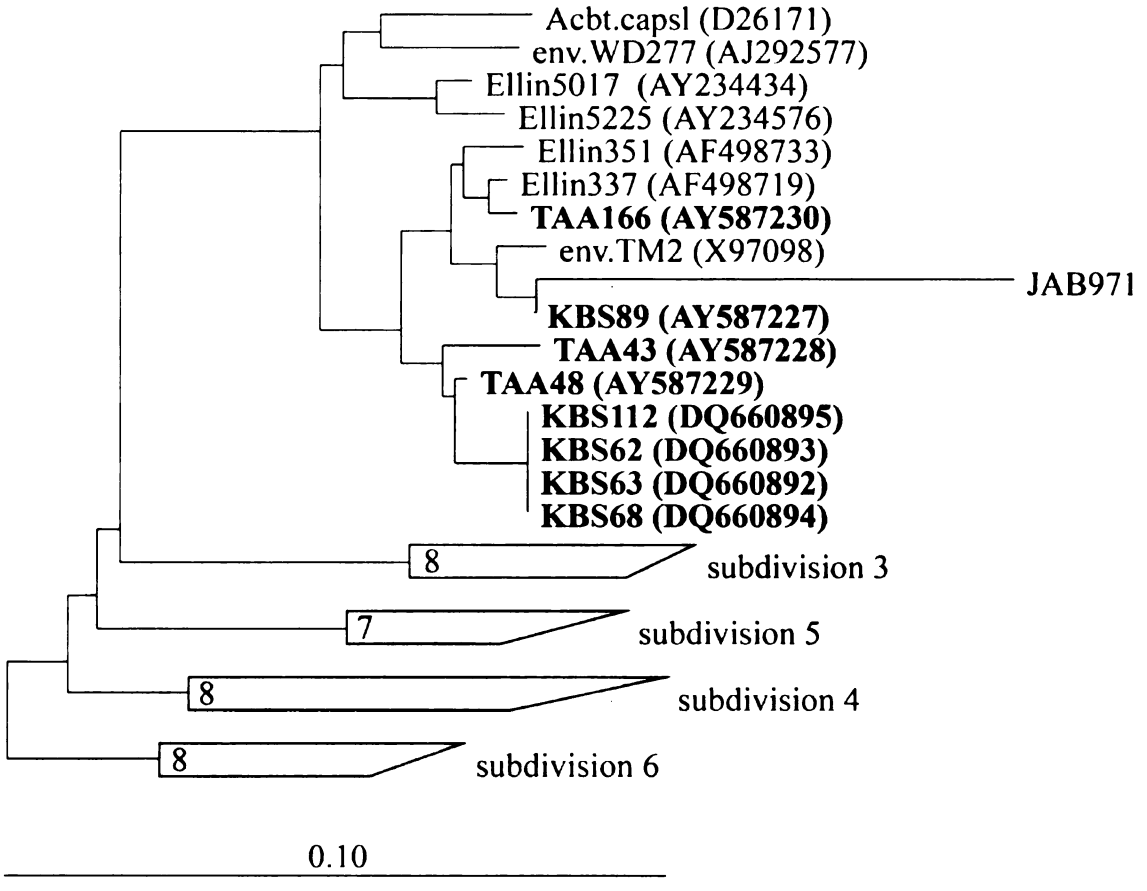
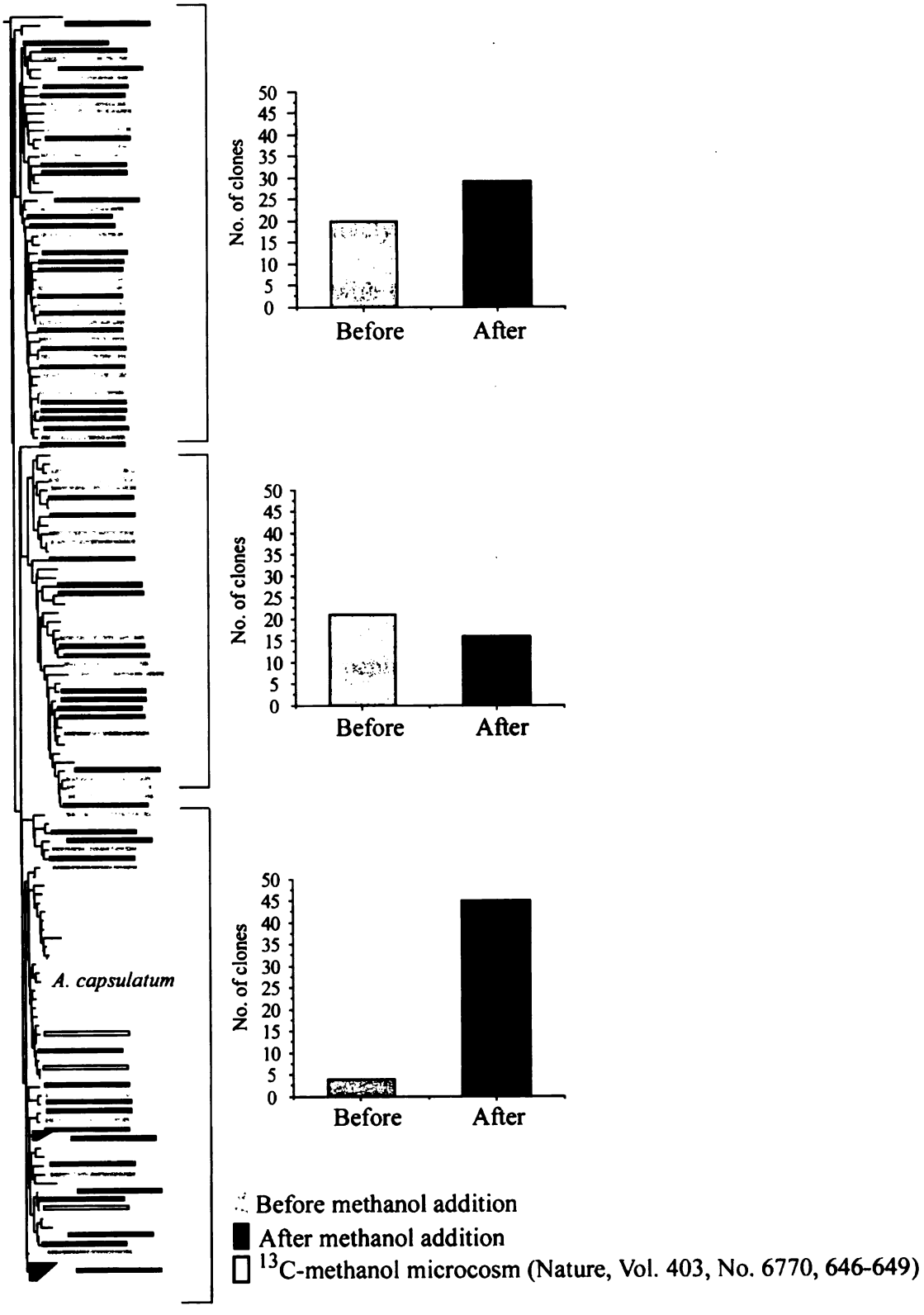


Figure 5.5. Neighbor-joining tree of the *Acidobacteria* subdivisions 1, 3, 4, 5, and 6 based on 16S rRNA gene using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as the outgroup (not shown). Strains from this study are in bolded text. JAB 971 in subdivision 1 is a partial 16S rRNA gene sequence from cultivation experiments using soil from the methanol microcosms as a source of inoculum. The scale bar indicates 0.10 changes per nucleotide.

Figure 5.6. Neighbor-joining tree of partial sequences of 16S rRNA gene clones in the phylum *Acidobacteria* obtained before and after incubation of KBS LTER treatment 8 soil with 350 mM methanol. Acidobacterial clones before and after the addition of methanol are represented in light gray and dark gray boxes, respectively. White boxes with black outlines represent *Acidobacteria* 16S rRNA gene clones obtained from oak forest soil incubated with ^{13}C-methanol, previously (26).


No. of clones
50
45
40
35
30
25
20
15
10
5
0
Before
After
A. capsulatum
Before methanol addition
After methanol addition
13C-methanol microcosm (Nature, Vol. 403, No. 6770, 646-649)

community, significantly selecting for members of subdivision 1; notably, the acidobacterial subdivision with the most success in cultivating to date (5, 7, 10, 16-18, 29, 30, 33) and harboring the putative, methanol-oxidizing clones (26). It is unclear if this was a result of acidobacteria oxidizing methanol or an indirect affect, such as a reduction in the nearby, competing communities thereby allowing the acidobacterial community to flourish. Alternatively, the acidobacterial community could have been interacting with or cross-feeding with the nearby methylotrophic community(s). Perhaps acidobacteria are capable of methylotrophy, but only under a symbiosis with nearby community(s) of known methylotrophs due to an auxotrophic requirement of some metabolite which can only be obtained through cross-feeding of nutrients.

Interestingly, during the course of these studies alternative explanations were realized about Radjewski and colleagues' claims of methanol oxidization in acidobacteria. At the time of the paper, stable isotope probing was a new technique and since then, scientists have revealed potential pitfalls of this technique that were not addressed at the time of this publication. The possibility exists that the DNA from the so-called heavy fraction might not have been heavy at all; rather the DNA could have been G + C rich ^{12}C-DNA. In stable isotope probing, DNA is separated based on its buoyant density in a cesium chloride gradient; if the non-heavy, ^{12}C DNA has a high proportion of G + C, it would settle around the area considered to be the heavy, ^{13}C DNA, thereby introducing unlabeled DNA in the presumed heavy fraction. This scenario is illustrated in Figure 5.7 (area of ambiguity) where non-labeled, ^{12}C DNA having a G + C content of ca. 62% has the same buoyant density at labeled, ^{13}C -DNA with a G + C content of ca. 20%. Recently isolated strains of subdivision 1 in the phylum *Acidobacteria* have a G +

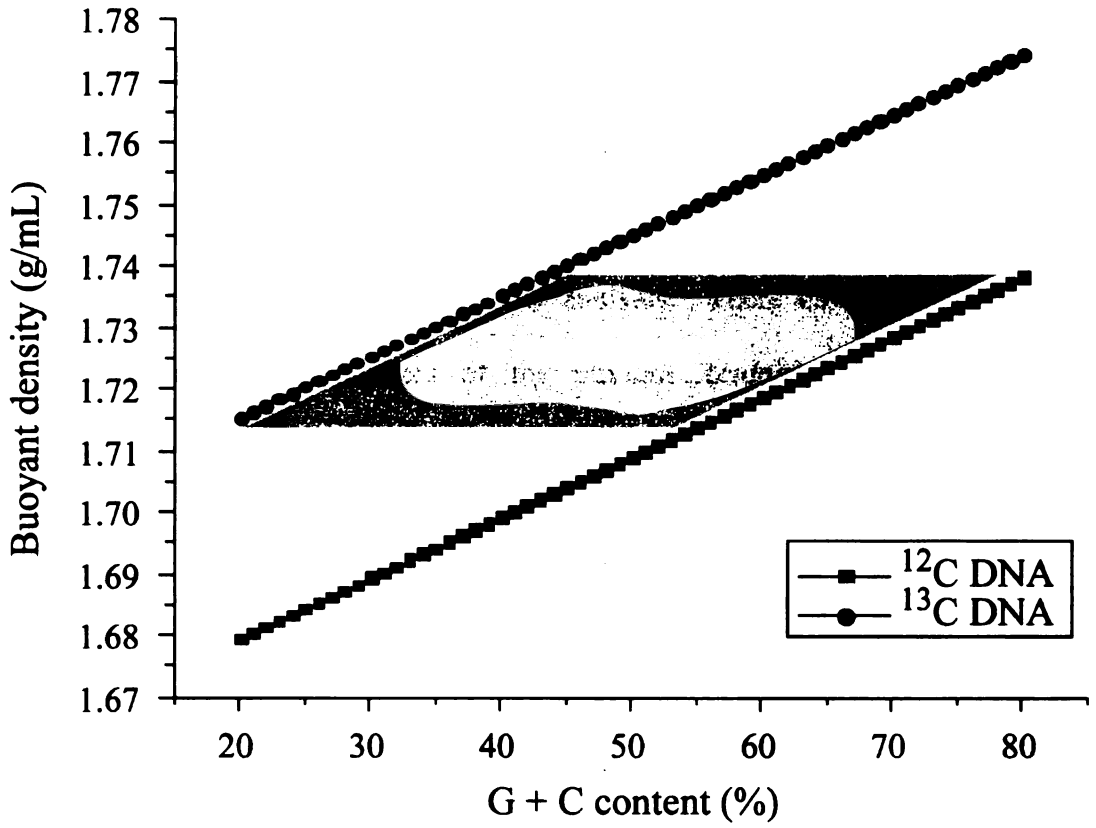


Figure 5.7. G + C content of ^{12}C- and ^{13}C- DNA in relation to its buoyant density on a cesium chloride gradient.

C content (%) ranging from 55 to 62% (Chapters 3 & 4), values within the area of ambiguity (Figure 5.7). In order to address this concern, researchers typically have a replicate microcosm with the non-labeled substrate, and a subtractive analysis is performed to truly determine the active community(s) oxidizing the tested substrate. Another possible explanation of Radajewski and colleagues' (26) results could be that the acidobacterial DNA became heavily-labeled not through the oxidization of the methanol, but by the acidobacteria cross-feeding on the byproducts of methanol oxidization of the nearby methylotrophic communities.

Fortunately, there is still hope to address this interesting physiology in the phylum *Acidobacteria* with the advent of genome analysis. There have been three, recently sequenced genomes of members of the phylum *Acidobacteria*: *Acidobacterium capsulatum* (subdivision 1), *Acidobacterium sp. strain* Ellin345 (subdivision 1), and *Solibacter usitatus strain* Ellin6076 (formerly, *Acidobacteria-3 sp.* Ellin6076) (subdivision 3). The recently annotated *Acidobacterium sp.* strain Ellin345 and *Solibacter usitatus strain* Ellin6076 genomes preliminary indicates the presence of a methanol dehydrogenase gene as well as the ability to oxidize other one-carbon compounds such as methane, methylamine, dimethylamine, trimethylamine, formaldehyde, and formate (http://www.jgi.doe.gov/). These data have provided more compelling evidence that members of the *Acidobacteria* phylum may have the potential to oxidize one-carbon compounds. The future direction of this research would be to ascertain if these genes are functional in *Acidobacterium sp.* strain Ellin345 and *Solibacter usitatus strain* Ellin6076 by testing their ability to oxidize these one-carbon compounds in culture. If these endeavors prove fruitful, the next step would be to

determine how ubiquitous this physiology is among members of the phylum by testing other currently, cultivated strains as well as designing alternative cultivation experiments to select for this novel physiology in this phylum. Eventually, one could assess if acidobacteria are truly one of the unexplored communities of methanotrophs responsible for the discrepancy in affinities between the environment and known methanotrophs.

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CHAPTER 6

SUMMARY and SIGNIFICANCE

The soil environment is a rich habitat of life with members of the *Bacteria*, *Archaea*, and *Eukarya*. However, this environment is largely unexplored since only 0.1 to 1% of the total microbial community has been cultivated. The exploration of this untapped microbial diversity will allow us to understand the role microorganisms play in the soil environment. This knowledge will enhance our understanding of nutrient cycling, biogeochemistry, and agricultural practices. A microbial community that is highly abundant in soil, but poorly understood is the phylum *Acidobacteria*. This research was a union of both cultivation and non-cultivation based approaches, which generated information pertaining to members of the phylum *Acidobacteria* in the soil environment.

We knew previous to this study that members of the phylum *Acidobacteria* were prevalent in a diverse collection of soils with varying degrees of relative abundance; however the distribution of the subdivisions in relation to the various edaphic properties was unknown. *Acidobacteria* comprise 1 to 6% of the total microbial community at the KBS LTER based on their abundance of rRNA. Basic questions were asked about the diversity of *Acidobacteria* at the KBS LTER, specifically how the structure of the community changed in relation to various edaphic properties.

Phylogenetic libraries of the 16S rRNA gene were made from the conventional agriculture (treatment 1), historically cultivated successional (treatment 7), and never

cultivated successional (treatment 8) plots. The practice of agriculture appears to influence the acidobacterial communities through a shift in the relative abundance of the different subdivisions. These data reveal that members of subdivision 1 and 6 are dominant in the never cultivated successional treatment, whereas members of subdivision 4 dominate the conventional agriculture treatment.

The relative percentage of clones of each subdivision varied with depth and treatment, suggesting that physical and chemical properties of the soil influenced the distribution of the various subdivisions. Subdivisions responded to soil moisture (subdivision 3), carbon concentrations (subdivision 4), soil pH (subdivision 1), methane fluxes (subdivision 1), and nitrous oxide fluxes (subdivision 4). In particular, the relative abundance of subdivision 1 clones in each treatment appeared to be related to soil pH which was consistent with the pH optima of recently isolated strain in subdivision 1 (below). Additionally, members of subdivision 4 are more prevalent in the conventional agriculture soil and respond positively to nitrous oxide fluxes suggesting that members of this subdivision are capable of denitrification or respond favorably to nearby denitrifing communities. These trends provide insight into the physiology of members of this phylum as well as generated information which can be used to develop cultivation strategies to isolate additional strains.

Successful cultivation attempts were made from the native and cultivated plots from the KBS LTER. The use of media compositions and incubation conditions that mimicked their native soil environment such as low nutrient media, atmospheric gas concentrations, and extended incubation times as well as the development of a novel screening method called Plate Wash PCR (PWPCR) was instrumental in isolating more

than a dozen strains from subdivisions 1 and 3. Using our novel cultivation approach, we recovered between 3.5 to 5 x 10^7 CFU/gram of soil (dry wt), which was ca. 2.5 to 6% of the microbial community based on direct counts. Extended incubation time, low concentrations of carbon, and soil inoculum with high moisture (significantly) improved total recovery of aerobic heterotrophs from soil. A facile, high-throughput method called Plate wash PCR was developed to rapidly screen a multitude of plates for the target organism(s) using group-specific primers. Additionally, PWPCR revealed that acidobacteria were more frequently detected with elevated levels of carbon dioxide (significantly), the presence of the catalase enzyme, low carbon, and low oxygen concentrations.

Characterization of these strains revealed the nature and importance of members of the phylum *Acidobacteria* in the soil environment. Typically, strains of subdivision 1 were isolated from soils using low-nutrient media incubated under elevated levels of carbon dioxide for extended periods. Such incubation conditions resulted in a slightly acidified medium that matched the pH optima of the strains (between 5 and 6), which also was similar to their native soil pH. All strains were Gram negative, aerobic, chemoorganotrophic, nonmotile rods that produced an extracellular matrix which causes cells to clump in liquid culture. The production of this extracellular material is believed to promote adhesion, prevent desiccation, and promote soil aggregation, all of which would be beneficial to a soil microorganism. Colonies were small, approximately 1 mm in diameter and either white, pale yellow or pink in color, the latter owing to the presence of a carotenoid(s) whose formation was much greater under 20% oxygen as opposed to 2%. The presence of a carotenoid(s) in these strains is believed to be an oxidative stress

response. Additionally, strain KBS 83 appears to be a microaerophile, growing optimally at oxygen concentrations between 4 to 16%. The ability to grow and/or grow better under lower concentrations of oxygen is a valuable trait in the soil environment, since oxygen can be depleted after rain events and in the inner layers of soil aggregates.

All strains contained either 1 or 2 copies of the 16S ribosomal RNA encoding gene which along with extended doubling time (10-15 hours at ca. 23°C), is suggestive of an oligotrophic lifestyle. Major differences in carbon utilization profiles were noted during routine characterization of the strains. Strains KBS 83 (subdivision 1) and KBS 96 (subdivision 3) were able to use more recalcitrant carbon source such as cellulose, syringate, ferulate, and pectin whereas members of the newly described *Terriglobus* genus (below) could not. The ability to oxidize these more recalcitrant carbon source(s) is a practical trait in the soil environment. The soil is typically defined as carbon limiting, however plant polymers and their products of degradation are major source of carbon for indigenous populations of microorganisms. Preliminarily, strain KBS 96 is capable of reducing nitrate to nitrite under anoxic conditions, but not able to denitrify. This strain was isolated from a conventional agriculture soil, high in nitrate concentrations. Recently isolated strains from subdivision 1 were not able to grow under these conditions; therefore it appears that this physiology could be used as a means of selection for isolating additional members in subdivision 3.

Previous work has suggested that members of subdivision 1 in the phylum *Acidobacteria* are capable of oxidizing one-carbon compounds such as methanol, a physiology known as methylotrophy. A molecular, growth, and cultivation-based approach were taken to explore this interesting physiology. The phylogenetic survey

revealed that members of subdivision 1 were positively influenced by methane, a one-carbon compound. Methanol-enriched soil selected for a phylogenetic clade of acidobacteria in subdivision 1, however no methanol-oxidizing acidobacteria were cultivated. Readily available strains were tested for their ability to growth on various one-carbon compounds, however under the conditions tested, they were not able to grow. At this time, our strains do not appear to have the capability to growth on one-carbon compounds under the conditions tested.

Six of the strains in subdivision 1 are sufficiently similar to one another, but distinct from previously named acidobacteria, to warrant creation of a new genus; *Terriglobus,* with *T. roseus* defined as the type species. The characteristics of members of the phylum *Acidobacteria* elucidated in this study are consistent with the widespread distribution of acidobacteria in soil. Type strain KBS 63 has been deposited into the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany and the USDA Agricultural Research Service Culture Collection to allow the our lab and the scientific community to work with these novel strains and continue to make future discoveries.

In conclusion, this research addressed some basic questions about acidobacteria in relation to their native environment using both molecular and growth-based approaches. Differences in the relative percentages of the different acidobacterial subdivisions in the various plots at the KBS LTER are suggestive of their niche or ecological role in the soil environment. The development of novel cultivation strategies including PWPCR along with the successful isolation more than a dozen members of the phylum *Acidobacteria* are major contributions to the scientific community. This research revealed not only

interesting properties of the acidobacteria, but also laid the foundation for further questions and research to continue on this diverse group of microorganisms.

APPENDIX A

OVERVIEW

This appendix is a brief description of the VSB-#, the "vitamins and salts base", and MHM-#, the "modified hyphomicrobium medium"; # indicates the respective pH of the basal solution.

1. VSB-#

1. 1X basal medium I
2. SL-10 trace element solution (1mL/liter)
3. Vitamin mixture (1mL/liter)
4. Vitamin B12 (1mL/liter)
5. Buffer (HEPES, MOPS, MES) (10mM final concentration)
6. distilled water (up to 1 liter)

Basal Medium I (2)

	Per liter	**Final Concentration**
NaCl	1.0g	ca. 17mM
$MgCl_2 \cdot 6H_2O$	0.62g	ca. 3.1mM
$CaCl_2 \cdot 2H2O$	0.15g	ca.1.0mM
NH_4Cl	0.25g	ca. 4.7mM
KH_2PO_4	0.2g	ca. 1.5mM
KCl	0.5g	ca. 6.7mM
Na_2SO_4	0.28g	ca. 2mM

NOTE: When making up the Basal Medium I, add each component one at a time and wait until it is completely dissolved before adding the next component. Follow the order of components exactly for successful preparation. In my experience, the addition of Na_2SO_4 in the Basal Medium I reduced the solubility of the salts. A separate stock of Na_2SO_4 was prepared at a concentration of 1.25M and 1600µL was added per liter.

SL-10 Trace Metals (1ml/liter) (2)

	Per liter
HCl (25%)	10ml
$FeCl_2 \cdot 4H_2O$	1.5g
$CoCl_2 \cdot 6H_2O$	190mg
$MnCl_2 \cdot 4H_2O$	100mg
$ZnCl_2$,	70mg
H_3BO_3	6mg

$Na_2MoO_4 \cdot 2H_2O$	36mg
$NiCl_2 \cdot 6H_2O$	24mg
$CuCl_2 \cdot 2H_2O$	2mg

NOTE: When making up the SL10 trace metals solution, add each component one at a time and wait until it is completely dissolved before adding the next component. Follow the order of components exactly for successful preparation. In my experience, the dissolving of $FeCl_2 \cdot 4H_2O$ was problematic, therefore, first dissolve $FeCl_2 \cdot 4H_2O$ in the HCl (25%) before addition of water or the other components.

Vitamin Mixture (1ml/liter)(2)

	Per 100ml
Sodium phosphate buffer (10mM; pH 7.1)	100ml
4-aminobenzoic acid	4mg
D (+)-biotin	1mg
Nicotinic acid	10mg
Calcium D (+)-pantothenate	5mg
Pyridoxine dihydrochloride	15mg

Vitamin B_{12} solution (50 mg/l) (1ml/liter)

2. MHM-#

1. 1X Freshwater base
2. 150mM mix of K_2HPO_4 & KH_2PO_4 (below) (pH 6) (1ml/liter)
3. SL-10 trace element solution (1mL/liter)
4. Vitamin mixture (1mL/liter)
5. Vitamin B12 (1mL/liter)
6. Buffer (HEPES, MOPS, MES) (20mM final concentration)
7. Desired carbon source
8. distilled water (up to 1 liter)

Freshwater Base (1)

	Per liter	**Final Concentration**
NaCl	1.0g	ca. 17mM
$MgCl_2 \cdot 6H_2O$	0.4g	ca. 2mM
$CaCl_2 \cdot 2H2O$	0.1g	ca. 0.7mM
NH_4Cl	0.25g	ca. 4.7mM
KH_2PO_4	0.2g	ca. 1.5mM
KCl	0.5g	ca. 6.7mM
Na_2SO_4	0.28g	ca. 2mM
KNO_3	5g	ca. 54mM

NOTE: When making up the MHM, add each component one at a time and wait until it is completely dissolved before adding the next component. Follow the order of components exactly for successful preparation. In my experience, the addition of Na_2SO_4 in the Basal Medium I reduced the solubility of the salts. A separate stock of Na_2SO_4 was prepared at a concentration of 1.25M and 1600µl was added per liter.

150mM mix of K_2HPO_4 & KH_2PO_4 (pH 6)

K_2HPO_4	6g
KH_2PO_4	19g

SL-10 Trace Metals (1ml/liter) (above) (2)
Vitamin Mixture (1ml/liter) (above) (2)
Vitamin B_{12} solution (50 mg/l) (1ml/liter)

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APPENDIX B

OVERVIEW

This appendix contains all of the raw data for the soil plating experiments from the KBS LTER including collection date, plating date, treatment & replicate(s), soil moisture (%), treatments, and average recovery in CFU/gram of soil (dry weight).

1. SOIL PLATING EXPERIMENT #1

SOIL COLLECTION DATA:
Collection Date: July 10, 2001
Plating Date: August 13, 2001
Treatment & Replicate: KBS LTER, Treatment 8, Replicate 1, sampling stations 1-5
Soil Moisture: 7.1 ± 1.2%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7)[†] After 24 days Air
Agarose	0.27 ± 0.04
Agar	1.06 ± 0.13
Agar, 0.001 R2A	1.09 ± 0.14
Agarose, 0.001 R2A	0.28 ± 0.01
NH_4Cl[a], aHSL[b], Fe[c], catalase[d]	0.81 ± 0.03
NH_4Cl[a], aHSL[b], Fe[c]	0.58 ± 0.12
NH_4Cl[a], Fe[c], catalase[d]	1.18 ± 0.13
NH_4Cl[a], Fe[c]	1.34 ± 0.09
NH_4Cl[a], aHSL[b], catalase[d]	0.84 ± 0.24
NH_4Cl[a], aHSL[b]	0.30 ± 0.13
NH_4Cl[a], catalase[d]	1.22 ± 0.39
NH_4Cl[a]	0.98 ± 0.21
aHSL[b], Fe[c], catalase[d]	0.69 ± 0.07
aHSL[b], Fe[c]	0.55 ± 0.16
Fe[c], catalase[d]	1.07 ± 0.49
Fe[c]	1.15 ± 0.04
aHSL[b], catalase[d]	0.54 ± 0.32
aHSL[b]	0.61 ± 0.03
catalase[d]	0.98 ± 0.24
no additives	1.06 ± 0.22
0.001R2A	1.18 ± 0.04
0.001 R2A, PEG[e]	0.01 ± 0.01

[†] Unless otherwise noted, medium was water Bacto™ agar (1.5%).
[a] Ammonium chloride (NH_4Cl) was at a final concentration of 1mM.
[b] aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.
[c] Ferric chloride (Fe) was at a final concentration
[d] Catalase was added at a final concentration of 65U/mL.
[e] Polyethylene glycol was added at a final concentration of 0.58M.

2. SOIL PLATING EXPERIMENT #2

SOIL COLLECTION DATA:
Collection Date: September 21, 2001
Plating Date: October 2, 2001
Treatment & Replicate: KBS LTER, Treatment 8, Replicate 1, sampling stations 1-5
Soil Moisture: 24.8 ± 0.7%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) After 28 days		
	Elevated CO_2	**Hypoxia**	**Air**
Washed agar[c]	4.57 ± 0.76	4.84 ± 0.17	3.12 ± 0.16
Washed agar[c] w/ VSB-7[a]	4.61 ± 0.39	6.02 ± 0.78	4.13 ± 0.42
Catalase[b] w/ VSB-7[a]	6.67 ± 4.15	4.20 ± 0.13	4.84 ± 1.38
AQDS[d] w/ VSB-7[a]	4.87 ± 0.61	4.04 ± 0.25	3.99 ± 0.54
AQDS[d], catalase[b] w/ VSB-7[a]	4.68 ± 1.17	3.69 ± 0.27	4.11 ± 0.11
VSB-7[a]	5.82 ± 2.30	4.38 ± 0.68	4.66 ± 1.67
AEA[c] w/ VSB-7[a]			
No additives	5.13 ± 1.24	5.06 ± 0.20	2.89 ± 0.11

[a] Unless otherwise noted, the medium consisted of VSB-7 (2) with HEPES (10mM), pH 7 as a buffering system, and aHSL cocktail. aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.
[b] Catalase was added at a final concentration of 65U/mL.
[c] "AEA" includes the addition of acidified ethyl acetate without the aHSL.
[d] AQDS had a final concentration of 25mM.
[e] Bacto™ agar was washed twice in distilled water.

3. SOIL PLATING EXPERIMENT #3

SOIL COLLECTION DATA:

Collection Date: November 27, 2001
Plating Date: November 30, 2001
Treatment & Replicate: KBS LTER, Treatment 8, Replicate 1, sampling stations 1-5
Soil Moisture: 32 ± 1.4%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) [a] After 22 days			
	Elevated CO_2	**Hypoxia**	**Air**	**Knallgas Mix(1)**
HEPES[b], catalase[c]	6.53 ± 0.61	6.20 ± 0.76	3.22 ± 0.38	2.96 ± 1.98
HEPES[b]	5.20 ± 0.58	4.96 ± 0.49	3.19 ± 0.59	-
HEPES[b], catalase[c], R2A[d]	4.94 ± 1.56	5.23 ± 1.06	4.11 ± 2.84	-
HEPES[b], R2A[d]	5.86 ± 1.11	5.03 ± 1.51	3.07 ± 0.63	-
HEPES[b], catalase[c], aHSL[e]	5.86 ± 1.85	6.55 ± 1.23	5.06 ± 0.50	-
HEPES[b], catalase[c], AEA[f]	4.29 ± 0.74	3.84 ± 0.59	4.36 ± 0.56	-
HEPES[b], catalase[c], Bacxell[g]	-	-	5.06 ± 1.33	-
HEPES[b], catalase[c], Bacxell[g], R2A[d]	-	-	4.24 ± 0.58	-
HEPES[b], catalase[c], Bacxell[g], aHSL[e]	-	-	5.30 ± 0.92	2.84 ± 2.18
HEPES[b], aHSL[e], Bacxell[g], R2A[d]	-	-	5.80 ± 1.10	-
catalase[c]	4.94 ± 0.68	4.99 ± 0.63	3.29 ± 0.38	-
Rainwater extract[h], catalase[c]	8.95 ± 6.03	4.81 ± 1.56	3.26 ± 0.43	2.32 ± 1.83

[a] Unless otherwise noted, the medium consisted of VSB-7 (2).
[b] HEPES (10mM), pH 7 was used as a buffering system.
[c] Catalase was added at a final concentration of 65U/mL.
[d] R2A carbon additive included all carbonaceous components of R2A medium except pyruvate. It was at a 0.1x concentration.
[e] aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.
[f] Acidified ethyl acetate was added as a control for the aHSL treatment.
[g] Bacxell was an additive given to us by another lab speculated to increase recovery of microorganisms.
[h] Rainwater extract was prepared via a cold extraction of soil from the KBS LTER.

4. SOIL PLATING EXPERIMENT #4

SOIL COLLECTION DATA:
Collection Date: June 10, 2002
Plating Date: June 11, 2002
Treatment & Replicate: KBS LTER Treatment 8, Replicate 4, sampling stations 1-5
Soil Moisture: 17.8 ± 1%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) [a] After 30 days		
	Elevated CO_2	**Hypoxia**	**Air**
aHSL[b], catalase[c]	5.21 ± 0.66	6.91 ± 0.25	7.02 ± 0.95
catalase[c]	5.8 ± 1.3	6.63 ± 0.81	9.67 ± 2.79
catalase[c], light	4.76 ± 0.50	-	7.75 ± 0.93
aHSL[b], catalase[c], R2A[d]	5.07 ± 0.53	6.66 ± 0.58	4.00 ± 3.63
aHSL[b]	4.61 ± 2.41	6.55 ± 0.78	-
No additives	6.25 ± 0.28	5.65 ± 1.93	-
aHSL[b], R2A[d]	5.36 ± 0.25	5.57 ± 2.33	-

[a] Unless otherwise noted, the medium consisted of VSB-7 (2) and HEPES (10mM), pH 6.8 as a buffering system.
[b] aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.
[c] Catalase was at a concentration of 65U/mL.
[d] R2A carbon additive included all carbonaceous components of R2A medium except pyruvate. It was at a 0.1x concentration.

5. SOIL PLATING EXPERIMENT #5

SOIL COLLECTION DATA:

Collection Date: November 12, 2002

Plating Date: November 22, 2002

Treatment & Replicate: KBS LTER Treatment 8, Replicate 3, sampling stations 1-5; Treatment 1, Replicate 3, sampling stations 1-3; Treatment 7, Replicate 2, sampling stations 1-3; Deciduous forest, Replicate 2, sampling stations 1-5

Soil Moisture: **T8,** 21.1 ± 0.7%; **T1 & 7** (reps. 1-3 for each), 17.5 ± 0.5%; **DF**, 17.2 ± 0.4%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) [a] 20 days of growth	
	Anoxia	**Hypoxia**
T8 soil		
aHSL[c], R2A[b]	6.26 ± 0.21	-
aHSL[c], Methyl cocktail [d]	0.19 ± 0.18	9.51 ± 0.76
aHSL[c], AQDS [e]	2.69 ± 0.39	8.0 ± 2.08
T1 & 7 soil (pooled)		
aHSL[c], R2A[b]	3.99 ± 0.53	5.11 ± 0.41
aHSL[c], Methyl cocktail [d]	0.44 ± 0.20	5.45 ± 1.10
aHSL[c], AQDS [e]	1.62 ± 0.34	5.16 ± 1.01
Deciduous forest soil		
aHSL[c], R2A[b]	0.89 ± 0.64	1.53 ± 0.31
aHSL[c], Methyl cocktail [d]	0.03 ± 0.03	0.84 ± 0.24
aHSL[c], AQDS [e]	0.38 ± 0.23	2.49 ± 1.51

[a] Unless otherwise noted, the medium consisted of VSB-7 (2) catalase (65U/mL), and HEPES (10mM), pH 6.8 as a buffering system.

[b] R2A carbon additive included all carbonaceous components of R2A medium except pyruvate. It was at a 0.1x concentration.

[c] aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.

[d] Methyl cocktail contained 5mM methanol, 3mM methylamine, and 1mM dimethylamine. This treatment was buffered with bicarbonate.

[e] AQDS had a final concentration of 25mM.

6. SOIL PLATING EXPERIMENT #6

SOIL COLLECTION DATA:
Collection Date: June 6, 2003
Plating Date: June 26, 2003
Treatment & Replicate: KBS LTER, Treatment 8, Replicate 3, sampling station 1-5
Soil Moisture: 10 ± 0.2%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) 47 days of growth			
	Elevated CO_2	**Hypoxia**	**Air**	**Anoxia**
12°C[a]				
No additives	0.51 ± 0.21	0.98 ± 0.16	0.94 ± 0.19	0.90 ± 0.43
aHSL[c]	0.61 ± 0.34	0.90 ± 0.21	0.84± 0.23	0.87 ± 0.10
AQDS[d]	0.54 ± 0.30	0.98 ± 0.30	0.99 ± 0.20	0.48 ± 0.13
R2A[b]	0.83 ± 0.09	0.84 ± 0.16	0.75 ± 0.17	0.90 ± 0.23
23°C[a]				
No additives	1.27 ± 0.17	1.14 ± 0.17	1.12 ± 0.28	0.58 ± 0.32
aHSL[c]	1.90 ± 0.52	1.16 ± 0.28	1.46 ± 0.35	0.81 ± 0.21
AQDS[d]	1.03 ± 0.17	1.00 ± 0.15	1.10 ± 0.32	1.08 ± 0.36
R2A[b]	1.12 ± 0.16	0.89 ± 0.49	1.20 ± 0.25	0.98 ± 0.37

[a] Unless otherwise noted, the medium consisted of VSB-7 (2) catalase (65U/mL), and HEPES (10mM), pH 6.8 as a buffering system.
[b] R2A carbon additive included all carbonaceous components of R2A medium except pyruvate. It was at a 0.1x concentration.
[c] aHSL cocktail included: ketocaproyl-, octanoyl-, hexanoyl-, butyryl-, heptanoyl-, and tetradecanoyl-DL- at a 1μM concentration each. The HSL cocktail stock was made in acidified ethyl acetate and added directly to cooled, molten agar medium immediately before pouring.
[d] AQDS had a final concentration of 25mM.

7. SOIL PLATING EXPERIMENT #7

SOIL COLLECTION DATA:
Collection Date: July 21, 2006
Plating Date: July 22, 2006
Treatment & Replicate: KBS LTER, Treatment 8, Replicate 1, sampling station 1-5; Treatment 1, Replicate 1, sampling station 1-5;
Soil Moisture: **T8,** 15.0 ± 0.0%; **T1**, 8.0 ± 0.0%

	Average CFU/gram of soil ± s.d (dry wt.) (10^7) After 30 days			
	Air		Hypoxia	
	pH 5.5	pH 6.5	pH 5.5	pH 6.5
T1 Soil				
VL55[b], plant polymers[d]	1.07 ± 0.27	0.98 ± 0.40	0.82 ± 0.02	0.87 ± 0.03
VL55[b], xylan[e]	0.91 ± 0.24	1.16 ± 0.21	0.94 ± 0.04	1.09 ± 0.01
VL55[b], R2B[c]	0.91 ± 0.15	1.23 ± 0.07	1.04 ± 0.01	1.07 ± 0.04
VSB[a], xylan[e]	0.00 ± 0.00	0.97 ± 0.11	0.02 ± 0.001	0.76 ± 0.03
VSB[a], R2B[c]	0.51 ± 0.19	0.36 ± 0.08	0.68 ± 0.04	0.54 ± 0.02
Soil extract medium[f]	-	-	-	-
R2A™	0.54 ± 0.09		0.97 ± 0.02	
T8 Soil				
VL55[b], plant polymers[d]	0.75 ± 0.19	0.55 ± 0.32	0.82 ± 0.05	0.59 ± 0.61
VL55[b], xylan[e]	0.77 ± 0.36	0.90 ± 0.27	1.02 ± 0.29	1.05 ± 0.50
VL55[b], R2B[c]	1.29 ± 0.45	0.80 ± 0.23	1.07 ± 0.47	0.98 ± 0.69
VSB[a], xylan[e]	0.00 ± 0.00	0.69 ± 0.17	0.29 ± 0.09	0.57 ± 0.30
VSB[a], R2B[c]	0.51 ± 0.19	0.69 ± 0.23	0.48 ± 0.23	0.44 ± 0.20
Soil extract medium[f]	-	-	-	-
R2A™	0.51 ± 0.15		0.61 ± 0.04	

[a] Unless otherwise noted, the medium consisted of VSB-# (2) catalase (65U/mL), and HEPES (10mM), pH 6.8 as a buffering system.
[b] VL55 is described previously (3).
[c] R2A carbon additive included all carbonaceous components of R2A medium except pyruvate. It was at a 0.1x concentration.
[d] Plant polymers mix consisted of xylan (oat spels), xanthan, pectin, xylan (Birchwood), methyl cellulose, and xylan (Larch) each at 0.008% (wt./vol) giving a final concentration of 0.05% (wt./vol).
[e] Xylan (Birchwood) was dissolved in water and added to a final concentration of 0.05% (wt./vol).
[f] Soil extract medium consisted of a mixture of equal volume of soil (T8 & T1) to water with agar added as the solidifying agent.

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APPENDIX C

OVERVIEW

This appendix is a list of primers, both 16S rRNA and functional genes, used throughout my dissertation.

PCR Primers Quick Reference			
Name	**Gene**	**Sequence**	**Ref.**
8F	16S rRNA	**5'** AGA GTT TGA TCC TGG CTC AG **3'**	(9)
ACD 31F	16S rRNA	**5'** GAT CCT GGC TCA GAA TC **3'**	(1)
1492R	16S rRNA	**5'** GGT TAC CTT GTT ACG ACT T **3'**	(9)
338R	16S rRNA	**5'** GCT GCC TCC CGT AGG AGT **3'**	(8)
338F	16S rRNA	**5'** CTC CTA CGG GAG GCA GCA GT **3'**	(8)
531R	16S rRNA	**5'** TAC CGC GGC TGC TGG CAC **3'**	(8)
810R	16S rRNA	**5'** GGC GTG GAC TTC CAG GGT ATC T **3'**	(8)
776F	16S rRNA	**5'** AGC AAA CAG GAT TAG ATA CCC TGG **3'**	(8)
1087F	16S rRNA	5' GGT TAA GTC CCG CAA CGA **3'**	(8)
modified M13F	TOPO TA vector	**5'** CAG TCA CGA CGT TGT AAA ACG ACG GC **3'**	(7)
modified M13R	TOPO TA vector	**5'** CAG GAA ACA GCT ATG ACC ATG **3'**	(7)
mxaf (MDH active site subunit)	mxa f1003 mxa f1561	**5'** GCG GCA CCA ACT GGG GCT GGT **3'** **5'** GGG CAG CAT GAA GGG CTC CC **3'**	(10)
mmoX (sMMO active site subunit)	mmoX 206F mmoX 886R	**5'** ATC GCB AAR GAA TAY GCS CG **3'** **5'** ACC CAN GGC TCG ACY TTG AA **3'**	(6)
mmoX (sMMO active site subunit)	mmoX F536 mmoX R898	**5'** CCG CTG TGG AAG GGC ATG AA **3'** **5'** CAC TCG TAG CGC TCC GGC T **3'**	(4)
pmoA (pMMO active subunit)	A189 mb661	**5'**GGN GAC TGG GAC TTC TGG **3'** **5'** CCG GMG CAA CGT CYT TAC C **3'**	(3)
pmoA & *amoA* (putative active site subunit of pMMO and AMO)	A189 A682	**5'**GGN GAC TGG GAC TTC TGG **3'** **5'** GAA SGC NGA GAA GAA SGC **3'**	(5)
Nitrogenase gene (2 sets; IGK & YAA and KAD & GEM)	nifH	*IGK*, **5'** AAR GGN GGN ATH GGN AA **3'** *KAD*, **5'** TGY GAY CCN AAR GCN GA **3'** *GEM*, **5'** AND GCC ATC ATY TCN CC 3' *YAA*, **5'** ATR TTR TTN GCN GCR TA **3'**	(11)

PCR Primers Quick Reference cont'd			
Name	**Gene**	**Sequence**	**Ref.**
Nitrite reductase gene	nirK1F nirK5R	**5'** GG(A/C) ATG GT(G/T) CC(C/G) TGG CA **3'** **5'** GCC TCG ATC AG(A/G) TT(A/G) TGG **3'**	(2)
Nitrite reductase gene	nirS1F nirS6R	**5'** CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T **3'** **5'** CGT TGA ACT T(A/G)C CGG T **3'**	(2)

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APPENDIX D

BACTERIAL GROWTH EFFICIENCY: UNDERSTANDING ECOLOGICAL STRATEGIES OF COPIOTROPHS AND OLIGOTROPHS

INTRODUCTION

Ecologists R.H. MacArthur and E.O. Wilson developed a theory of r and K selection that classifies organisms based on their life history including traits such as development, growth, reproductive strategies, and life span. R-strategists are defined as species found in low population densities where growth can be exponential and substrate availability is high (21), therefore growth is regulated independent of population density. They are greatly affected by catastrophic events in the physical environment, and their characteristics include high growth rate, reduced body size, and reduced intraspecific competition (2, 16). K-strategists are typically found in high population densities at or near the carrying capacity (K) where there is intense intraspecific competition for scarce resources (21) and growth is regulated by the population density. Characteristics of a K-strategists include reduced growth rate, increased body size, and presumed higher efficiency in the utilization of resources and competition (2, 16). There is a systematic trade-off when applying this theory: an organism can either be a r-strategist or a K-strategist, but not both (20, 21, 39, 40) because it contradicts all notions of evolution of a generalist versus a specialist (39).

It is relatively easy to observe this theory in plants and animals when one considers traits such as clutch size or parental care. But how can this theory be observed

and applied to bacteria? Andrew and Harris (1986) hypothesized that bacteria with high maximum growth rates and a requirement for abundant resources during growth are r-strategists, where as bacteria with low maximum growth rate and require few resources for growth are K-strategists (1). Depending on their metabolic attributes, one can classify these organisms as being more oligotrophic (K-selected) or copiotrophic (r-selected). Copiotrophs are microbes that grow and multiply in the presence of an abundance of carbon, whereas oligotrophs are defined as microbes that are isolated on media containing 1-15mg organic C/liter (30).

The classic definition of oligotrophy as defined by Poindexter states that the bacteria must be isolated on medium containing 1-15 mg/L of carbon (30). This definition might be too stringent. The requirement to grow at low concentrations of carbon might be a characteristic to some more obligate oligotrophic bacteria, but its absence does not exclude the possibility of other strains to be considered oligotrophic as suggested in an article by Arthur Koch (14). Koch suggested there are four categories of oligotrophy; 1. bacteria that cannot be cultivated; 2. bacteria cultivated on poor medium; 3. bacteria isolated on poor medium, but are able to grow on rich medium; and 4. bacteria only initially cultivated (14). The third category is consistent with the growth dynamics of *Sphingopyxis alaskensis* strain RB2256 (formerly *Spingomonas alaskensis*), the model oligotroph, which was isolated on synthetic seawater medium containing less that 1 mg of carbon per liter, but subsequently adapted in the lab to grow on high nutrient medium (33). Recently, a more inclusive definition has been suggested by Fierer and colleagues that includes attributes such as relatively slow growth rate, high affinity uptake systems, and better growth under nutrient-poor conditions (9) which has a genetic marker of the

16S rRNA-encoding gene in the rrn operon (13, 37). It is this more inclusive definition of oligotrophy which will be used in this chapter.

The 16S rRNA-encoding gene in the rrn operon appears to be a good genetic marker to preliminarily categorize a bacterium as being more copitrophic or oligotrophic, specifically when discussing growth rate (13, 37). The genes encoding the 5S, 16S, and 23S rRNAs in bacteria are typically organized into an operon (Figure D.1), and the number of 16S-rRNA encoding gene(s), *rrs*, are determined using Southern hybridization with specific probes. In general, bacteria with few *rrs* copies ($\leq$ 2 copies) are found in low nutrient environments and are slow-growing such as *Terriglobus roseus* strain KBS 63 and *Sphingopyxis alaskensis* strain RB2256, where as bacteria with multiple copies of the *rrs* ($\geq$ 3 copies) are found in nutrient rich environment and are fast-growing such as *E.coli*. This correlation between high number of *rrs* and faster growth rate is clearly illustrated when growing different species of *Mycobacterium* and *Rhizobium* that differ in *rrs* copy number (17, 36), presumed to be a result of these strain increased ability to respond to changes in the environment. Since the *rrs* appears to be a good genetic marker for assessing bacteria's ecological strategy, fast-growing bacteria with a high *rrs* copy number ($\geq$ 3 copies) will be referred to as copiotrophs, and slow-growing bacteria with a low *rrs* copy number ($\leq$ 2 copies) will be referred to as oligotrophs.

There are systematic differences in metabolic efficiency between copiotrophs and oligotrophs. The idea of efficiency has been discussed in the literature with such terms as Y_{ATP} and bacterial growth efficiency. Y_{ATP} is a term more frequently used by microbial physiologists, and it is defined as the amount of ATP required for the formation of

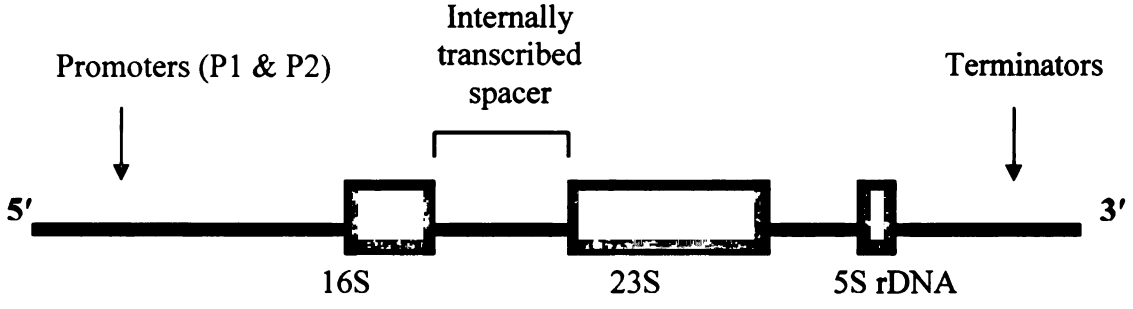

Figure D.1. Classic organization of the rrn operon.

cellular material (38), where yield is ultimately dependent on the amount of ATP produced by the organism. Y_{ATP} can vary dramatically with the type of carbon source (10^4 ATP mole/grams cell formed): glucose and pyruvate, 28.8 and 13.5, respectively) as well as medium supplemented with amino acids (31.9 x 10^4 ATP mole/grams cell formed) and nucleic acid bases (28.8 x 10^4 ATP mole/grams cell formed) (38). Bacterial growth efficiency or BGE is a term used by microbial ecologists, and it is defined as the amount of new biomass produced (bacterial production or BP) per unit organic carbon substrate consumed (BP + bacterial respiration (BR)) (6). BGE has been explored primarily in natural planktonic ecosystems where it increases along with productivity, reaching an asymptote at a BGE of 0.5 (5). Hereafter, this concept of efficiency will be referred to as bacterial growth efficiency (BGE), and it will be defined as moles of carbon in biomass/moles of carbon consumed.

The goal of this study was to determine if there is a difference in BGE among a diverse collection of bacteria which differ in the *rrs* copy number. Previous work has illustrated that there is a strong, positive correlation between *rrs* copy number and growth rate indicating that the *rrs* copy number is a suitable genetic marker for ecological strategy (13). In addition, other studies revealed differences in Y_{ATP} or BGE of communities and/or pure cultures suggesting systematic differences among strains' ability to utilize substrate. The next logical question is to determine if there is a correlation between BGE and *rrs* copy number.

Copiotrophs' strategy is to maximize their growth rate, typically having a high number of offspring per unit time; therefore, it is plausible to assume that when maximizing growth rate, it would be less efficient (decrease in cells per mole of carbon)

or wasteful with their resources. On the other hand, an oligotroph's strategy is to limit their population density based on the amount of limited resources available; in order to do so, it would demand the most efficient (increase in cells per mole of carbon) use of resources. Oligotrophs focus in on quality then quantity therefore they would be less likely to be wasteful with their substrate. Based on these strategies, the prediction is that there is a negative correlation between BGE and *rrs* copy number.

This model was tested on a collection of soil microorganisms isolated from the KBS LTER and isolates from rice paddy soils from Tsutomu Hattori (Institute of Genetic Ecology, Tohoku University, Sendai, Japan). *Terriglobus roseus* strain KBS 63 is a recently cultivated and characterized members of the phylum *Acidobacteria* that harbor traits suggesting an oligotrophic lifestyle such as low *rrs* copy number (1 or 2), slow growth rate, and abundance in low nutrient environments such as soil (8). As a reference, *Escherichia coli* REL 607 (γ -Proteobacteria), our model copiotroph, and *Spingopyxis alaskensis* RB2256 (α- Proteobacteria), our model oligotroph, were used in the analysis. The *rrs* copy number of the test strains has previously been determined as well as their phylogeny. When possible, related pairs of the isolates (i.e. a copiotroph & oligotroph of the β-*Proteobacteria*) were used to reduce the threat of shared evolutionary histories.

MATERIALS and METHODS

Description of bacterial strains Strains used in this study were obtained from long term plating experiments from either soil at the W.K. Kellogg Biological Station Long Term Ecological Research site (KBS LTER) or rice paddy (10, 12, 13). *Escherichia coli* strain REL 607, a derivative of *E.coli* B/r was a gift obtained from Richard E. Lenski

(19), EC2 & LC9 were gifts obtained from Joel A. Klappenbach isolated from the KBS LTER (12, 13), HF3 and HF2 were gifts obtained from T. Hattori isolated from rice paddy soils (10), and *Sphingopyxis alaskensis* RB2256 (formally *Sphingomonas*), a marine ultramicrobacterium, was a gift obtained from R. Cavicchioli and M. Ostrowski (7, 25, 34, 35). These phylogenetically diverse strains were divided into two categories, copiotrophs and oligotrophs: copiotrophs were defined as having a *rrs* copy number of three or more, whereas oligotrophs were defined as having a *rrs* copy number of two or less (Table D.1).

Medium Composition and Incubation Conditions Replicate 750 mL side-arm flasks of all the strains were grown at room temperature (ca. 23°C), in 30mL of VSB-6 (*Terriglobus roseus* KBS 63) or -7 (all other strains) (8) with glucose (2.5mM) as the primary carbon and energy source under yield-reduced conditions, supplemented with 5μg/mL yeast extract (BD, Franklin Lakes, NJ), and shaken on an orbital shaker (ca. 225 rotations/min). Yield-reduced conditions are defined as the limiting factor of cellular yield where the amount of growth is proportional to the amount of carbon. This ensured a decreased chance of a build-up of storage materials such as glycogen and polyhydroxybutarates and promoted complete utilization of the ^{14}C glucose label. A magnetic stir bar was present during the growth of all strains in the 750 mL side-arm flask to ensure successful processing of the sample (below). Typical inocula consisted of a 1:100 dilution of cultures in mid/late log phase (ca. $1x10^8$ cells/mL) growing on the VSB-6,7 with 2.5 mM glucose and 5μg/mL yeast extract. The optical density was monitored periodically with a Thermo™ Spectronic 20D+ at 600 nm.

Table D.1. Efficiency experimental strains and their growth characteristics.

Strain	Taxonomy	rrs	Growth Rate (hr^{-1}) †	Final O.D. (600nm) †	Cell yield (µg carbon in biomass/flask)‡
Copiotrophs					
REL 607	*λ-Proteobacteria Enterobacteriaceace*	7	0.33 ± 0.02	0.48 ± 0.00	2006 ± 56.9
HF3	*λ-Proteobacteria Pseudomonas*	4	0.37 ± 0.01	0.50 ± 0.00	1807 ± 90.6
EC2	*Bacteroidetes Spingobacteriaceae*	6	0.18 ± 0.00	0.45 ± 0.00	1905 ± 0.5
HF2	*β-Proteobacteria*	5	0.27 ± 0.02	0.83 ± 0.01	2393 ± 66.9
Oligotrophs					
RB2256	*α-Proteobacteria Spingomonadaceae*	1	0.14 ± 0.00	0.57 ± 0.00	2481 ± 154.3
LC9	*Bacteroidetes Spingobacteriacease*	2	0.08 ± 0.00	0.31 ± 0.01	1708 ± 111.8
KBS 63	*Acidobacteria Terriglobus*	2	0.04 ± 0.00	0.33 ± 0.03	1360 ± 52.2
Verrucomicrobium spinosum	*Verrucomicrobia Verrucomicrobium*	1	0.10 ± 0.00	0.45 ± 0.03	2259 ± 87.8

† - minimal medium is VSB-6,7 with 2.5mM glucose, 5µg/mL yeast extract.
‡ - measurement based on ^{14}C incorporation from labeled glucose

Addition of ^{14}C label The ^{14}C labeled-glucose was added to the culture vessel once the culture reached early-log phase, typically having an optimal density of ca. 0.05 at 600 nm. Uniformly labeled 0.1 μmoles of glucose, ^{14}C-UL-glucose (Sigma-Aldrich, St. Louis, Missouri), was added with a time zero activity of ca. 444,000 counts per minute (CPM). After three, 100 μL samples from each vessel were taken for the time zero measurement; the flasks were sealed and allowed to grow until they reached the transition point between log and stationary phase.

Processing of ^{14}C Glucose The replicate flasks were processed to separate and quantify the labeled biomass, carbon dioxide, and spent-medium once the culture reached the transition point between log and stationary phase. The flasks were connected to a series of hoses to vent the $^{14}CO_2$ in the headspace into three CO_2 trap in tandem consisting of phenylethylamine:methanol (1:1 vol/vol) (Figure D.2). The headspace of each flask was vented with nitrogen gas, briefly, for ca. 5 to 10 minutes to ensure that any labeled $^{14}CO_2$ in the headspace was trapped. Venting was stopped, and a 5 mL aliquot of the medium was removed from the flask and distributed into five, 1 mL aliquots in 1.5 mL microcentrifuge tubes. These microcentrifuge tubes were spun at 4°C at 16,000 g for 25 minutes which allowed separation of the biomass from the spent-medium. The medium was decanted into scintillation vials and the biomass was resuspended in the scintillate. Simultaneously, venting with nitrogen gas continued and trichloracetic acid (final concentration of 2% (vol/vol)) was added to the culture to acidify the medium to convert any dissolved CO_2 in the medium to gaseous form thereby allowing it to be trapped in the phenylethylamine:methanol CO_2 trap. The replicate flasks were vented for

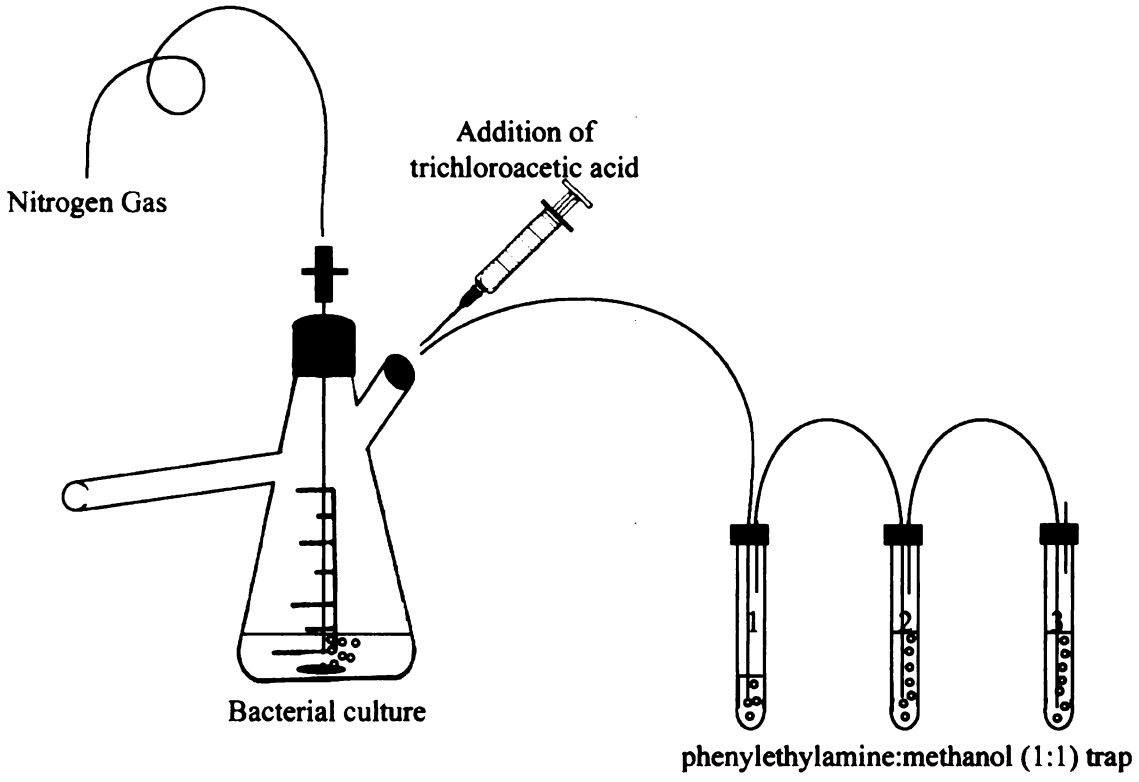

Figure D.2. Methodology for trapping ^{14}C-labeled carbon dioxide in the bacterial growth efficiency experiments. Approximately 5mL of a 10% (vol/vol) tricholoracetic acid solution was added to the 30 mL of culture to acidify, thereby allowing the dissolved carbon dioxide to convert into a gaseous form. The gaseous carbon dioxide was trapped with a carbon dioxide trap (phenylethylamine and methanol, 1:1 (vol/vol)) upon venting the headspace with nitrogen gas.

a period of 3.5 to 4 hours. Triplicate one milliliter aliquots of each of the CO_2 traps were measured for activity.

The labeled biomass, carbon dioxide, and spent-medium was quantified using a Beckman Coulter LS 6000TA Liquid Scintillation System (Fullerton, California), courtesy of the Dr. Ronald J. Patterson lab using Biosafe II (Research Products International Inc., Mt. Prospect, Illinois) as the scintillate. All solutions in which the ^{14}C radioactive label was present were assessed for their capacity to quench the signal. The only solution which indicated a significant affect ($p < 0.002$) of quenching was the phenylethylamine:methanol CO_2 trap, and a correction factor of 3.38% was used.

Data Analysis In order to fully account for the ^{14}C label, the biomass, carbon dioxide, and spent-medium were summed and no analysis was run unless $\geq$ 96% of the label could be recovered. During the development and use of this technique, attaining full recovery of the ^{14}C label was problematic. Numerous replicates of the strains were processed to achieve $\geq$ 96% recovery. Typically, incomplete recovery was due to the loss of carbon dioxide either during incubation of the strains or processing of the ^{14}C-labeled carbon dioxide, since the biomass and spent-medium counts were consistent.

BGE was determined using the values in the biomass and carbon dioxide measurements. Briefly, the activity per µmole of carbon (CPM/µmole C) was determined by taking the time zero measurement, and dividing it by the total amount of carbon added in µmoles (2.5mM glucose = ca. 75 µmoles). It was then divided by six since there are six carbons in glucose, in order to determine the specific activity of one

μmole of carbon, which was then used to determine the μmoles of carbon in the biomass and μmoles of carbon in the carbon dioxide by dividing the total activity for each by the specific activity of one μmole of carbon. The amount of carbon in μg could then be determined from μmole of carbon, which was the value used to determine BGE. BGE was determined using the following equation: (μg of carbon in biomass)/ (μg of carbon in biomass + μg of carbon in carbon dioxide).

RESULTS

Description of bacterial strains Bacterial strains were obtained from the previous soil cultivation experiments from the KBS LTER (8, 13) and rice paddy soils from Tsutomu Hattori (Institute of Genetic Ecology, Tohoku University, Sendai, Japan). Additional strains including *Verrucomicrobium spinosum*, *Escherichia coli* strain REL 607 (γ -Proteobacteria) (our model copiotroph) and *Spingopyxis alaskensis* (formely *Spingomonas alaskensis*) RB2256 (α- Proteobacteria) (our model oligotroph) were used in the analysis. Strains were selected based on their phylogenetic affiliation, *rrs* copy number, and ability to grown in VSB-6 or 7. These strains were separated into two groups: oligotrophic bacteria and copiotrophic bacteria. Oligotrophs were defined as having an *rrs* copy number of two or less, whereas copiotrophs were defined as having a *rrs* copy number of three or more. The number of *rrs* operons per genome were experimentally determined previously (8, 13) using non-radioactive Southern hybridizations. Specific phylogenetic affiliations included members of the following phyla: *Acidobacteria*, *Verrucomicrobia*, *Bacteriodetes*, and α, β, and γ *Proteobacteria* (Figure D.3). When possible, related pairs of the isolates (i.e. a copiotroph & oligotroph

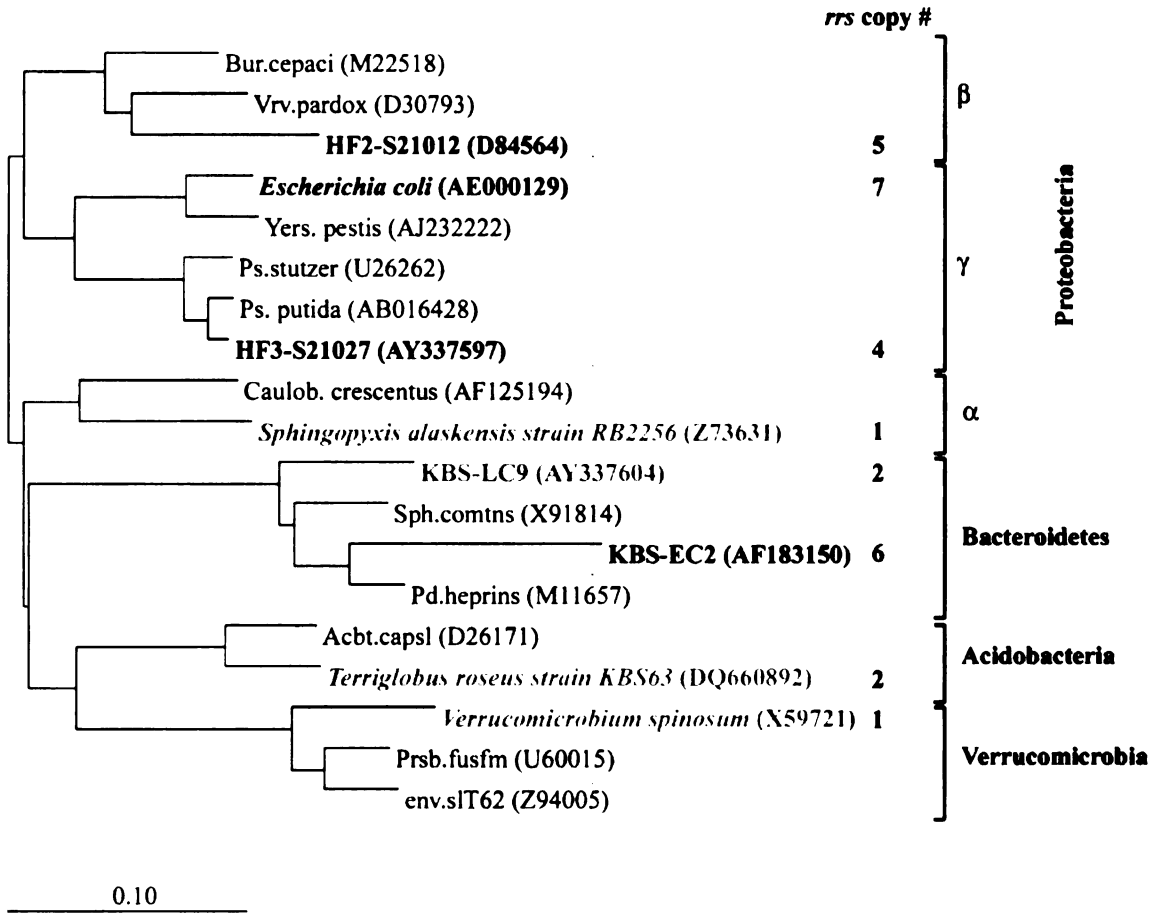

Figure D.3. Neighbor-joining phylogenetic tree of the 16S rRNA gene of strains used to determine bacterial growth efficiency. Strains from this study are in bolded text; bolded in black represent copiotrophs (n=4) whereas bolded in gray represents oligotrophs (n=4). The *rrs* copy number and major phylogenetic affiliations are illustrated to the right of the phylogenetic tree. The scale bar indicates 0.10 changes per nucleotide.

of the β-Proteobacteria) were used to reduce the potential of shared evolutionary histories; however this was not successful for some genera due to several strains' inability to grow in the medium.

Bacterial growth efficiency, cell yield, and growth rates Strains were grown on VSB-6 or 7 with glucose as the sole carbon and energy source under yield-reduced conditions to determine bacterial growth efficiency, cell yield, and growth rates. The results are illustrated in Table D.1. In general, the oligotrophs had growth rates ranging from 0.04 – 0.14 hr^{-1} whereas the copiotrophs growth rates ranged from 0.18 – 0.37 hr^{-1}. A significant ($p < 0.05$) positive correlation existed between bacterial growth rates and the *rrs* copy number, where 50% of the data could be explained by the *rrs* copy number (Figure D.4).

The BGE for each organism was determined under saturating conditions of glucose (final concentration, 2.5mM glucose) by comparing the amount of ^{14}C in the biomass over the total ^{14}C consumed. BGE among all organisms tested ranged between 0.29 to 0.52. There was no relationship between bacterial growth efficiency and *rrs* copy number (Figure D.5). As expected, there was a significant relationship ($p < 0.006$) between cellular yield and bacterial growth efficiency where 87% of the differences in cellular yield could be explained by the organism's BGE.

Phylogenomic analyses of ATP-dependent genes and response regulators Oligotrophs harbor traits including, but not limited to, reduced growth rate, increased

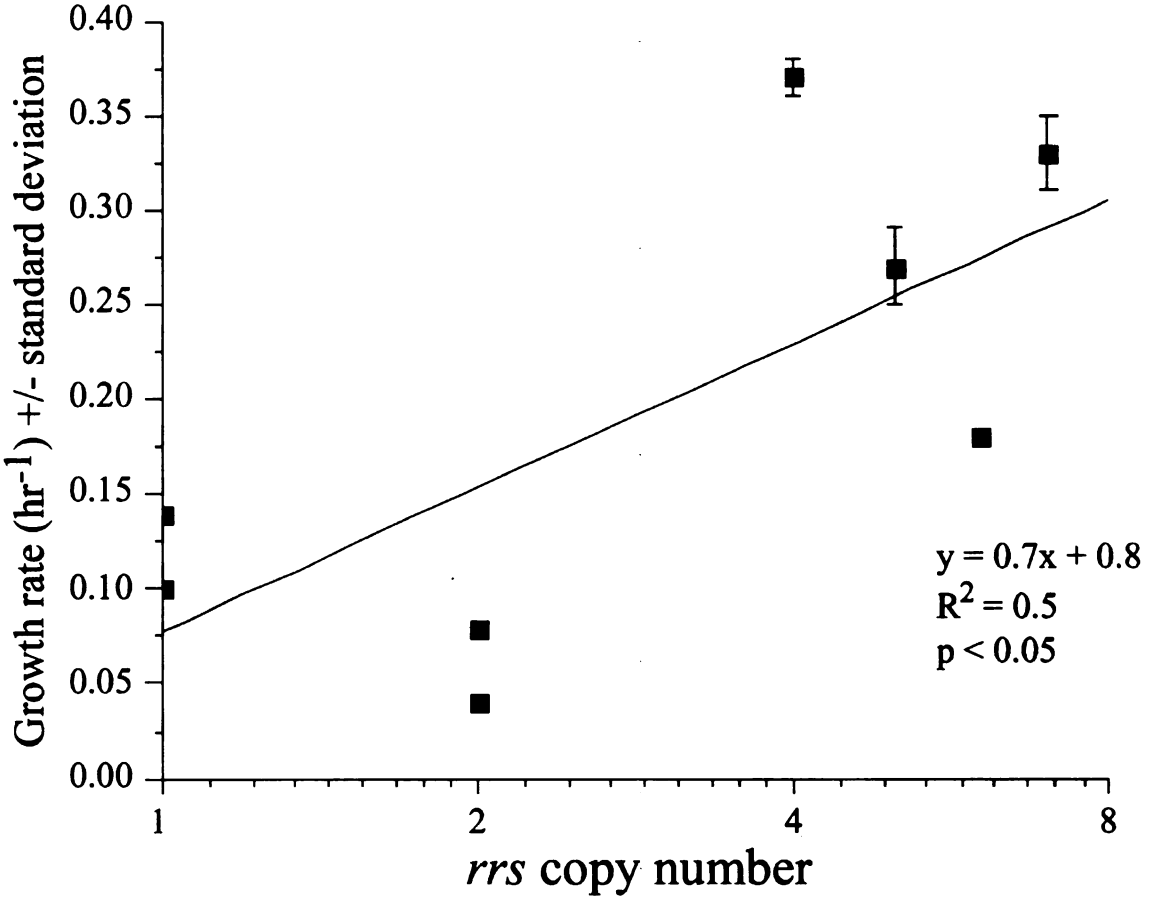

Figure D.4. Relationship between bacterial growth rate and *rrs* copy number. Each point represents the average growth rate from two replicates grown in the VSB-6,7 with 2.5mM glucose and 5μg/mL yeast extract at room temperature.

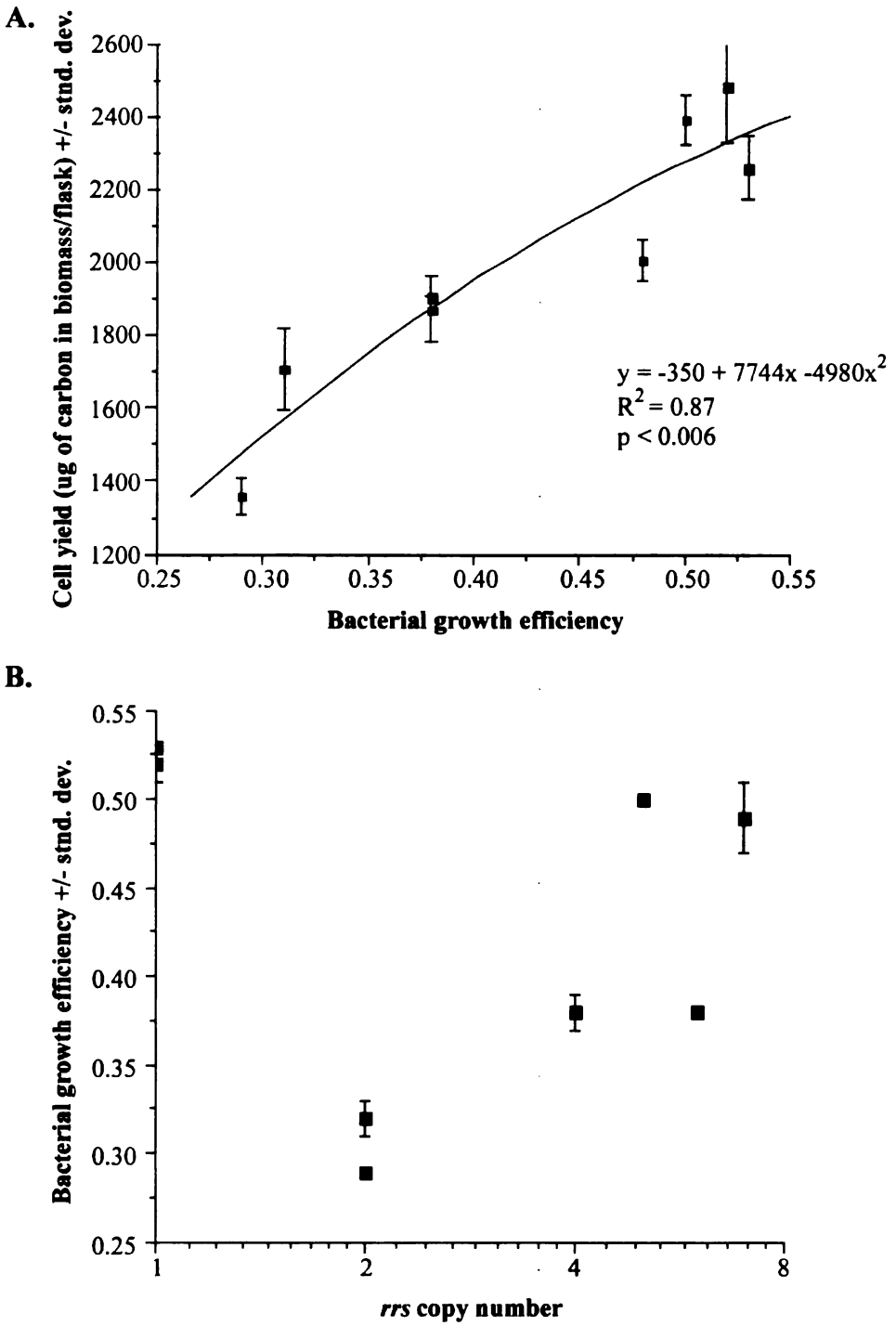

Figure D.5. Panel A depicts the relationship between bacterial growth efficiency and cell yield. Panel B depicts the relationship between bacterial growth efficiency and *rrs* copy number.

body size, and presumed higher efficiency in resource consumption. Their presumed high metabolic efficiency in resource utilization results from adapting to environments where carbon is scarce. One adaptation to this environment would be their high affinity uptake systems allowing these oligotrophs to take up carbon when they are scarce; in order to transport carbon against the gradient into the cell for growth, active transport systems such as ATP-binding cassette (ABC) transporters, are required. The relationship between the proportion of ATP-dependent proteins out of the total protein versus *rrs* copy number was evaluated. The hypothesis was that transporters in the genomes of these oligotrophs would have a higher percentage of ATP-binding cassette (ABC) transporters, and there would be a negative correlation between *rrs* copy number and the percentage of ATP-dependent transporters out of the total proteins. The ability of oligotrophs to transport carbon for growth provides them with the capacity to uptake and therefore use this carbon, however it comes with an energetic cost which will decrease efficiency. The trade-off is that copiotrophs with less energy-dependent transporters would not be able to survive in these low nutrient environments because of the diminished capacity to uptake carbon.

This hypothesis was tested by analyzing 133 readily available data of sequenced non-symbiotic, heterotrophic, bacterial genomes from the TransportDB (http://www.membranetransport.org) (31), specifically calculating the total number of ATP-dependent proteins out of the total number of proteins. There was a significant negative correlation with the percent abundance of ATP-dependent proteins and *rrs* copy number ($p < 0.0001$). However, only 18% of the increase in ATP-dependent proteins can be explained by a decrease in *rrs* copy number (Figure D.6). Bacteria harboring a low

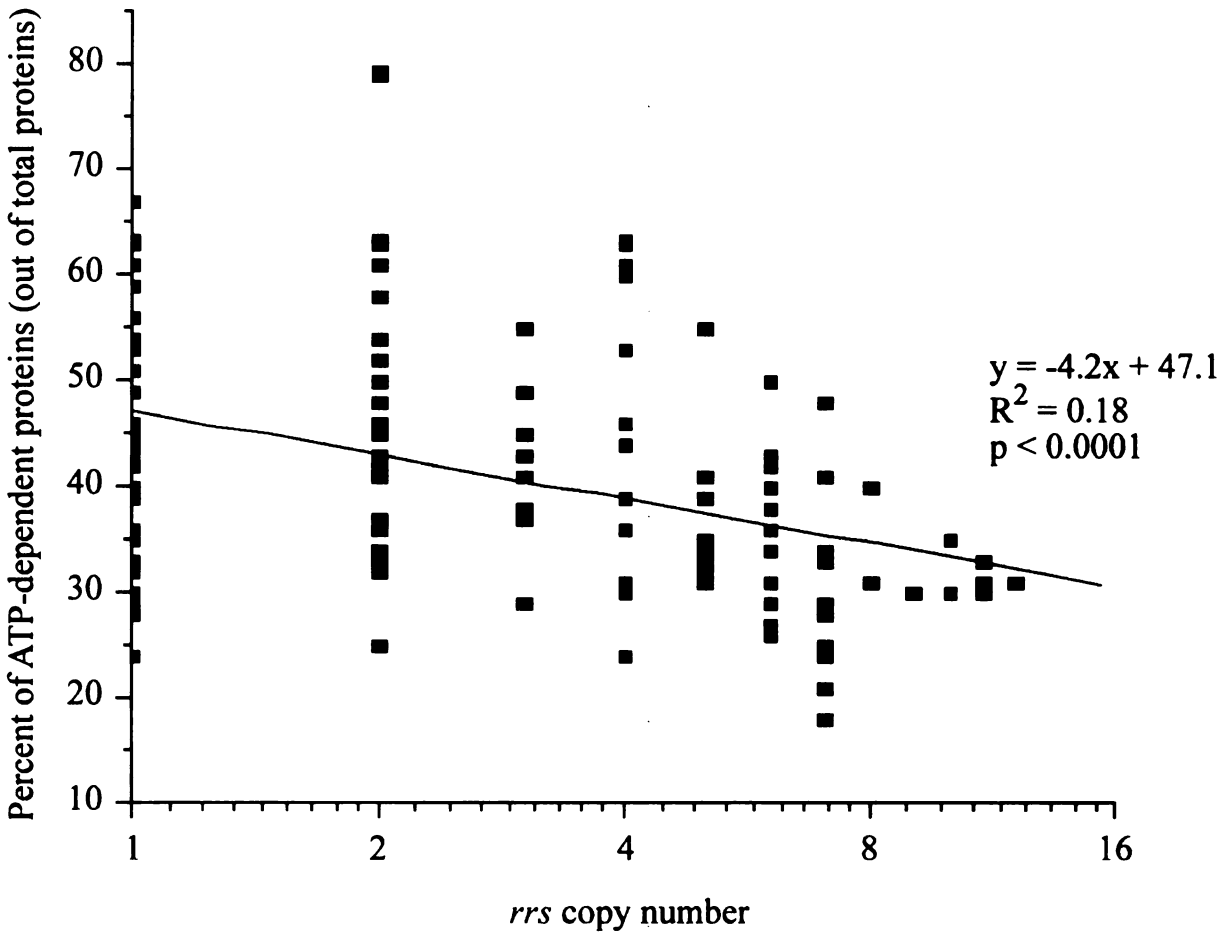

Figure D.6. Percent of ATP-dependent proteins (out of the total proteins) versus *rrs* copy number for non-symbiotic heterotrophs. Data obtained from 133 sequenced genomes from the TransportDB (http://www.membranetransport.org) (31).

rrs copy number have a higher portion of their genome dedicated to ATP-dependent transporters which allow them to survive in low nutrient environments. This relationship clearly illustrates an intrinsic property, ATP-dependent proteins, which reflect a different ecological strategy for bacteria.

Another intrinsic property that was assessed to determine any correlation with *rrs* copy number was the number of response regulators in two-component systems in bacterial genomes, not associated with chemotaxis. Two-component systems are pathways some bacteria use to sense environmental stimuli through the use of a histidine kinase sensor protein typically in the periplasm that transmits a signal to the carboxyl-terminal of the response regulator (22). The response regulators typically are DNA-binding, transcriptional regulators which allow the transcription of the necessary genes in response to the environmental stimuli (3). Copiotrophs harbor traits including, but not limited to, high growth rate and abundance in nutrient rich and fluxing environments. It was hypothesized that these bacteria would have to dedicate a high portion of their genome to sense changes in these fluxing environments such as response regulator-encoding genes, which would lend to a positive correlation between *rrs* copy number and the percent of the genome devoted to response regulator genes. Response regulators associated with chemotaxis are excluded in the analysis because the property of chemotaxis allows them to physically change their surrounding environment, thereby not directly linking gene function with environmental stimuli. This hypothesis was tested by analyzing 187 readily available data of sequenced non-symbiotic, bacterial genomes from Response Regulator Census on the NCBI website

(http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html), specifically calculating the percent of response regulator genes out of the total genes in each of the respective genome. There was a significant positive correlation with the percent abundance of response regulator genes and *rrs* copy number ($p < 0.0001$). Bacteria harboring a high *rrs* copy number have a higher portion of their genome dedicated to sensing changes in the environment. The increase in *rrs* copy number explains ca. 20% of the increase in response regulators, which is as hypothesized, where the bacteria with high *rrs* copy number have the capability of sensing a fluctuating environment (Figure D.7).

DISCUSSION and FUTURE DIRECTIONS

The primary goal of this study was to determine if there was a negative correlation between bacterial growth efficiency and *rrs* copy number, the genetic marker for ecological strategies in bacteria, among a phylogenetically diverse group of bacteria. Under saturating concentrations of glucose, there was not an observable trend between bacterial growth efficiency and *rrs* copy number (Figure D.5). One explanation for this result could be due to intrinsic differences in the metabolic pathways to oxidize carbon, synthesize vitamins, enzymes, co-factors, and create reducing power for energy production (Table D.2). There is no available metabolic information on these organisms to address this concern. For the purposes of these experiments, it was assumed that all matters pertaining to growth among these strains was equal. Furthermore, the environmental conditions in which these strains were grown, in particular pH and temperature, could be metabolically costly for some organisms to grow due to a possible,

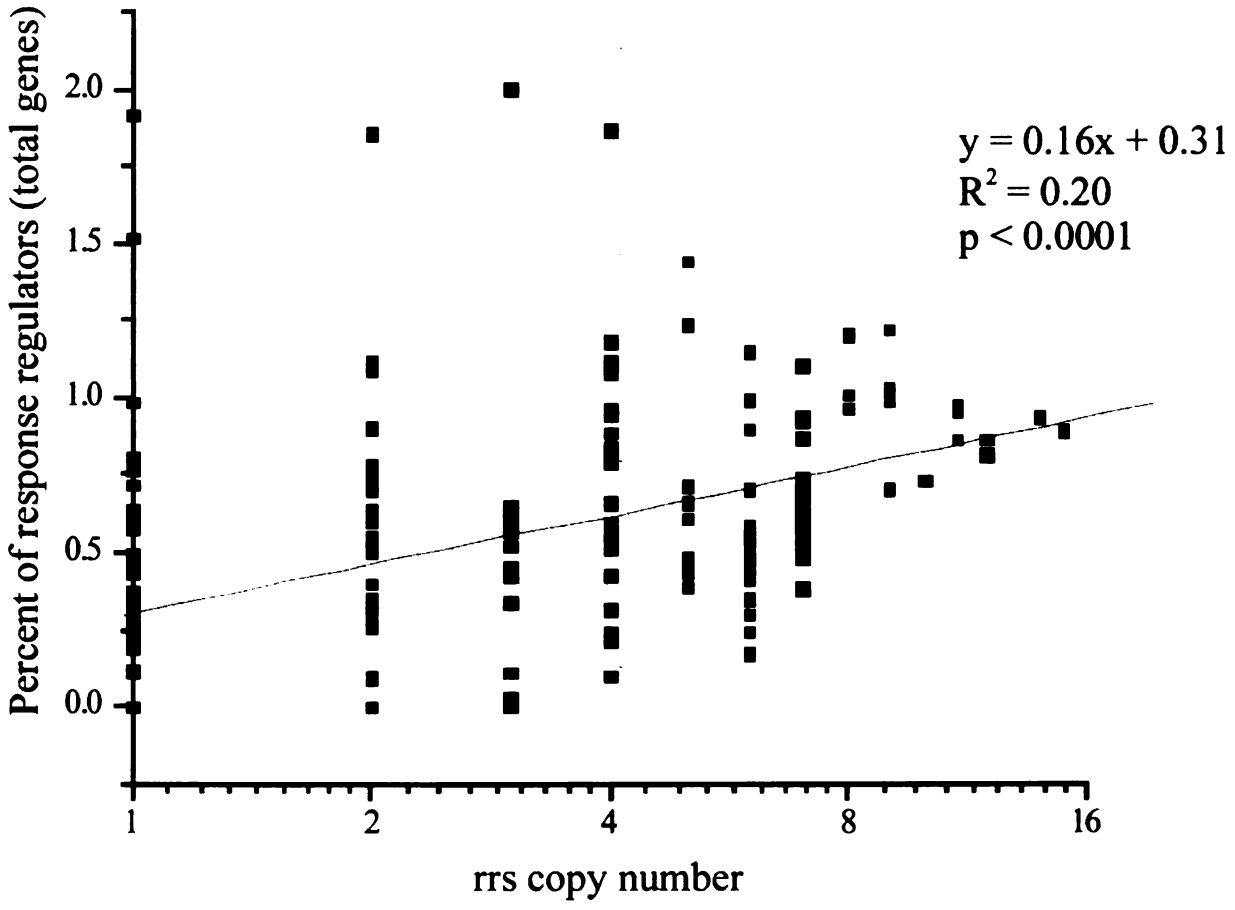

Figure D.7. Percent of response regulators in two-component systems not associated with chemotaxis, versus *rrs* copy number for bacteria and archaea. Data obtained 187 sequenced genomes obtained from TIGR (http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html).

Table D.2. Intrinsic metabolic features and environmental factors that influence bacterial growth efficiency.

Metabolic Features	***Proposed Mechanisms***	***Reference(s)***
Active transport	Varying proportions of transporters that consume ATP or IMF	(23)
Motility	Consumption of IMF in flagellar motility or synthesis of excreted compounds in gliding motility	(29)
rrs copy number	Fast (high rrs) growth vs. slow (low rrs) growth	(13, 37)
Translational machinery	Tradeoff between speed and accuracy of translational machinery influences amount of functional protein synthesized per mole of ATP	(18)
Pathway utilization	Tradeoff between rate and efficiency of analogous pathways	(11)
Maintenance of ion gradient	Consumption of IMP or ATP to maintain internal pH or intracellular ion concentrations	(29)
Synthesis of extracellular macromolecules	Extracellular enzymes or matrix materials are not typically measured as biomass	(24)
Turnover of macromolecules	Half life of proteins and ribosomes dictates demand for new synthesis of these macromolecules	(32)
Synthesis and utilization of storage compounds	Variable energy costs or savings associated with intracellular storage of C, S, N, and P	(6)
ATP-producing pathways	Trade-off between rate and yield of ATP	(27)
Environmental Factors		
Free energy of substrates	Limits maximal ATP yield	(6)
Oxidation state of substrates	Cost of assimilatory reduction of C, S, N, and P for biosynthesis	(6)
Substrate concentration	Increase ATP demand for transport at low substrate concentrations; energy spilling at excess concentrations	(4)
Carbon: Nitrogen	High ratio demands carbon is oxidized before nitrogen can be used for biosynthesis	(15)
Substrate availability	Demand for synthesis of compounds not available from the environment	(6)
Deviation from a microbe's optimal growth conditions	Synthesis of macromolecules required for growth/survival in suboptimal conditions; costs associated with removing or degrading inhibitory compounds	(26)

deviation from their optimal conditions. The varying degrees of these demands could negatively influence the bacterial growth efficiency calculation by indicating an adversely low value since more carbon would be diverted to non-growth related functions to compensate for these non-ideal conditions. One could speculate that the sample size would need to be rather large in subsequent experiments to overcome this variability to reveal true differences reflected by an organism's *rrs* copy number.

Another explanation for the lack of correlation between bacterial growth efficiency and *rrs* copy number is due to the saturating glucose conditions under which the efficiency measurements were made. Oligotrophs are typically found in stable environments where carbon concentrations are low; therefore, one could predict it would be under these low, but stable nutrient concentrations where an oligotroph would maximize their efficiency. The efficiency experiments were performed under saturating concentrations of carbon, therefore masking any meaningful relationship between efficiency and low *rrs* copy number.

In order to address this concern, the next step in this research would be to grow these strains under stable, low nutrient concentrations and measure their bacterial growth efficiency. Oligotrophs are typically more abundant in low nutrient environments; therefore, they have evolved to be more fit under these conditions. If oligotrophs are more efficient at utilizing substrates, it would be plausible to assume that they would do so under conditions typical of their native environment. Furthermore oligotrophs typically have a higher portion of ATP-dependent transporters (Figure D.6) which would allow them to uptake carbon in carbon-poor environments. Strains would need to be grown using a chemostat whereby the population density is controlled by the dilution rate

which is a reflection of the nutrient concentration in the system. The hypothesis would be that oligotrophs reach their maximum growth efficiency at lower dilution rates, reflective of a low nutrient environment, as compared to copiotrophs reaching their maximum efficiency at high dilution rates or nutrient rich environments (Figure D.8, panel A). The model would be that there is a positive correlation between *rrs* copy number and resource concentration (as measured by dilution rate in a chemostat) at which the organism reaches its maximum growth efficiency (Figure D.8, panel B).

To further understand this proposed study, it would be beneficial to predict why copiotrophs have lower bacterial growth efficiencies at low nutrient concentrations. When a bacterium oxidizes carbon, the energy obtained is either use for growth or non-growth. Energy used for non-growth related functions is typically defined as maintenance energy. It is believed that the demand for maintenance energy increases the further away from optimal conditions. Maintenance energy can be quantified through the use of Pirt's double reciprocal plots (1/yield versus 1/growth rate) (28, 29).

Under steady state, low nutrient concentrations, the maintenance demands would have a greater effect in terms of efficiency in copiotrophs than oligotrophs because it is an environment not conducive to fast growth. The proposed hypothesis is that maintenance energy is lower in oligotrophs than copiotrophs as measured by a double reciprocal plot of the inverse of yield and growth rate under steady state, low nutrient conditions (Figure D.9, panel A) at dilution rates below maximum efficiency. Copiotrophs would have a larger slope than oligotrophs because the demand for maintenance energy would be higher under these low nutrient conditions. The model

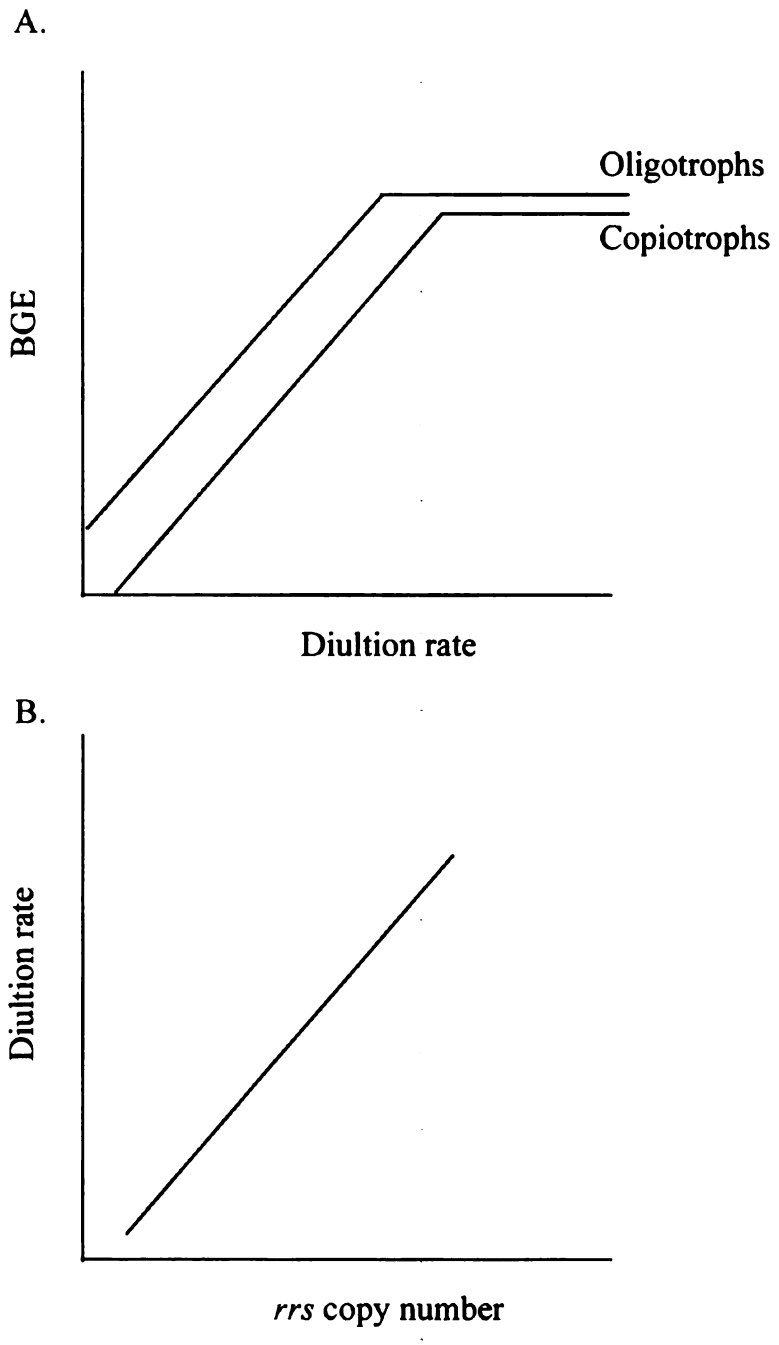

Figure D.8. Proposed model for future bacterial growth efficiency experiments. Panel A depicts the proposed relationship between bacterial growth efficiency (BGE) versus dilution rate. Panel B depicts the proposed linear relationship between dilution rate, a proxy for resource concentration indicating rate at which the organism reaches its maximum growth efficiency (Panel A) measured in a chemostat, versus *rrs* copy number.

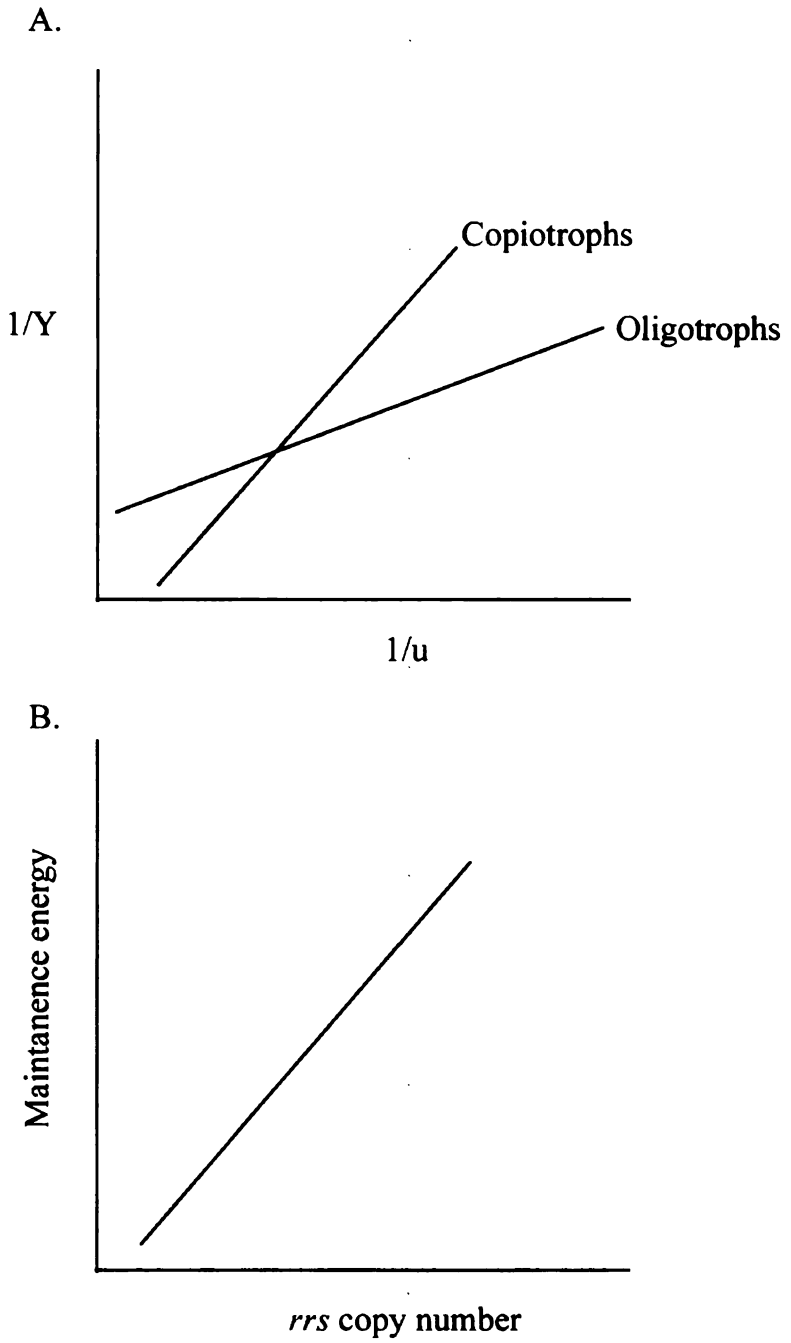

Figure D.9. Proposed model for future bacterial growth efficiency experiments. Panel A depicts the proposed relationship for maintenance energy for oligotrophs and copiotrophs as illustrated by Pirt's double reciprocal plots of the inverse of yield (1/Y) versus the inverse of growth rate (1/μ) at steady state. Panel B depicts the proposed linear relationship between maintenance energy of oligotrophs and copiotrophs grown at steady state dilution rates versus *rrs* copy number.

would be that there is a positive correlation between maintenance energy, as measured by the slope of the double reciprocal plot, and *rrs* copy number (Figure D.9, panel B) meaning there would be a lower demand in maintenance energy in oligotrophs when growing under their ideal conditions The juxtaposition of these experiments, as mentioned above, is that one could speculate that the sample size would need to be rather large in subsequent experiments to overcome the variability of different physiological as well as environmental attributes of the strains to reveal true differences in efficiency reflected by *rrs* copy number.

The foundation of this research brings an ecological theory into a microbe's perspective. It is a unifying step of multiple disciplines: microbiology, ecology, and evolution. Understanding bacteria's ecological strategies in the environment is necessary to appreciate the community's dynamics especially when only a small fraction of the soil microbial community has been cultivated, such as the members of the phylum *Acidobacteria*. By knowing a bacterium's efficiency as well as the environmental regime, we will be able to make predictions of the impacts certain communities have on nutrient cycling, biogeochemistry, and agricultural practices.

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