MICHELLE ANN FURLONG
Bacterial communities in natural ecosystems: groundwater, soil, earthworm casts and burrows
(Under the Direction of WILLIAM B. WHITMAN)

Bacterial isolates from fresh earthworm casts were phenotypically and taxonomically different than isolates from soil and earthworm burrows. While most of the cast isolates grew on MacConkey media, reduced nitrate and utilized acetate and Casamino acids for carbon, the majority of the soil and burrow isolates did not. The majority of the soil and burrow isolates belonged to the high G + C gram-positive bacteria (54% and 48%, respectively). The other isolates were distributed among the low G + C gram positives and α-, β- and γ- proteobacteria. The majority of the cast isolates were γ-proteobacteria (78%). The remainder of them grouped with the high and low G + C gram positive, α- and β- proteobacteria and cytophaga/flexibacter groups.

The cast isolates were also less diverse than the soil and burrow isolates. The casts contained fewer biotypes (groups of organisms containing the same phenotype), and those biotypes were more similar to each other than were those of the bulk soil or burrow isolates. After standardizing the sample size, soil and burrow isolates each contained twice as many OTUs (operational taxonomic units as defined by rDNA) and had diversity and evenness values that were much greater than the cast isolates.

Cloned rDNA from TCE contaminated and uncontaminated groundwater was compared. The uncontaminated groundwater contained one ribotype, which represented 26 clones and was very similar to *Pseudomonas gessardii*. The contaminated groundwater contained seventeen ribotypes, which represented forty clones. Sixty-five, thirty-three, and three percent of these clones were related to the β- and γ- proteobacteria and an unknown group, respectively. The majority of the γ- proteobacteria (85%)

grouped with the Methylococcaceae family, and the remainder grouped with the pseudomonads. Forty-six percent of the β-proteobacteria grouped with methylotrophs, and the remainder grouped with various genera in the order Burkholderiales. Archaeal DNA was detected only in the contaminated water. One ribotype, which represented fifteen clones, grouped with the Crenarchaeota.

INDEX WORDS: Bacterial community, Prokaryotic community, Earthworm cast, Earthworm burrow, Groundwater, 16S rDNA, TCE degradation

BACTERIAL COMMUNITIES IN NATURAL ECOSYSTEMS: GROUNDWATER, SOIL, EARTHWORM CASTS AND BURROWS

by

MICHELLE ANN FURLONG

B.S., Georgia State University, 1994

A Dissertation Submitted to the Graduate Faculty of

The University of Georgia in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2000

BACTERIAL COMMUNITIES IN NATURAL ECOSYSTEMS

GROUNDWATER, SOIL, EARTHWORM CASTS AND BURROWS

by

MICHELLE ANN FURLONG

Approved:

Major Professor: William B. Whitman

Committee Members: David C. Coleman
Paul F. Hendrix
Mary Ann Moran
Lawrence J. Shimkets

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
August 2000

DEDICATION

To Joey, for your love and support and for understanding my love of earthworms.

ACKNOWLEDGMENTS

I would like to thank Prof. William B. Whitman for his superior knowledge on the subjects of bacterial physiology and phylogeny that he has shared with me, and for his extreme patience while guiding my research. I would like to thank Prof. David C. Coleman, who introduced me to the wonders of soil and helped me with the ecological aspects of my project. I would like to thank David R. Singleton, who has helped me with molecular techniques and developing specific aspects of my research. I would also like to thank Prof. Paul F. Hendrix, Prof. Mary Ann Moran and Prof. Lawrence J. Shimkets for their help with earthworm ecology, microbial diversity, and general microbiology, respectively. Finally, I would like to thank my laboratory coworkers for their guidance and support and for making life in the lab interesting.

TABLE OF CONTENTS

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Microbial ecology is the study of prokaryotic, fungal and protist communities in their natural habitats. Microbial ecologists are faced with three challenges when examining a microbial community. They must reveal the microbial diversity, or the types of microorganisms that exist in the habitat, calculate the abundance of each to determine the community structure, and describe the activity of these organisms, or community function. To obtain a complete set of ecological data for a natural microbial community is a huge undertaking. Typically these studies begin with determining the microbial diversity, because this frequently reveals information important to community structure and function.

There are many reasons why it is important that the prokaryotic diversity of natural environments be revealed. Understanding the effect of disturbance on bacterial diversity and function in the environment contributes to the understanding of soil and water quality and the development of sustainable ecosystems. Because prokaryotes are sensitive to disturbances, such as pollution and agricultural practices, knowledge of changes in prokaryotic diversity can help monitor disturbances and help explain the transformation of chemicals in the environment. For example, if a habitat were contaminated with trichloroethylene (TCE) then the prokaryotic diversity could be monitored throughout the ecosystem to track movement or spreading of the TCE. Also, if it were known which prokaryotes were present in the habitat, which were capable of degrading the TCE, they could be targeted in bioremediation experiments. Another reason why prokaryotic diversity studies are important is because they expand the possibility of finding new prokaryotes in the environment, which increases the chance of

finding novel antibiotics or enzymes that can be used to improve technology. These are just a few examples of how increased knowledge of prokaryotic diversity can affect mankind.

Although prokaryotic diversity studies have progressed greatly in the past three centuries, it is still quite obvious that there is much to learn. Antonius Van Leeuwenhoek first observed prokaryotic cells, which he called "wee animacules", in 1676. From observations with his primitive lenses he classified these organisms using a handful of terms, such as round, bacillus, spiral, or motile [Van Leeuwenhoek, 1677]. With every new technological advancement since the microscope, knowledge about prokaryotic diversity has expanded. Prokaryotes are now described by a number of different traits and a complex classification system has been designed for taxonomic purposes. The same technology that has been used to explain the extreme diversity of prokaryotic organisms in the environment has also caused a realization that little is known about the origins of many of these prokaryotes and about the structure of natural prokaryotic communities. Currently, it is believed that there are about 10^{30} prokaryotic cells on earth [Whitman *et al.*, 1998]. Although it has been estimated that there are 10^{9} bacterial species [Dykhuizen, 1998], only 5,000 different species, derived from Bergey's Manual [1999], have been described [Bull *et al*., 1992]. Fuhrman [1999] described this lack of knowledge elegantly when he said, “Everyday we go outside, we may be stepping on a kingdom that hasn’t been identified.”

Unlike eukaryotic organisms, which can be classified by easily observed physical properties, prokaryotes are classified by indistinct morphological and physiological properties. The methods used presently to observe most of these properties usually

require having the organisms in culture, which is not always easy to do. Greater than 85% of bacteria in environmental samples are not capable of being cultured on standard laboratory media [Amann *et al*., 1995;Bakken and Olsen, 1987;Giovannoni *et al*., 1990; Torsvik *et al*., 1990; Ward *et al*., 1990]. Therefore, before prokaryotes in nature can be cultured and described, some initial information about their physiology must be known first to design the culture conditions and media. Because of this, it is unrealistic for a microbiologist to expect to be able to culture and describe every living prokaryotic organism in a natural ecosystem in a lifetime. Therefore, a number of different strategies for determining microbial diversity in natural ecosystems have been developed.

2. Methods for Determining Prokaryotic Diversity

One approach used to examine the prokaryotic diversity in natural environments is based on the ability to culture representative organisms. Two directions are typically taken with this approach. First, substrate utilization patterns of the community have been used to determine the functional diversity in a number of different habitats [Garland and Mills, 1991; Winding, 1994; Zak *et al*., 1994; Garland, 1996; Degens and Harris, 1997; Lindstrom *et al*., 1998]. This method does not attempt to infer phylogeny or classify organisms in a taxonomic manner. Second, isolation of representative prokaryotes has been used to determine the functional and/or taxonomic diversity of many environments [Buyer and Drinkwater, 1997; Buyer and Kaufman, 1996; Chandler *et al*., 1997; Chin *et al*., 1999; Gonzalez *et al*., 1996; Gorlach, *et al*., 1994; Gosink and Staley, 1995; Kaneko and Atlas, 1977; Mahaffee and Kloepper, 1997; Nold and Ward, 1995; Nold *et al*., 1996 Schut, *et al*., 1993; Suzuki *et al*., 1997]. Organisms can actually be identified by phenotypic and phylogenetic analysis using this method.

Another approach which has been taken to determine natural microbial diversity is the characterization of the extractable fatty acid methyl esters (FAME) and phospholipid fatty acid (PFA) contents [Bossio *et al*., 1998; Buyer and Drinkwater, 1997; Cavigelli *et al*., 1995; Griffiths *et al*., 1999; Mancuso *et al*., 1990; Ritchie *et al*., 2000; Rajendran *et al*., 1992; Smith *et al*., 1986; Zelles and Bai, 1994]. This approach offers little information concerning microbial function and is limited in the taxonomic information that may be obtained [Haack *et al*., 1994].

The third approach used to characterize microbial diversity is the characterization of extracted nucleic acids. This approach can be performed by many different methods. Extracted nucleic acids have been analyzed using guanine plus cytosine composition [Nusslein and Tiedje, 1998; Holben and Harris, 1995], thermal denaturation and reassociation [Torsvik *et al*., 1990], nucleic acid hybridization [Zarda *et al*., 1997; Liesack and Stackebrandt, 1992], reverse sample genome probing (RSGP) [Voordouw *et al*., 1991], fingerprinting with denaturing gradient gel electrophoresis [Muyzer *et al*., 1993], restriction fragment length polymorphism (RFLP) [Laguerre *et al*., 1994; Moyer *et al*., 1994] or low molecular weight RNA patterns [Hölfle, 1992], terminal restriction fragment length polymorphisms (T-RFLP) [Liu *et al*., 1997], length heterogeneity-PCR (LH-PCR) [Suzuki *et al*., 1998; Ritchie *et al*., 2000], phylogeny of PCR amplified and cloned ribosomal (16S, 23S or 5S) DNA or combinations of any of these. Limited information concerning microbial function can be obtained by any of these molecular methods, and the only methods that offer phylogenetic information are the RSGP, nucleic acid hybridization, and phylogenetic analyses of cloned rDNA.

Each of these three approaches will be described in the following sections. However, only the methods specific to this dissertation, i.e. isolation and analysis of culturable prokaryotes, FAME and PFA analyses, and the phylogenetics of amplified 16S ribosomal DNA clones, will be explained in detail.

2.1 Culture Based Approach to Microbial Diversity

The isolation and characterization of culturable prokaryotes from environmental samples is one method used to observe environmental microbial diversity. This method involves taking representative samples from the habitat, inoculating appropriate media that can grow a wide variety of prokaryotes, collecting and maintaining pure cultures of all isolates, and characterizing them with biochemical and/or molecular methods. This method has been used by many researchers to examine the prokaryotic diversity in soils, seawater, sediments, rhizosphere and microbial mats [Buyer and Kaufman, 1996; Chandler *et al*., 1997; Chin *et al*., 1999; Gilbert *et al*., 1993; Gonzalez *et al*., 1996; Gorlach, *et al*., 1994; Gosink and Staley, 1995; Kaneko and Atlas, 1977; Mahaffee and Kloepper, 1997; Nold and Ward, 1995; Nold *et al*., 1996 Schut, *et al*., 1993; Suzuki *et al*., 1997]. Although it sounds simple and straightforward, many special considerations have to be taken into account when using this method.

The first step to determining the prokaryotic diversity in an environment using an isolate collection is to obtain samples that represent the habitat. Considering the fact that prokaryotes are usually less than 1 μm in diameter in natural environments, microbial ecologists must be careful to consider their immediate environment (microenvironment), which consist of the nutrients available to them and their competitors, as well as their entire habitat. For example, a heterogeneous environment, like soil, contains a multitude

of microenvironments for microorganisms. It has been shown that soil microorganisms are not randomly or uniformly distributed throughout soil. They tend to congregate in pores and near suitable food sources, like fecal pellets, cellular remains and amorphous organic material (Foster, 1988). By homogenizing large samples (i.e. 1 to 10 grams), these microenvironments may be mixed causing competition in growth media of organisms that would not normally compete with each other in the natural environment. Studying numerous small samples that reflect many different microenvironments within the habitat would represent the microbial community more accurately than a few large samples. Another consideration when taking representative samples in the environment are the spatial distributions of nutrients and energy sources along a profile gradient. For example, sunlight and oxygen usually decrease in concentration with depth in a soil or water column. Since certain populations of prokaryotes are dependent on these, the microbial community structures probably also change with depth. Therefore, these gradients should also be considered when obtaining representative samples in a water or soil column.

The next step is to isolate representative prokaryotes from the samples. Several factors should also be considered when choosing a culture method. A growth medium should contain nutrients that are found in the habitat sampled. Media supplemented with seawater, soil extract, or spring water have been used to isolate representative bacteria from marine, soil and hot spring microbial mat environments [Button *et al*., 1993; Gonzalez *et al*., 1996; Gorlach, *et al*., 1994; Gosink and Staley, 1995; Nold and Ward, 1995; Nold *et al*., 1996 Schut, *et al*., 1993; Suzuki *et al*., 1997]. Other media have included specific carbon sources that are unique to the habitat [Nold *et al*., 1996;

Gonzalez *et al*., 1996; Chin *et al*., 1999]. It has been shown that when samples were serially diluted in growth media before incubation some bacteria were detected in the higher dilutions that were not detected in the lower dilutions (dense with inoculum) after incubation [Jackson *et al*., 1998; Santegoeds *et al*., 1996; Schut *et al*., 1993]. Some researchers have shown that many slowly growing bacteria exist in natural samples and require periods as great as 2 weeks to grow at discernable levels [Santegoeds *et al*., 1996; Kataoka *et al*., 1996; Schut *et al*., 1993]. Incubation temperature has also been proven to affect which bacteria from a sample grow in the culture [Santegoeds *et al*., 1996]. It is important to observe properties of the habitat when designing a scheme to isolate representative bacteria from environmental samples.

Before the diversity can be examined, the last step is to identify or characterize the isolates in the collection. This can be accomplished by analyzing their ribosomal DNA, fatty acids, and/or biochemical properties. The ribosomal DNA methods can offer the most efficient phylogenetic information, and some inferences can be made about functional properties if the isolates can be grouped with described organisms at the genus or species level. This technique has been used by many laboratories recently to describe the prokaryotic diversity of cultivated strains from the subsurface (Pedersen *et al*., 1996; Chandler *et al*., 1997), soil (Chin *et al*., 1999; Felske *et al*., 1999; Dunbar *et al*., 1999), seawater (Gonzalez *et al*., 1996; Suzuki *et al*., 1997; Benlloch *et al*., 1995), and hot spring microbial mats (Nold *et al*., 1996). These methods will be described in more detail in the molecular approach section below. The fatty acid methyl ester (FAME) analysis has also been used in recent soil microbial diversity studies (Buyer and Kaufman, 1996; Mahaffee and Kloepper, 1997). Organisms can only be grouped

accurately to the genus level using this method; thus making it difficult to infer functional properties as well. Biochemical and morphological characterization has also been used recently to examine the diversity of soil isolates by function (Chin *et al*., 1999; Gorlach *et al*., 1994; Gilbert *et al*., 1993). Relatively little phylogenetic or taxonomic information can be obtained efficiently by this method, since it requires a large number of tests to obtain enough information suitable for taxonomic or phylogenetic purposes. However, this is the most accurate way of obtaining information concerning potential functional significance in the community. The best way to obtain accurate phylogenetic and potential functional properties of community isolates is to use a combined approach. For instance, phylogenetic and phenotypic characterization of members of numerically abundant, culturable bacteria in soils utilized both 16S rDNA and biochemical analyses (Chin *et al*., 1999).

2.2 Fatty Acid Profile Approach to Microbial Diversity

In the past, measurements of fatty acids from environmental samples were simply used to estimate microbial biomass. Since many bacteria have unique signature fatty acid profiles, they have been used more recently to examine microbial community structure. Fatty acids can be extracted from environmental samples, and the abundance of each type in the complex mixtures can be determined by gas chromatography. This method has been used to describe microbial communities in soils [Bossio *et al*., 1998; Cavigelli, *et al*., 1995; Griffiths *et al*., 1999; Zelles and Bai, 1994], sediments [Mancuso *et al*., 1990; Rajendran *et al*., 1992] and subsurface environments [Smith *et al*., 1986]. This approach involves three relatively rapid steps (figure1).

The first step is to obtain an environmental sample that represents the habitat and has a large amount of microbial biomass. Because of the requirement for a large sample size, it is difficult to use this method to observe microbial communities associated with micro-environements, such as rhizosphere soil.

The next step is to extract and separate the fatty acids from the sample. First, the lipids are obtained using a one-phase extraction with chloroform and methanol. The lipids are then separated into glyco-, neutral and phospho- (polar) portions using silicic acid columns. The neutral, and glyco-lipid portions are usually ignored in bacterial diversity studies since they are not useful for identifying bacteria. The phospholipid fatty acids (PLFAs) are hydrolyzed in a mild alkaline and methanolic solution. The extracted fatty acids (FA) are separated into hydroxy substituted (OHFA), unsubstituted and unsaponifiable components using amino propyl solid phase columns. The unsubstituted FAs are separated into saturated (SATFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids using silver ion chromatography. The unsaponifiable components must be extracted with acid hydrolysis before they are separated into unsubstituted FAs and OHFAs using the solid phase columns. The lipopolysaccharaides in the original sample must also be extracted. These are processed by treating the sample residue after the initial one phase extraction with HCl, extracting with chloroform and methylating. The OHFA methyl esters (ME) are separated from the unsubstituted FAs from this portion using a solid phase column [Zelles and Bai, 1994].

The third step is identifying the fatty acids using gas chromatography and mass spectroscopy (GC-MS) and determining which organisms they might represent. All of the resulting OHFAs must be trimethylsilylised (TMsi) before they can be separated by

GC-MS. The positions of unsaturation in the MUFAs and PUFAs can be determined if they are first derivatized with dimethyl disulphide (DMDS) and dimethyl oxide (DMOX), respectively [Zelles and Bai, 1994].

The FA assignments are based on databases of fatty acid profiles from cultured and described organisms. Since there may be significant quantitative changes in proportions of certain fatty acids from organisms grown under different conditions [Haack *et al*., 1994], assignments based on a database of cultured organisms can be highly inaccurate. Also, since fatty acid profile information in the literature is limited, it may be difficult to make assumptions concerning identification [Haack *et al*., 1994]. However, since this method is reproducible and can be performed quickly, it can be used to determine the effect of season, agricultural management, and soil type on the diversity of a microbial community [Bossio *et al*., 1998]. Fatty acid analyses have also been useful for comparing community profiles based on the presence or absence of large and well-characterized taxonomic groups (i.e. Gram-negative bacteria or sulfate-reducing bacteria). For instance, Zelles and Bai [1994] were able to show that forest soil contained more fungi and less Gram negative organisms as compared to salt marsh sediment.

2.3 Molecular Phylogenetic Approach to Microbial Diversity

The molecular phylogenetic approach to microbial diversity is based on the use of macromolecular sequence comparisons to define relationships. This practice is dependent on the fact that all organisms contain macromolecular sequences that are conserved enough to be compared in a pair-wise manner, yet have diverged enough to reflect evolutionary change. In the past this was accomplished by comparing conserved protein sequences, such as cytochrome C, ribonucleases and globins. While rewarding

for higher eucaryotes, it has been difficult to apply protein phylogenies to prokaryotes because of the extent of their phylogenetic and biochemical diversity [Demoulin, 1979]. The investigations by Carl Woese and his colleagues have shown that ribosomal RNA sequences are especially useful for phylogenetic comparisons between organisms because: (1) they are present in all organisms, (2) conserved enough to make alignments and diverged enough to show evolutionary change (properties of an effective chronometer), (3) easily recovered from all cells, and (4) lengthy enough for statistical comparisons [Stackebrandt and Woese 1981]. The world's knowledge about evolution and diversity has exploded since phylogenetic comparisons based on rRNA were introduced, and the application of these methods to study microbial diversity in natural environments has been well established (Figure 2).

One of the first natural microbial diversity studies using rRNA analyses involved the examination of a relatively simple microbial community (hot spring) and analysis of the 5S rRNA [Stahl *et al*., 1985]. Although 5S rRNA functions as an effective chronometer for phylogenetic studies, its small size (~ 120 nucleotides) makes it difficult to make statistically significant phylogenetic comparisons in complex communities [Pace *et al*., 1986]. Recent studies of this type involve the examination of more complex communities and the analysis of 16S ribosomal sequences. The 16S sequences are much larger (~1500 nucleotides); therefore, they contain more phylogenetic information. With recent sequencing technology, partial 16S ribosomal sequences are as easy to obtain as the 5S sequences. Because of this, enormous databases that contain thousands of 16S ribosomal sequences have been developed and are used extensively to determine phylogeny. The 23S sequences are even larger (~3,000 nucleotides) and also act as

excellent chronometers. However, they are not as useful as the 16S sequences because of a limited number of published sequences. Thus, most of the molecular methods used to study microbial diversity focus on the 16S rRNA.

The first step in examining the microbial diversity of natural samples using phylogenetic methods is obtaining a representative collection of pure nucleic acids. There are two important objectives in this step; cell lysis and nucleic acid purification. All prokaryotic cells and spores should be lysed within the sample without extensively shearing the DNA. If certain cell types are not lysed, then the nucleic acid collection will be biased, thus distorting the microbial diversity [Wintzingerode *et al.*, 1997]. If the DNA is too fragmented, artifacts will be introduced when the nucleic acids are analyzed [Wintzingerode *et al.*, 1997]. The most effective methods for cell lysis seem to be SDS, hexadecyltrimethylammonium bromide and proteinase K treatments in high salt buffers at 70 °C or SDS treatment at 70 °C with subsequent bead mill homogenization (Kuske *et al.*, 1998; Zhou *et al.*, 1996). Once the cells are lysed, the nucleic acids can be phenol-chloroform extracted and/or ethanol precipitated by standard methods. The next important objective is to separate the nucleic acids from humic substances, since they interfere with most nucleic acid analyses. This objective has been achieved effectively by agarose gel electrophoresis and separation of nucleic acids from the agarose by a DNA-purification minicolumn (Zhou *et al.*, 1996). These methods are not as successful for RNA, which is highly susceptible to degradation by RNases. Extraction and purification of RNA has been most successful with lysozyme lysis and extraction with low pH-buffered hot phenol, followed by gel filtration in a Sephadex G-75 spin column (Moran *et al.*, 1993). Importantly, if all of the cells in the sample are not lysed or the

extracted nucleic acids are fragmented or impure, it will result in a biased analysis that does not necessarily represent that community.

Three basic methods have been used to recover ribosomal sequence information from extracted nucleic acids for phylogenetic analysis. The analysis of cloned PCR products has been used to determine microbial diversity in seawater [Benlloch *et al*., 1995; Britschgi and Giovannoni, 1991; DeLong *et al*., 1993; Field *et al*., 1997; Massana *et al*., 1997; Suzuki *et al*., 1997], deep subsurface sediments and water [Chandler *et al*., 1997; Pedersen *et al*., 1996], soil [Borneman *et al*., 1996; Felske *et al*., 1999; Dunbar *et al*., 1999; Hengstmann *et al*., 1999; Kuske *et al*., 1997; McCaig *et al*., 1999; Stackebrandt *et al*., 1993] and hot springs [Hugenholtz *et al*., 1998 (b)]. The shotgun cloning method, which involves directly cloning total DNA and screening for rDNA, has been used by Schmidt *et al*. [1991] to analyze the diversity of a marine picoplankton community. The analysis of cDNA libraries, which were synthesized from 16S rRNA, has been used to examine natural microbial communities in hot spring microbial mats [Ward *et al*., 1990; Weller *et al*., 1991; Weller and Ward, 1989]. Although it has been suggested that the shotgun cloning and cDNA library methods avoid potential PCR biases (see below), their application in community diversity studies has been limited. It is often difficult to obtain RNase-free RNA of high purity, which makes the cDNA library method unappealing for most. Screening for rDNA from thousands of recombinants obtained from total community DNA is also tedious, which makes the shotgun cloning technique inefficient. Since DNA extractions do not suffer from the same pitfalls as RNA extractions and rDNA can be obtained most efficiently by PCR amplification, the PCR method has been utilized most often.

There are several factors to consider when designing a scheme to amplify general 16S rDNA from mixed microbial communities. First, nucleic acid primers must be designed that will not introduce any unnecessary bias. Typically the primers designed for prokaryotic diversity studies are either universal (can amplify most eukaryotic and prokaryotic rDNA) or domain specific. A number of universal, bacterial and archaeal primers have been published [Lane, 1991]. Brunk *et al*. [1996] found that some published universal or domain specific primers had lower binding affinities for some specific groups, which was due to mismatched nucleotide binding between primer and template. Suzuki and Giovannoni [1996] have observed that a bias in amplification of mixed environmental DNA was dependent on the choice of primer pairs and the number of cycles of replication. The bias associated with some primers can be reduced by decreasing the number of replication cycles during PCR [Suzuki and Giovannoni, 1996]. Programs such as CHECK_PROBE in the RDP database [Maidak *et al*., 1999] are used to screen primer specificity when selecting or designing oligonucleotides for diversity studies. Primers are also selected based on the desired region of the 16S gene to be amplified. The amplified region usually contains at least one variable region, or region that is not highly conserved in all organisms. This is necessary to achieve an accurate measure of phylogeny. The primer pairs 27F and 1522R, 1492R or 1392R [Giovannoni, 1991; Lane, 1991] have been frequently used in recent studies [Benlloch *et al*., 1995; Chandler *et al*., 1997; Dunbar *et al*., 1999; Hengstmann *et al*., 1999; Hugenholtz *et al*., 1998 (b); Kuske *et al*., 1997; Pedersen *et al*., 1996; Suzuki *et al*., 1997]. The formation of chimeras during PCR amplification is another concern of using this method. Chimeras occur when two different homologous DNA sequences recombine in the reaction tube

causing the formation of PCR products that don't reflect the true community. Chimeras can be reduced by increasing the elongation time and reducing the number of cycles during PCR [Wang and Wang, 1986]. Programs, such as CHECK_CHIMERA [Maidak *et al*., 1999], are often used to detect chimeras in 16S gene libraries. Designing a PCR protocol to amplify representative prokaryotic rDNA from environmental samples requires careful consideration of every step.

The PCR amplified rDNA must be cloned and sequenced before it can be phylogenetically analyzed. Typically, commercial cloning kits, such as the TA cloning kit (Invitrogen Corporation, San Diego, Calif.), the pGEM-T cloning kit (Promega, Madison, Wis.), or pBluescript KSII- (Stratagene, La Jolla, Calif.) are used to clone the amplified DNA. Hundreds of clones typically result from a single ligation and transformation event from amplified DNA.

Since it is possible that the nucleic acids from some organisms are more abundant in the original sample than others, many of the 16S rDNA clones may be duplicates. Frequently, a screening step is useful to determine the number clones in the rDNA library that contain redundant information. These screening steps often increase the efficiency of the sequencing and phylogenetic analyses.

One common screening method is the restriction fragment length polymorphism analysis (RFLP). This involves digesting the DNA inserts from the clones with one or more tetrameric restriction enzymes and using gel electrophoresis to examine their banding patterns. Phylotypes, or clones that have similar DNA, will produce identical banding patterns. It has been reported that RFLPs obtained with 2 tetrameric enzymes have been used to represent organisms with a median genetic similarity of 95.6% [Moyer

et al., 1996]. This method has been used by many laboratories to screen their clonal libraries prior to sequencing [Britschgi and Giovannoni, 1991; Chandler *et al*., 1997; DeLong *et al*., 1993; Dunbar *et al*., 1999; Hugenholtz *et al*., 1998 (b); Massana *et al*., 1997; Suzuki *et al*., 1997]. Dunbar *et al*. found 498 phylotypes in a library of 801 clones, which dramatically decreased the number of clones that were actually sequenced and analyzed [Dunbar *et al*., 1999].

PCR amplified rDNA can also be analyzed by denaturing gradient gel electrophoresis (DGGE). This technique can be applied directly after the PCR step (it does not require cloning the DNA first). Using this technique, DNA fragments of the same length but of different sequences can be separated according to their melting properties. Double-stranded DNA amplified from the community is electrophoresed through a linearly increasing gradient of denaturants until it reaches conditions that cause the two strands to separate and cease moving. This creates a fingerprint of the community DNA that can be used to make conclusions about the microbial diversity. Each of the resulting bands can be extracted from the gel and sequenced. The application of this method in microbial ecology was first described by Muyzer *et al*. [Muyzer *et al*., 1993] and has been used extensively to examine microbial communities in a variety of environmental samples [Felske *et al*., 1999; Ferris *et al*., 1996; Henckel *et al*., 1999; Heur *et al*., 1997; MacNaughton *et al*., 1999; Oveas *et al*., 1997; Wise *et al*., 1999]. It has made the process of examining PCR amplified community DNA extremely efficient since it does not require a cloning step.

Once the representatives of each amplified phylotype or DNA clone are sequenced they can be analyzed using phylogenetic analyses. Phylogenetic assignments

can be made by first comparing the 16S rDNA sequence to a database of previously retrieved sequences. The RDP has an on-line program called SIMILARITY_RANK [Maidak *et al*., 1999]. The Genetics Computer Group (GCG, Wisconsin, USA) and the National Center for Biotechnology Information (NCBI) both have programs called FastA and BLAST that can all be used to determine which sequences are most similar to the submitted sequence. These programs base their similarity rankings on number of conserved bases within the two sequences and the total number of bases being compared. Frequently, sequences that are most similar to environmental clones are clones from other environmental libraries and are not classified. Phylogenetic trees based on DNA distance can be constructed to determine if a clone is related to a previously described taxonomic group. A computer software package called Phylip [Felsenstein, 1993] is typically used for this task. This software requires a file containing an alignment of the DNA sequences from the clones and known organisms. Phylip uses algorithms, such as Jukes Cantor [Jukes and Cantor, 1969], to determine distance and others, like Neighbor-joining [Saitou and Nei, 1987] and Fitch-Margoliash [Fitch and Margoliash, 1967], to determine branching order within the trees. Algorithms used in phylogenetic analyses have been reviewed by Hillis *et al*. [Hillis *et al*., 1993]. If a clone clusters within a taxonomic group of related sequences, then that information can be useful for determining the community structure of that environment. Likewise, if a clone clusters within group of organisms that have a trait in common, then it is possible to infer some functional properties of the microorganism that gave rise to the clone. However, the ability to classify a clone phylogenetically is limited by the amount of sequence used in the analysis and by the

number of described organisms available for comparison. Frequently, there is not enough information to place a clone in a phylogenetic group.

2.4 Polyphasic Approach

Each approach described above for investigating microbial community diversity has an important limitation. The culture approach neglects a large portion of the microbial community or the unculturable portion. The fatty acid approach is limited in its accuracy, since there are a number of organisms whose fatty acid profiles are unknown. The problem with the molecular approach is that many of the detected sequences do not group with described organisms, which makes it difficult to draw conclusions about the physiology of the organisms. The best strategy to obtain a more comprehensive dataset is to use a polyphasic approach, which combines molecular, fatty acid, and/or isolate data of the same environment.

2.5 Diversity Indices

In addition to inferring diversity from phylogenetic and descriptive data, microbial diversity can also be measured using diversity indices. Traditionally, all biological diversity measurements are based on the concept of species richness and evenness [Krebs, 1989]. Richness refers to the number of species present. Evenness refers to the distribution of individuals within the species designations. Since it is sometimes difficult to determine the number of species occurring in a habitat using the three approaches described above, diversity indices have been used in prokaryotic diversity studies using a different concept [Buyer and Kaufman, 1996; Dunbar *et al*., 1999; Kaneko and Atlas, 1977; Mahaffee and Kloepper, 1997; McCaig *et al*., 1999; Zak *et al*., 1994]. These diversity measurements are based on the concept of an operational

taxonomic unit (OTU) or phylotype, ribotype or biotype. Using this concept, OTU richness and evenness can be measured by using a variety of formulas which were reviewed by Kennedy and Smith [1995]. Buyer and Kaufman [1997] used these indices to show that microbial diversity in the rhizosphere changes as the season changes. Many of these formulas can be affected by sample size. When comparing the microbial diversity in two different environments where the sample sizes are different, rarefaction should be used as an alternative or an additive formula [Hurlbert, 1971]. Rarefaction measures the expected number of OTUs in a random sub-sample of n individuals. This method was used by Dunbar *et al*. to show differences in richness of microbial communities in 4 soil types using data collected from clone libraries and culture collections [Dunbar *et al*., 1999]. Although diversity indices were created for eukaryotic organisms they have been useful for describing prokaryotic diversity.

3. Soil Diversity

Soil is the most diverse microbial habitat on earth. It contains more genera and species of microorganisms than any other habitat [Stotzky, 1997]. It has been estimated that approximately 4,000 different species of bacteria exist in one gram of soil [Torsvik *et al*., 1990]. Because of this great diversity and the importance of microbes in nutrient cycling and plant growth, soil microbial diversity has been well studied in the past two centuries.

The great diversity in soil microbial communities can be attributed to the fact that it is an extremely complex environment and consists of a multitude of microhabitats that have varying chemical and physical properties [Figure 3]. This heterogeneity can be understood if soil were visualized as a complex of aggregates or different sized mineral

particles, which vary in their chemical properties and associate with soil organic matter (SOM), gases, and water in a variety of ways. While some of these microhabitats in soil have low concentrations of available nutrients and/or little water, there are "hot spots" where nutrients and water are plentiful [Paul and Clark, 1996; Stotzky, 1997]. It is believed that microorganisms are most abundant in these "hot spots", because there is less environmental stress. Populations of microorganisms form guilds around these "hot spots" and, to a lesser extent, elsewhere. Within these guilds, organisms interact with each other in competitive and beneficial ways [Brock and Madigan, 1988]. Collectively, these guilds make up the microbial communities found in soil. Because of this complex network of interactions and associations within these microbial communities, there is great diversity.

Prior to the use of molecular techniques to observe prokaryotic diversity in soil, soil scientists relied solely on the examination of cultured populations. These traditional methods have shown that most of the culturable soil bacteria belong to the *Actinobacteria* division [Chin *et al*., 1999, Felske *et al*., 1999; Goodfellow *et al*., 1968; Kuster *et al*., 1968; Paul and Clark, 1996; Mahaffee and Kloepper, 1997]. *Actinobacteria* is a metabolically diverse group of organisms. About 90% of all *Actinobacteria* cells isolated from soil are of the genus *Streptomyces*. These organisms are saprobic and many are capable of degrading recalcitrant substrates abundant in soil, such as lignin, chitin, pectin, keratin and complex aromatics and humic acids. *Arthrobacter* is another genus of *Actinobacteria* found commonly in soil. These organisms can degrade a variety of organic molecules, including herbicides and pesticides [Paul and Clark, 1996]. Although they are not as abundant as the *Actinobacteria*, low G + C gram positive bacteria, such as

the *Clostridium* spp. and *Bacillus* spp. have also been frequently found in soil [Chin *et al*., 1999; Felske *et al*., 1999; Mahaffee and Kloepper, 1997; Paul and Clark, 1996]. It is believed that these organisms survive well in soil, because they are capable of forming spores. Spore formation allows these cells to survive throughout periods of stress or low nutrient availability [Paul and Clark, 1996]. Some gram negative bacteria such as, α-, β- and γ- Proteobacteria are also frequently found in soil using culture techniques. Their presence is likely due to their ability to grow on a wide range of substrates [Paul and Clark, 1996].

The use of molecular methods has introduced a different picture of soil prokaryotic diversity. The bacterial divisions that are detected frequently in most soils using molecular methods are the α-*Proteobacteria*, *Actinobacteria, Acidobacterium,* and *Verrucomicrbium*. Although the α-*Proteobacteria* and *Actinobacteria* divisions have been observed in soil using culture methods, the *Acidobacterium* and *Verrucomicrobium* groups typically are not. These are newly recognized divisions that contain only a few cultured and described members. Not much is known about their physiology. Members of the *Euryarchaeota* and *Crenarchaeota* divisions have also been detected in soil [Buckley, *et al*., 1998; Grobkopf, *et al*., 1998] using molecular techniques. Organisms within these two archaeal divisions have never been isolated from soil. Other divisions that are frequently found in soil using culture techniques, such as the β- and γ- Proteobacteria and low G + C gram positive, have also been observed using molecular techniques. However, molecular methods indicate that these taxa are not as abundant as expected from the culture studies. It is difficult to identify many of the prokaryotes in soil using only molecular methods, because the sequences that represent them frequently

have no similarity to any previously described division. Also, sequences that represent organisms that were previously isolated from the same environment are rarely found. The overall diversity of soil appears much greater when molecular methods are used. [reviewed by Hugenholtz *et al*., 1998]

The differences in prokaryotic diversity that occur when these two methods are used could be due to biases that are associated with each method. The culture methods are selective for prokaryotes that are capable of growing under a particular set of conditions. The molecular methods are biased by choice of PCR primers, DNA extraction methods, and PCR artifacts [Wintzingerode, *et al*., 1997]. However, it is generally accepted that the culture bias has a stronger influence and greatly underestimates the true diversity. This conclusion does not mean that the culture method has been abandoned. It is commonly used with molecular methods [Chin *et al*., 1999; Dunbar *et al*., 1999; Felske *et al*., 1999; Stackebrandt *et al*., 1993] to help identify the function of significant groups found in soil clone libraries. The widespread occurrence of the *Acidobacterium* and *Verrucomicrobium* groups in clone libraries is a perfect example. Their constant presence in soil suggests they are ecologically significant, but the limited amount of physiological information makes it difficult to make assumptions about their role. Although the culture method offers a biased view of prokaryotic diversity it is a necessary part of any ecological study.

4. Importance of Earthworms

It is well recognized that earthworms are important to plant litter decomposition and fertility of soil. As long ago as 1789, Gilbert White, a European naturalist, postulated the importance of earthworm burrowing and feeding on the fertility of soil

[White, 1877]. In 1881, Charles Darwin was the first to actually document increased plant litter decomposition due to earthworm activity [Darwin, 1881]. Since Darwin's observations, the role of earthworms in litter decomposition and fertility of soil has been well studied. It is now known that this role is complex, which involves the assistance of microorganisms. Before the relationship between earthworms and microorganisms can be explained, earthworm physiology, and eating and burrowing habits must first be described.

4.1 Earthworm Physiology and Eating and Burrowing Habits.

The influence of earthworms on soil processes and microorganisms is not uniform. It varies with their ecological categories. There are three categories of earthworms, epigeic, endogeic, and anecic [Brown, 1995; Bouché, 1977; Edwards and Bohlen, 1996]. Epigeic worms live just beneath the litter layer on the soil surface. They consume decomposing litter and soil. They do not live in permanent burrows. Some examples of epigeic species are *Eisenia fetida*, *Lumbricus rubellus* and *Lumbricus festivus*. Endogeic worms reside in extensive burrows in the mineral soil horizons. They consume more soil than epigeic and anecic worms. Therefore, their nutrition is derived from more humified material. Some examples of endogeic species are *Aporrectodea caliginosa*, *A. trapezoides*, *Pontoscolex corethrurus*, *Octolasion cyaneum*, and *O. lacteum*. Anecic worms live in permanent, vertical burrows, which open at the soil surface. They consume soil and decaying litter from the surface. They are typically larger than epigeic and endogeic worms and consume more soil than epigeics. Some examples of these species are *Lumbricus terrestris* and *Aporrectodea longa*. Since the diet and burrows of each of these three types of worms differ, their effects on soil

processes and microorganisms also differ [Brown, 1995; Bouché, 1977; Edwards and Bohlen, 1996].

4.1a. Digestion

The digestive systems of different species of earthworms differ in detail, but their basic structure and function are common. The digestive system is relatively simple, consisting of a buccal chamber, pharynx, esophagus, crop, gizzard, and intestine (Figure 4). The digestive process has been described in most detail for the worm *E. fetida* [van Gansen, 1963; Edwards and Bohlen, 1996]. Although the descriptions to follow will be based mainly on that species of epigeic worm, they can be applied to most other species. There is relatively little information on the specific enzymes used to digest the variety of different materials that pass through the digestive tract. This review will cover the ones that are known.

The foregut, or the reception zone, of the earthworm consists of the buccal cavity, pharynx and esophagus. Materials enter the buccal cavity, which is flanked by a prostomium, and is passed to the pharynx. The pharynx is often not differentiated from the buccal cavity. The pharynx contains muscles and glands that are stimulated by sensory structures that detect the presence of food. The muscles in the pharynx act as a suction pump, which pull the materials down the pharynx to the esophagus [Edwards and Bohlen, 1996; Edwards and Fletcher, 1988].

The pharyngeal glands secrete mucus, which is a water-soluble mixture of amino acids and low molecular weight glycosides and a glycoprotein [Martin et al., 1987]. Two purposes have been proposed for this mucus. One is to lubricate and protect the intestines [Lavelle, 1983]. The other purpose could be to act as a microbial priming

mechanism. The rich organic matter and high water content of the mucus may stimulate the microorganisms in the ingested materials, making them capable of metabolizing the organic matter, ingested by the worm, at an increased rate. The products of this external digestion are then absorbed in the posterior part of the alimentary canal [Lavelle and Gilot, 1994; Lavelle et al., 1995]. However, it is also believed that not all of the organic matter in the mucus is metabolized by microorganisms because the loss of energy would be greater than the energy derived from the soil organic matter (SOM) intake by the worm [Lavelle et al, 1995]. Therefore, some of this mucus is probably also reabsorbed in the intestine. An amylase is also present in this mucus and is probably used to break down some of the simple carbohydrates found in the ingested materials [Edwards and Bohlen, 1996; Edwards and Fletcher, 1988].

Calciferous glands in the esophagus secrete calcium carbonate coated in mucus. There are two possible explanations for the function of this secretion. One possibility is that it influences the pH of the intestinal fluid, making the pH neutral. Experimental removal of these glands results in lowering of the coelomic pH [Edwards and Bohlen, 1996; Robertson, 1936]. Another possibility is that it is used to rid the body of excess calcium, since many worms live in calcium rich soils [Edwards and Bohlen, 1996]. Regardless of their functions, these secretions probably affect the microorganisms in the earthworm digestive tract.

Once the ingested material leaves the esophagus it passes to the midgut, or the secretory zone, of the earthworm. This zone contains the crop and gizzard and the anterior intestines. The crop is a thin-walled storage chamber, which is situated in front of the gizzard. The folds of the crop regulate the rate of movement of material into the

gizzard, prevent regurgitation, and mix the food. The gizzard is a thick-walled, muscular chamber, which is used to grind the food and pump the materials into the anterior intestine. Food is moved along to the anterior intestine by contraction of a sphincter of circular muscles [Edwards and Fletcher, 1988].

Most of the digestive enzymes are found in the mid-gut. One amylase and two proteases are known to be active in the crop and gizzard. It is uncertain whether these are secreted by the earthworm or microorganisms within the alimentary canal of the earthworm [van Gansen, 1962]. A cellulase and a chitinase are found in the anterior intestine [Tracy, 1951; Parle, 1963]. It is uncertain whether these enzymes are secreted by the worm or by microorganisms in the digestive tract. Cellulase, which is normally present in *P. corethrurus*, was not detected in the gut tissues in the absence of microorganisms [Zhang et al., 1993], suggesting that cellulase is secreted by microorganisms. Parle [1963] suggested that most of the cellulase was probably not secreted by microorganisms, because cellulase activity was detected in gut contents of *A. longa, L. terrestris*, and *A. calignosa* after rinsing the digestive tract with water. Since it is unlikely that all of the bacteria were eliminated from the digestive tract and any cellulases secreted by bacteria may have been absorbed by the alimentary canal tissues, the bacterial origin of the enzymes cannot be ruled out by Parle's work. It is also possible that microorganisms secrete the chitinase. Parle [1963] showed that there is a ten-fold increase in chitin-degrading bacteria from the foregut to the hindgut. Because of the diverse nature of the food of earthworms, it is likely that a variety of other enzymes are present in the digestive tracts of different worms. It is uncertain, however, if the earthworms or the microorganisms inside of the worms are the source of the enzymes.

The last zone of the earthworm digestive tract, which contains the remainder of the intestine, is called the hindgut or absorption zone. Food is moved through the tube-like intestine by rhythmic expansions and contractions, which are influenced by the body wall movements during locomotion [Arthur, 1965]. The intestine is lined with a peritrophic membrane, which is excreted with the undigested material, or cast [Edwards and Bohlen, 1996]. The details of the absorption of nutrients are unknown. However, it is known that water and mucus, which were abundant throughout the fore- and mid-gut are less abundant in the hindgut, suggesting that absorption occurs [Edwards and Bohlen, 1996; Edwards and Fletcher, 1988; Lavelle et al., 1995].

4.1b. Excretion

The principal excretory organs for earthworms are nephridia (Figure 4). Nephridia extract waste material from the coelomic fluid, in which they lie. All nitrogenous waste of earthworms is in the form of urea or ammonia. The waste is excreted in two ways. Approximately one half of it is excreted through the body wall as mucus. This is significant for earthworms that have permanent burrows (anecic), because the mucus accumulates within the walls of the burrow. This nitrogen-rich mucus probably affects the soil microbial community. The mechanism of excretion of the other half of the nitrogenous waste is unclear. Eventually it ends up in the alimentary canal as mucus, and it is deposited with the cast material. So in addition to the organic matter that is ingested and processed, the digestive tract also contains nitrogenous waste [Edwards and Bohlen, 1996].

4.2a. Role of Microorganisms in the Earthworm

There is some debate as to the role of microorganisms in the digestive tract of earthworms. One possibility is that the earthworms are simply digesting the microorganisms. Another possibility is that there is a mutualistic relationship between microorganisms and worms. Experimental and circumstantial evidence exists for both scenarios. The explanations seem to depend on the species of worm and the type of microorganisms involved.

Increase in the number of microorganisms throughout the digestive tract of the earthworm suggests that earthworms are not eating the microorganisms. There is a large amount of evidence that bacteria multiply within the alimentary canals of most worms and are probably not digested. There is also evidence that fungi, algae, protozoa and nematodes are actually reduced in number during passage and may be very important in the diet of earthworm [Yeates, 1981].

Most studies have shown that numbers of bacteria are much higher in fresh casts (0 to 9 hours old) or posterior digestive tract contents from worms with long retention times (6-20 hours) as compared to soil [Daniel and Anderson, 1992; Fischer et al., 1995; Karsten and Drake, 1995; Kristufek et al., 1992; Kristrufek et al., 1993; Parle, 1963; Pedersen and Hendriksen, 1993; Schönholzer, et al., 1999; Wolter and Scheu, 1999]. A few studies have shown conflicting results, however. Tiunov and Scheu [2000] showed that there was no difference in bacterial numbers from casts and ingested material (soil and litter). However, since the casts were at least 1 day old, this study is not really comparable to the others. Likewise, Kristufek et al. [1992] showed that numbers of bacteria in the posterior digestive tracts of *A. caliginosa* were nearly the same as soil.

Since this earthworm has a gut retention time of only 1 hour [Scheu, 1992], there may not be enough time for bacterial multiplication. The fact that bacterial numbers either increase or remain the same suggests that bacteria are not a likely food source for earthworms.

It is possible that earthworms eat some eukaryotic microorganisms, such as species of fungi, yeast, protozoa and nematodes. A decrease in the hyphal lengths or numbers of fungi or yeast after passage through the digestive tract of earthworms has been observed [Domsch and Banse, 1972; Parle, 1963; Dash et al., 1980; Kozlovskaya and Zhdannikova, 1961]. *L. terrestris* worms actually preferentially ingest certain species of fungi, such as *Fusarium oxysporum*, *Alternaria solani*, and *Trichoderma viride* over others (*Cladosporium cladosporoides, Poronia piliformis, Chaetomion globosum* and *Penicillium digitatum*) [Cooke, 1983]. The extent to which earthworms select for different fungal species is not well established, however. Numbers of algae, such as cyanobacteria and diatoms generally decrease during passage through the earthworm digestive tract [Atlavinyté and Pocené, 1973], suggesting that algae is also significant in the earthworm diet. Atlavinyté and Pocené [1973] also showed that earthworms grow best in soil with green algae and cyanobacteria. Free living protozoa may be a necessary requirement in the diet of some earthworms. In some experiments, earthworms actually grow better when fed protozoa [Flack and Hartenstein, 1984; Miles, 1963]. Yeates [1981] observed that nematode populations decreased by 66% in the presence of earthworms, suggesting that worms also digest nematodes. Although earthworms probably do not utilize bacteria as a food source, there is evidence that they do eat fungi, yeast, algae, protozoa, and nematodes.

It is uncertain why earthworms digest microorganisms, such as fungi, algae, yeast, protozoa, and nematodes. Earthworms cannot synthesize tryptophan and methionine, and these amino acids are not very abundant in the soil or litter that earthworms ingest. Therefore, it is believed that microorganisms are an important sources of these essential amino acids for earthworms [Pokarzhevskii et al., 1997]. It is possible that earthworms could be digesting microorganisms to obtain these amino acids. There have been no other explanations for the importance of microorganisms in the nutrition of worms.

It has been proposed that earthworms have a mutualistic relationship with microorganisms [Barois and Lavelle, 1986; Lavelle et al., 1995; Trigo and Lavelle, 1993]. Two observations have been made that suggest that this relationship exists. First, it has been shown repeatedly that microbial respiration is enhanced in ingested materials after passage through the digestive tract [Binet et al., 1998; Daniel and Anderson, 1992; Scheu, 1987; Tiunov and Scheu, 2000; Trigo and Lavelle, 1993; Winding et al., 1997]. The conditions in the anterior portion of the alimentary canal, such as high water content, neutral pH, and a readily assimilable organic matter (mucus), are suitable for increased microbial activity [Barois and Lavelle, 1986; Barois, 1992]. Therefore, it seems natural that microorganisms would benefit from these conditions. The second observation is that some of these microorganisms contain enzymes, such as chitinase [Parle, 1963], that break down complex organic matter into simple carbohydrates that can be assimilated by the worm. However, the benefits from this type of activity have not been clearly demonstrated. Although more evidence is needed to elucidate the details of this mutualistic relationship between earthworms and microorganisms, it seems to be a clear possibility (Figure 5).

4.2b. General Effects of Burrowing on Microorganisms

Since one half of the nitrogenous waste of an earthworm is excreted through the body surface, it presumably accumulates in earthworm burrows, or drilosphere, and affects the soil microbial community in those areas (Figure 3). All studies that have examined these effects have been performed with *L. terrestris*, which forms permanent burrows. Therefore, the burrows are easily recognized and the effects are probably more dramatic.

There are fewer studies that look at the effects of burrows on the microorganisms. Generally, the effects on microorganisms in the drilosphere are similar to those found for the earthworm digestive tract. Polyanskaya and Tiunov [1996] showed that fungal hyphae were less dense and bacteria were more abundant in the drilosphere than bulk soil. Another study showed that nematodes, microbial respiration, and inorganic nitrogen were more abundant in the drilosphere when compared to bulk soil, and microbial biomass C was lower [Gorres et al., 1997]. There have been no assumptions made about the role of microorganisms in the drilosphere. However, the above results suggest that the earthworm excretion products do influence the microbial community.

4.3a. Microorganisms Found in the Earthworm Digestive Tract or Fresh Casts

It seems likely that the digestive tract of the earthworm, with its rich organic matter, moisture content, and neutral pH, would contain a different microbial community structure than ingested materials (soil, dung or litter). One of the earliest studies that looked at bacterial communities inside earthworm digestive tracts and fresh casts suggested that this was not the case. Bassalik isolated more than 50 species of bacteria from *L. terrestris* digestive tract and cast samples and found the same bacteria in soil

[Bassalik, 1915]. Some of the recent studies, however, have found that the gut flora is different from that of soil and/or ingested material [Contreras, 1980; Fischer, et al., 1995; Jolly et al., 1993; Karsten and Drake, 1997; Karsten and Drake, 1995; Kristufek et al., 1993; Marialigeti, 1979; Pedersen and Hendriksen, 1993]. Four explanations for the differences have resulted from these studies. (1) The alimentary canal may be stimulating or reducing the growth of select organisms. (2) The worms may be feeding selectively. (3) The alimentary canal may be conducive for spore germination as demonstrated by Fischer et al. [1997]. (4) The culturability of the organisms may be increased after passage through the alimentary canal. These explanations are discussed below with respect to experimental data.

Using whole cell hybridization Fischer et al. [1995] were able to show that gram-negative proteobacteria increase in number between the foregut and the hindgut sections of earthworms. The α-, β- and γ- proteobacteria increased 2-, 4-, and 28- fold, respectively. Because the bacterial numbers were based on dry weight of the gut contents, these increases were not due to an increase in particle concentration after fluid absorption in the hindgut. Since this study did not look at abundance of these bacteria in soil surrounding the worms it is unknown if selective feeding played any role. It is possible that the increase in bacteria could have resulted from activation of some dormant bacteria. Dormant bacteria usually have a low copy number of DNA, which means the fluorescent probe would not be detected easily in dormant cells. Stimulation of the growth of proteobacteria in the earthworm gut is always a possibility as well.

In another experiment, Pedersen and Hendriksen [1993] observed the effects of gut passage on some select gram negative organisms. They kept detritivore worms in

pots with soil and dung that were inoculated with viable gram negative bacteria *Pseudomonas putida, Escherichia coli, Enterobacter cloacae,* and *Aeromonas hydrophila*. They found that the numbers of culturable *E. coli* and *P. putida* decreased significantly after passage through the earthworm pharynx and esophagus and either increased slightly or maintained constant numbers from the crop to the hindgut. The numbers of culturable *A. hydrophila* remained about the same in all areas of the digestive tract. This was expected, since *A. hydrophila* has been isolated from earthworm feces on a few occasions [Hendriksen, 1995; Marks and Cooper, 1977; Ramteke and Gopal, 1991]. The numbers of *E. cloacae* dropped 10,000-fold after passage through the pharynx, and increased 10,000-fold in the hindgut [Pedersen and Hendriksen, 1993]. The reduction of some of the bacteria suggests that there is a selection process (selective digestion or adverse environmental conditions for some bacteria) occurring within the pharynx that disappears in the mid- and hind- gut sections.

Ramteke and Gopal [1991] found that *A. hydrophila* constituted 70% of isolates found in the worm *Pheretima posthuma*. Marialigeti [1979] found that 75% of the bacteria isolated from the digestive tract of *Eisenia. lucens* were related to the gram-negative genus, *Vibrio*, which is very similar to *Aeromonas*. The experiments of Marialigeti and Ramteke and Gopal are significant, since *Aeromonas* and *Vibrio* spp. are typically not isolated from soil that is not fertilized with dung. Therefore, their presence in the worms cannot be explained by selective feeding. Also, due to the fact that these bacteria are not spore formers, increased spore germination in the digestive tract does not explain these results. This stimulation of *Aeromonas* and *Vibrio* in the worm casts

suggests that the gut environment is either selective for certain groups of microorganisms or enables increased culturability.

Proportions of populations of some gram-positive, culturable organisms are also different inside the earthworm digestive tract as compared to soil. Kristufek et al. [1993] isolated actinomycetes from soil and *L. rubellus* digestive tracts. They found a higher density of *Micromonospora* strains in the digestive tract of *L. rubellus* (13%) than in soil (1%). This study also showed that many of the actinomycete strains that are found in soil are not found in the digestive tract [Kristufek et al., 1993]. Contreras [1980] also isolated actinomycetes from the digestive tract of *E. lucens*. A single species (*Streptomyces lipmanii*) dominated the flora of this worm. This observation was interesting since *S. lipmanii* is relatively uncommon to soil or the rotting wood that these worms eat. The results of this study do not favor the selective feeding hypothesis, since *S. lipmanii* is not abundant in ingested material. Since none of these studies actually enumerated the organisms in different regions of the alimentary canal, it is difficult to distinguish whether or not enrichment or increased culturability explains the differences between the bacteria in the digestive tract and ingested materials without further evidence. Also since the actinomycetes in these studies are spore formers it is possible that increased spore germination in the digestive tract accounts for the changes.

There is also some evidence that the earthworm digestive tract may be enriched in microbes capable of anaerobic growth and activity. The anaerobic potential of the earthworm digestive tract has been poorly explored. However, it makes sense that a closed system, like that of the earthworm alimentary canal, would contain some regions where oxygen was below normal levels. Karsten and Drake [1995] found that, on a dry

weight basis, the ratio of bacteria capable of growth under anaerobic conditions to those under aerobic conditions was higher in worm gut homogenates than soil. In another study they found that under anaerobic and aerobic conditions gut homogenates supplemented with nitrate (NO_3) produced nitrous oxide (N_2O) at rates exceeding that of soil homogenates. The rates of N_2O production decreased by 80% when the homogenates were preincubated with antibiotics [Matthies et al., 1999], suggesting that the N_2O is coming from microorganisms. Some anaerobic and many facultative anaerobic bacteria use NO_3 as a terminal electron acceptor and reduce it to N_20. It is quite possible that the digestive tract of the earthworm has anaerobic regions that act to select for nitrate-reducing bacteria. A previous study, which showed earthworm casts produced five times higher N_2O than unfertilized soil [Elliot et al., 1990], suggests that the nitrate reducing bacteria from earthworms may have an impact on nitrogen transformations in soil.

In addition to the changes that occur in microbial communities as they pass through the earthworm digestive tract, there is evidence that the alimentary canal may contain an indigenous micro-flora. Scanning and transmission electron microscopy of the alimentary canal wall of *O. cyaneum* and *L. terrestris* showed that there are many bacteria that seem to be attached to the alimentary canal wall via a mucopolysaccharide-like material [Jolly et al., 1993]. One of these attached bacteria had a specific morphology that has been observed in other vertebrate and invertebrate digestive tract microflora [Bruorton et al., 1991; Harris et al., 1991; Klaasen et al., 1991; Klug and Kotarski, 1980]. These bacteria, which have been described as segmented filamentous bacteria, were seen attached to the epithelium in the hindgut by a “socket-like” structure

[Jolly et al., 1993]. The permanence or transience of these organisms is unknown, however it is possible that they have a significant purpose since energy was expended to produce an attachment mechanism.

4.3b. Microorganisms Found in Earthworm Burrows

Another way in which earthworms may influence microorganisms is in their burrow walls. Specific microorganisms are also found to be more abundant in the drilosphere than bulk soil. There are fewer studies to support this claim. When bacteria were isolated from burrows by Bhatnagar [1975] 55% of them were capable of fixing nitrogen and 16% of them were denitrifying. Similarly, Parkin and Berry [1999] found elevated populations of nitrifying and denitrifying bacteria and increased rates of nitrification and denitrification in the drilosphere as compared to bulk soil [1999]. The excretion products that the earthworms secrete are nitrogen rich and are probably responsible for the elevated levels of nitrogen transformation in the drilosphere.

4.4. Conclusion

There is a great deal of information suggesting that earthworm activity changes the microbial community structure of soil. Some of the details of these structural changes are still unclear. For instance, future studies should focus on total microbial community structure rather than distinct taxa or physiological groups. There does not seem to be one simple explanation for the differences in microbial community structures of the digestive tract and worm habitat. These are dependent on the species of worm and the type of habitat. The microbial communities in the drilosphere have not been studied extensively. Perhaps this is due to the difficulty involved in sampling the burrow. More data about the drilosphere should be obtained before any precise conclusions can be made.

References

1. **Atlavinyté O., and C. Pociené**. 1973. The effect of earthworms and their activity on the amount of algae in the soil. Pedobiologia **13:** 445-455.
2. **Amann, R. I., W. Ludwig, and K. Schleifer**. 1995. Phylogenetic identification and In situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:** 143-169.
3. **Arthur, D.R.** 1965. Form and function in the interpretation of feeding in lumbricid worms. Viewpoints Biol. **4:** 204-251.
4. **Bakken, L.R. and R.A. Olsen.** 1987. The relationship between cell size and viability of soil bacteria. Microb. Ecol. **13:** 103-114.
5. **Barois, I.** 1992. Mucus production and microbial activity in the gut of two species of *Amynthas* from cold and warm tropical climates. Soil Biol. Biochem. **24:** 1507-1510.
6. **Barois, I., and P. Lavelle.** 1986. Changes in respiration rate and some physiochemical properties of a tropical soil during transit through *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta). Soil Biol. Biochem. **18:** 539-541.
7. **Bassalik, K.** 1913. Uber Silikatzersetzung durch bodenbakterien. Z. Gärungs-Physiol. **2:** 1-32.
8. **Beare, M.H., D.C. Coleman, D.A. Crossley Jr., P.F. Hendrix, and E.P. Odum.** 1995. Hierarchical approach to evaluating the significance of soil biodiversity to biogeochemical cycling. Plant and Soil **170:** 5-22.
9. **Benlloch, S., F. Rodríguez-Valera, A.J. Martinez-Murcia.** 1995. Bacterial diversity in two coastal lagoons deduced from 16S rDNA PCR amplification and partial sequencing. FEMS Microbiol. Ecol. **18:** 267-280.
10. **Bhatnagar, T.** 1975. Lombriciens et humification: un aspect nouveau de l'incorporation microbienne d'azote induite par les vers de terre, pp. 169-182. In G. Kilbertus, O. Reisinger, A. Mourey, and J.A. Cancela de Fonseca (ed.), Biodegradation et Humification. Pierron, Sarreguemines, France.
11. **Binet, F., L. Fayolle, and M. Pussard.** 1998. Significance of earthworms in stimulating soil microbial activity. Biol. Fertil. Soils **27:** 79-84.
12. **Borneman, J., P.W. Skroch, K.M. O'sullivan, J.A. Palus, N.G. Rumjanek, J.L. Jansen, J.Neinhuis, and E.W. Triplett**. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:** 1935-1943.
13. **Bossio, D.A., K.M. Scow, N. Gunapala, and K.J. Graham.** 1998. Determinants of soil microbial communitites: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microb. Ecol. **36:** 1-12.
14. **Bouché, M. B.** 1977. Soil organisms as components of ecosystems. Ecol. Bull. (Stockholm). **25:** 122-132.
15. **Britschgi, T.B. and S.J. Giovannoni.** 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. Appl. Environ. Microbiol. **57:** 1707-1713.
16. **Brock, T.D., and M.T. Madigan.** 1988. Biology of Microorganisms. Prentice Hall, Englewood Cliffs, New Jersey.
17. **Brown, G.G.** 1977. How do earthworms affect microfloral and faunal community diversity? Plant and Soil **170:** 209-231.

18. **Brunk, C.F., E. Avaniss-Aghajani, C.A. Brunk.** 1996. A computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. Appl. Environ. Microbiol. **62:** 872-879.
19. **Bruorton, M.R., C.L. Davis, and M.R. Perrin.** 1991. Gut microflora of vervet and samango monkeys in relation to diet. App. Environ. Microbiol. **57:** 573-578.
20. **Buckley, D.H., J.R. Graber, and T.M. Schmidt.** 1998. Phylogenetic analysis of nonthermophilic members of the kingdom *Crenarchaeota* and their diversity and abundance in soils. Appl. Environ. Microbiol. **64:** 4333-4339.
21. **Bull, A.T., M. Goodfellow, and J.H. Slater.** 1992. Biodiversity as a source in innovation in biotechnology. Annu. Rev. Microbiol. **46:** 219-252.
22. **Button, D.K., F. Schut, P. Quang, R. Martin, and B.R. Robertson.** 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures and initial results. Appl. Environ. Microbiol. **59:** 881-891.
23. **Buyer, J.S. and L.E. Drinkwater.** 1997. Comparison of substrate utilization and fatty acid analysis of soil microbial communities. J. Microbiol. Methods **30:** 3-11.
24. **Buyer, J. S. and D. D. Kaufman.** 1996. Microbial diversity in the rhizosphere of corn grown under conventional and low-input systems. Appl. Soil Ecol. **5:** 21-27.
25. **Cavigelli, M.A., G.P. Robertson, and M.J. Klug.** 1995. Fatty acid methyl ester (FAME) profiles as measures of soil micrbial community structure. Plant Soil **170:** 99-113.
26. **Chandler, D. P., S. Li, C. M. Spadoni, G. R. Drake, D. L. Balkwill, J. K. Fredrickson, and R. J. Brockman.** 1997. A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. FEMS Microbiol. Ecol. **23:** 131-144.
27. **Chin, K., D. Hahn, U. Hengstmann, W. Liesack, P.H. Janssen.** 1999. Characterization and identification of numerically abundant culturable bacteria from anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. **65:** 5042-5049.
28. **Contreras, E**. 1980. Studies on the intestinal actinomycete flora of *Eisenia lucens* (Annelida, Oligochaeta). Pedobiologia **20:** 411-416.
29. **Cooke, A.** 1983. The effects of fungi on food selection by *Lumbricus terrestris* L, 365-373. In J.E. Satchell (ed.), Earthworm Ecology. Chapman and Hall, New York.
30. **Daniel, O. and J.M. Anderson.** 1992. Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus Rubellus* Hoffmeister. Soil Biol. Biochem. **24:** 465-470.
31. **Darwin, C.R.** 1881. The formation of vegetable mould through the action of worms with observation on their habitats. Murray, London.
32. **Dash, M.C.** 1980. Nematode feeding by tropical earthworms. Oikos **34:** 322-325.
33. **Degens, B. P., J. A. Harris.** 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol. Biochem. **29:** 1309-1320.
34. **Delong, E.F., D.G. Franks, and A.L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:** 924-934.

35. **Demoulin, V.** 1979. Protein and nucleic acid sequence data and phylogeny. Science **205:** 1036-1039.
36. **Domsch, K.H. and H.J. Banse.** 1972. Mykologische untersuchungen am regenwurm exkrementen. Soil Biol. Biochem. **4:** 31-38.
37. **Dunbar, J., S. Takala, S.M. Barns, J.A. Davis, and C.R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65:** 1662-1669.
38. **Dykhuizen, D.E.** 1998. Santa Rosalia revisted: why are there so many species of bacteria? Antonie van Leeuwenhoek **73:** 25-33.
39. **Edwards, C. A. and P. J. Bohlen.** 1996. Biology of Earthworms. Chapman and Hall, London.
40. **Edwards, C.A. and K.E. Fletcher.** 1988. Interactions between earthworms and micro-organisms in organic-matter breakdown. Agric. Ecosyst. Environ. **24:** 235-247.
41. **Elliot, P.W., D. Knight, and J.M. Anderson.** 1990. Denitrification in earthworm casts and soil from pastures under different fertilizer and drainage regimes. Soil Biol. Biochem. **22:** 601-605.
42. **Felsenstein, J.** 1993. PHYLIP: phylogeny inference package. University of Washington, Seattle.
43. **Felske, A., A. Wolterink, R. van Lis, W.M. de Vos, A.D.L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:** 137-145.
44. **Ferris M.J., G. Muyzer, and D.M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62:** 340-346.
45. **Field, K.G., D. Gordon, T. Wright, M. Rappé, E. Urbach, K. Vergin, and S.J. Giovannoni.** 1997. Diversity and depth-specific distribution of sar11 cluster rRNA genes from marine planktonic bacteria. Appl. Environ. Microbiol. **63:** 63-70.
46. **Fischer, K., D. Hahn, R.I. Amann, O. Daniel, and J. Zeyer.** 1995. In situ analysis of the bacterial community in the gut of the earthworm *Lumbricus terrestris* L. by whole-cell hybridization. Can. J. Microbiol. **41:** 666-673.
47. **Fischer, K., D. Hahn, R.W. Hönerlage, and J. Zeyer.** 1997. Effect of passage through the gut of the earthworm *Lumbricus terrestris* L. on *Bacillus megaterium* studied by whole cell hybridization. Soil Biol. Biochem. **29:** 1149-1152.
48. **Fitch, W.M., and E. Margoliash.** 1967. Construction of phylogenetic trees. Science **155:** 279-284.
49. **Flack, F.M. and R. Hartenstein.** 1984. Growth of the earthworm Eisenia foetida on microorganisms and cellulose. Soil Biol. Biochem. **16:** 491-495.
50. **Foster, R. C.** 1988. Microenvironments of soil microorganisms. Biol. Fertil. Soils. **6:** 189-203.
51. **Fuhrman, J.** 1999. ASM Conference on Microbial Diversity. Chicago, IL.
52. **Garland, J. L.** 1996. Patterns of potential C source utilization by rhizosphere communities. Soil Biol. Biochem. **28:** 223-230.

53. **Garland, J. L. and A. L. Mills.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. **57:** 2351-2359.
54. **Gilbert, G.S., J.L. Parke, M.K. Clayton, and J. Handelsman.** 1993. Effects of an introduced bacterium on bacterial communities on roots. Ecology **74:** 40-854.
55. **Giovannoni, S.** 1991. The polymerase chain reaction, 177-203. *In* Nucleic Acid Techniques in Bacterial Systematics, E. Stackebrandt and M. Goodfellow (ed.). John Wiley & Sons Ltd., New York.
56. **Giovannoni, S.J., T.B. Britschgi, C.L. Moyer, and K.G. Field.** 1990. Genetic diveristy in Sargasso Sea bacteriaplankton. Nature (London) **345:** 60-63.
57. **Gonzalez, J.M., W.B. Whitman, R.E. Hodson, M.A. Moran.** 1996. Identifying numerically abundant culturable bacteria from complex communities: an example from a lignin enrichment culture. Appl. Environ. Microbiol. **62:** 4433-4440.
58. **Goodfellow, M., I.R. Hill, and T.R.G. Gray.** 1968. Bacteria in a pine forest soil, pp.500-515. *In* T.R.G. Gray and D. Parkinson (ed.), The Ecology of Soil Bacteria. University of Toronto Press, Toronto,
59. **Gorlach, K., R. Shingaki, H. Morisaki, and T. Hattori.** 1994. Construction of eco-collection of paddy field soil bacteria for population analysis. J. Gen. Appl. Microbiol. **40:** 509-517.
60. **Görres, J.H., M.C. Savin, and J.A. Amador.** 1997. Dynamics of carbon and nitrogen mineralization, microbial biomass, and nematode abundance within and outside the burrow walls of anecic earthworms (*Lumbricus terrestris*). Soil Science **162:** 666-671.
61. **Gosink, J.J., and J.T. Staley.** 1995. Biodiversity of gas vacuolate bacteria from Antarctic sea ice and water. Appl. Environ. Microbiol. **61:** 3486-3489.
62. **Griffiths, B.S., K. Ritz, N. Ebblewhite, and G. Dobson.** 1999. Soil microbial community structure: effects of substrate loading rates. Soil Biol. Biochem. **31:** 145-153.
63. **Grobkopf, R., S. Stubner, and W. Liesack**. 1998. Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl. Environ. Microbiol. **64:** 4983-4989.
64. **Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug.** 1994. Accuracy, reproducibility and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl. Environ. Microbiol. **60:** 483-2493.
65. **Harris, J.M., L.J. Seiderer, and M.I. Lucas.** 1991. Gut microflora of two salt marsh detritivore thalassinid prawns *Upogebia africana* and *Callianassa draussi*. Microb. Ecol. **21:** 277-296.
66. **Henckel, T., M. Friedrich, and R. Conrad.** 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol. **65:** 1980-1990.
67. **Hendriksen, N.B.** 1995. Effects of detritivore earthworms on dispersal and survival of the bacterium *Aeromonas hydrophila*. Acta Zool. Fennica **196:** 115-119.
68. **Hengstmann, U., K. Chin, P.H. Janssen, and W. Liesack.** 1999. Comparative phylogeneitc assignment of environmental sequences of genes encoding 16S

rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. 65: 5050-5058.

69. **Heuer, H., M. Krusek, P. Baker, K. Smalla, and E.M. Wellington.** 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. **63:** 3233-3241.
70. **Hillis, D.M., M.W. Allard, M.M. Miyamoto.** 1993. Analysis of DNA sequence data: phylogenetic inference. Methods in Enzymology **224:** 456-487.
71. **Höfle, M.G.** 1992. Identification of bacteria by low molecular weight RNA profiles: a new chemotaxonomic approach. J. Microbiol. Methods **8:** 235-248.
72. **Holben, W.E. and D. Harris.** 1995. DNA-based monitoring of total bacterial community structure in environmental samples. Molec. Ecol. **4:** 627-631.
73. **Hugenholtz, P., B.M. Goebel, and N.R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:** 4765-4774.
74. **Hugenholtz, P. D. Pitulle, K.L. Hershberger, and N.R. Pace.** 1998. Novel division level bacterial diversity in a yellowstone hot spring. J. Bacteriol. **180:** 366-376.
75. **Hurlbert, S.H.** 1971. The nonconcept of diversity: a critique and alternative parameters. Ecology. **52:** 577-586.
76. **Jackson, C.R., E.E. Roden, and P.F. Churchill.** 1998. Changes in bacterial species composition in enrichment cultures with various dilutions of inoculum as monitored by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **64:** 5046-5048.
77. **Jolly, J.M., H.M. Lappin-Scott, J.M. Anderson, and C.D. Clegg.** 1993. Scanning electron microscopy of the gut microflora of two earthworms: *Lumbricus terrestris* and *Octolasion cyaneum.* Microb. Ecol. **26:** 235-245.
78. **Jukes, T.H., and C.R. Cantor.** 1969. Evolution of protein molecules. pp. 21-132. *In* H.N. Munro (ed.), Mammalian protein metabolism. Academic Press Inc., New York.
79. **Kaneko, T. and R.M. Atlas.** 1977. Diversity of bacterial populations in the Beaufort Sea. Nature **270:** 596-599.
80. **Karsten, G.R. and H.L. Drake.** 1997. Denitrifying bacteria in the earthworm gastrointestinal tract and in vivo emission of nitrous oxide (N_2O) by earthworms. Appl. Environ. Microbiol. **63:** 1878-1882.
81. **Karsten, G.R. and H.L. Drake.** 1995. Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. Appl. Environ. Microbiol. **61:** 1039-1044.
82. **Kataoka, N., Y. Tokiwa, Y. Tanaka, K. Takeda, and T. Suzuki.** 1996. Enrichment culture and isolation of slow-growing bacteria. Appl. Microbiol. Biotechnol. **45:** 771-777.
83. **Kennedy, A.C. and K.L. Smith.** 1995. Soil microbial diveristy and the sustainability of agricultural soils. Plant and Soil **170:** 75-86.
84. **Klaasen, H.L.B.M., J.P. Koopman, M.E. Van den Brink, H.P.N. Van Wezel, and A.C. Beynen.** 1991. Monoassociation of mice with noncultivable, intestinal, segmented, filamentous bacteria. Arch. Microbiol. **156:** 165-180.

85. **Klug, M.J., and S. Kotarski**. 1980. Bacteria associated with the gut tract of larval stages of the aquatic cranefly *Tipula abdominalis* (Diptera:Tipulidae). App. Environ. Microbiol. **40:** 408-416.
86. **Kozlovskaya, L.S. and E.N. Zhdannikova.** 1961. The combined activity of earthworms and the microflora in forest soils. Doklady Akademii Nauk, SSSR **139:** 574-576.
87. **Krebs, C.J.** 1989. Ecological Methodology. Harper and Row, New York.
88. **Kristufek, V., K. Ravasz, and V. Pizl.** 1993. Actinomycete communities in earthworm guts and surrounding soil. Pedobiologia **37:** 379-384.
89. **Kristufek, V., K. Ravasz, and V. Pizl.** 1992. Changes in densities of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea calignosa*. Soil Biol. Biochem. **24:** 1499-1500.
90. **Kuske, C.R., K.L. Banton, D.L. Adorada, P.C. Stark, K.K. Hill and P.J. Jackson.** 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. **64:** 2463-2472.
91. **Kuske, C.R., S.M. Barns, and J.D. Busch.** 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. . Appl. Environ. Microbiol. **63:** 3614-3621.
92. **Küster, E.** 1968. Taxonomy of soil actinomycetes and related organisms, pp. 322-336. In The Ecology of Soil Bacteria. University of Toronto Press, Toronto.
93. **Laguerre, G., M. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identifcation of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:** 56-63.
94. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Technology in Bacterial Systematics. John Wiley and Sons, New York.
95. **Lavelle, P. and C. Gilot.** 1994. Priming effects of macroorganisms on microflora: a key process of soil function, p. 173-180. *In* K. Ritz, J. Dighton, and K. Giller (ed.) Beyond the Biomass. Wiley-Sayce, UK.
96. **Lavelle, P., J. Kanyonyo, and R. Rangel.** 1983. Intestinal mucus production by two species of tropical earthworms: *Millsonia lamtoiana* (Megascolecidae) and *Pontoscolex corethrurus* (Glossoscolecidae), pp. 405-410. *In* P. Lebrun (ed.) New Trends in Soil biology. Dieu-Brichart, Louvain-la-Neuve.
97. **Lavelle, P., C. Lattaud, D. Trigo, and I. Barois.** 1995. Mutualism and biodiversity in soils. Plant and Soil **170:** 23-33.
98. **Liesack, W. and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bact. **174:** 5072-5078.
99. **Lindstrom, J. E., R. P. Barry, and J. F. Braddock.** 1998. Microbial community analysis: a kinetic approach to constructing potential C source utilization patterns. Soil Biol. Biochem. **30**: 231-239.
100. **Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney.** 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. **63**: 4516-4522.

101. **Mahaffee, W. F. and J. W. Kloepper.** 1997. Temporal chnages in bacterial communities of soil , rhizosphere, and endorhiza assoicated with field-grown cucumber (*Cucumis sativus* L.). Microb. Ecol. **34:** 210-223.
102. **Maidak, B.L., J.R. Cole, C.T. Parker Jr., G.M. Garrity, N. Larsen, B. Li, T.G. Lilburn, M.J. McCaughey, G.J. Olsen, R. Overbeek, S. Pramanik, T.M. Schmidt, J.M. Tiedje, and C.R. Woese.** 1999. A new version of the RDP (Ribosomal Database Project). Nucleic. Acids Res. **27:** 171-173.
103. **Mancuso, C.A., P.D. Franzmann, H.R. Burton, and P.D. Nichols.** 1990. Microbial community structure and biomass estimates of methanogenic Antarctic lake ecosystem as determined by phospholipid analysis. Microbiol. Ecol. **19:** 73-96.
104. **Marialigeti, K.** 1979. On the community-structure of the gut-microbiota of *Eisenia lucens* (Annelida, Oligochaeta). Pedobiologia **19:** 213-220.
105. **Marks, D.H. and E.L. Cooper.** 1977. *Aeromonas hydrophila* in the coelomic cavity of the earthworms *Lumbricus terrestris* and *Eisenia foetida*. J. Invert. Pathol. **29**: 382-383.
106. **Martin, A., J. Cortez, I. Barois, P. Lavelle.** 1987. Les mucus intestinaux de ver de terre, moteur de leurs ineractions avec la microflore. Rev. Ecol. Biol. Sol **24:** 549-558.
107. **Massana, R., A.E. Murray, C.M. Preston, and E.F. DeLong.** 1997. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara channel. Appl. Environ. Microbiol. **63:** 50-56.
108. **Matthies, C., A. Griebhanner, M. Schmittroth, and H.L. Drake**. 1999. Evidence for involvement of gut-associated denitrifying bacteria in emission of nitrous oxide (N_2O) by earthworms obtained from garden and forest soils. Appl. Environ. Microbiol. **65:** 3599-3604.
109. **McCaig, A.E., L.A. Glover, and J.I. Prosser.** 1999. Molecular anlaysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. **65:** 1721-1730.
110. **MacNaughton, S.J., J.R. Stephen, A.D. Venosa, G.A. Davis, Y. Chang, and D.C. White.** 1999. Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. **65:** 3566-3574.
111. **Miles, H.B.** 1963. Soil protozoa and earthworm nutrition. Soil Sci. **95:** 407-409.
112. **Moran, M.A., V.L. Torsvik, T. Torsvik, and R.E. Hodson.** 1993. Direct extraction and purification of rRNA for ecological studies. Appl. Environ. Microbiol. **59:** 915-918.
113. **Moyer, D.L., F.D. Dobbs, and D.M. Karl.** 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi seamount, Hawaii. Appl. Environ. Microbiol. **60:** 871-879.
114. **Moyer, C.L., J.M. Tiedje, F.C. Dobbs, and D.M. Karl.** 1996. A computer simulated restriction fragment length polymorphism anlysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl. Environ. Microbiol. **62:** 2501-2507.

115. **Muyzer, G., E.C. DeWall, and A.G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:** 695-700.
116. **Nold, S.C., E.D. Kopczynski, and D.M. Ward.** 1996. Cultivation of aerobic chemoorganotrophic proteobacteria and gram-positive bacteria from a hot spring microbial mat. Appl. Environ. Microbiol. **62:** 3917-3921.
117. **Nold, S.C. and D. M. Ward.** 1995. Diverse *Thermus* species inhabit a single hot spring microbial mat. System. Appl. Microbiol. **18:** 274-278.
118. **Nüsslein, K. and J.M. Tiedje.** 1998. Characterization of the dominant and rare members of a young hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. Appl. Environ. Microbiol. **64:** 1283-1289.
119. **Ovreas, L., L. Forney, F.L. Daae, and V. Torsvik.** 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. **63:** 3367-3373.
120. **Pace, N.R., D.A. Stahl, D.J. Lane, and G.J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences, p.1-55. *In* K.C. Marshall (ed.), Advances in Microbial Ecology. Plenum Press, New York.
121. **Parle, J.N.** 1963. Micro-organisms in the intestines of earthworms. J. Gen. Microbiol. **31:** 1-11.
122. **Parkin, T.B., and E.C. Berry**. 1999. Microbial nitrogen transformations in earthworm burrows. Soil biol. Biochem. **31:** 1765-1771.
123. **Paul, E.A., and F.E. Clark.** 1996. Soil Microbiology and Biochemistry, 2nd edn. Academic Press, San Diego.
124. **Pedersen, K., J. Arlinger, S. Ekendahl, and L. Hallbeck.** 1996. 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the access tunnel to the Äspö hard rock laboratory, Sweden. FEMS Microbiol. Ecol. **19:** 249-262.
125. **Pedersen, J.C. and N.B. Hendriksen.** 1993. Effect of passage through the intestinal tract of detritivore earthworms (*Lumbricus* spp.) on the number of selected gram-negative and total bacteria. Biol. Fertil. Soils **16:** 227-232.
126. **Polyanskaya, L.M., and A.V. Tiunov.** 1996. Microflora of the walls of galleries formed by the earthworm *Lumbricus terrestris*. Microbiol. **65:** 88-90.
127. **Pokarzhevskii, A.D., D.P. Zaboyev, G.N. Ganin, and S.A. Gordienko.** 1997. Amino acids in earthworms: are earthworms ecosystemivorous? Soil Biol. Biochem. **29:** 559-567.
128. **Rajendran, N., O. Matsuda, N. Imamura, Y. Urushigaway.** 1992. Variation in microbial biomass and community structure in sediments of eutrophic bays as determined by phospholipid ester-linked fatty acids. Appl. Environ. Microbiol. **58:** 562-571.
129. **Ramteke, P.W. and K. Gopal.** 1991. Microbial flora of gut of earthworm *Pheretima posthuma*. J. Adv. Zool. **12**: 66-67.

130. **Ritchie, N.J., M.E. Schutter, R.P. Dick, and D.D. Myrold.** 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Appl. Environ. Microbiol. **66:** 1668-1675.
131. **Robertson, J.D.** (1936). The function of the calciferous glands of earthworms. J. Exp. Biol. **13:** 279-297.
132. **Saitou, N. and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.
133. **Santegoeds, C.M., S.C. Nold, and D.M. Ward.** 1996. Denaturing gradient gel electrophoresis used to moniter enrichment culture of aerobic chemoorganotrohic bacteria from a hot spring cyanobacterial mat. Appl. Environ. Microbiol. **62:** 3922-3928.
134. **Scheu, S.** 1987. Microbial activity and nutrient dynamics in earthworm casts. Biol. Fertil. Soils **5:** 230-234.
135. **Schmidt, T.M., E.F. DeLong, and N.R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacterol. **173:** 4371-4378.
136. **Schönholzer, F., D. Hahn, and J. Zeyer**. 1999. Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studied by image analysis. FEMS Microbiol. Ecol. **28:** 235-248.
137. **Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59:** 2150-2160.
138. **Smith, G.A., J.S. Nickels, B.D. Kerger, J.D. Davis, S.P. Collins, J.T. Wilson, J.F. McNabb, D.C. White.** 1986. Quantitative characterization of microbial biomass and community structure in subsurface material: A prokaryotic consortium responsive to organic contamination. Can. J. Microbiol. **32**: 104-111.
139. **Stackebrandt, E., W. Liesack, and B.M. Goebel.** 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB. **7:** 232-236.
140. **Stackebrandt, E., and C.R. Woese.** 1981. The evolution of prokaryotes, p.1-31. *In* M.J. Carlisle, J.R.Collins, and B.E.B. Moseley (ed.), Molecular and Cellular Aspects of Microbial Evolution. Cambridge University Press, Cambridge.
141. **Stahl, D.A., D.J. Lane, G.J. Olsen, and N.R. Pace.** 1985. Characterization of a yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. **49:** 1379-1384.
142. **Stotzky, G.** 1997. Soil as an environment for microbial life, p. 1-20. In J.D. van Elsas, J.T. Trevors, and E.M.H. Wellington (ed.), Modern Soil Microbiology. Marcel Dekker, Inc., New York.
143. **Suzuki, M.T. and S.J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA gnes by PCR. Appl. Environ. Microbiol. **62:** 625-630.
144. **Suzuki, M.T., M.S. Rappe, and S.J. Giovannoni.** 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl. Environ. Microbiol. **64:** 4522-4529.

145. **Suzuki, M.T., M.S. Rappe, Z.W. Haimberger, H. Winfield, N. Adair, J. Strobel, and S.J. Giovannoni.** 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. **63:** 983-989.
146. **Tiunov, A.V. and S. Scheu.** 2000. Microbial biomass, biovolume and respiration in *Lumbricus terrestris* L. cast material of different age. Soil Biol. Biochem. **32:** 265-275.
147. **Torsvik, V., J. Goksoyr, and F.L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:** 782-787.
148. **Tracy, M.V.** 1951. Celluslase and chitinase in worms. Nature (London) **167:** 776-777.
149. **Trigo, D. and P. Lavelle.** 1993. Changes in respiration rate and some physiochemical properties of soil during gut transit through *Allolobophora molleri* (Lumbricidae, Oligochaeta). Biol. Fertil. Soils **15:** 185-188.
150. **Van Gansen, P.** 1962. Structure and function of the digestive canal of the earthworm Eisenia foetida Savigny. Annales de la Societe Royal Zoologique de Belgique **93:** 1-20.
151. **Van Leeuwenhoek, A.** 1677. Observations communicated to the publisher by Mr. Antony Van Leeuwenhoek in a Dutch letter of the 9th of October 1676 here english'd: concerning little animals by him observed in rain-well-sea and snow water; as also in water where in pepper had lain infused. Philosophical Transactions. **12**: 821-831.
152. **Voordouw, G., J.K. Voordouw, R.R. Karkhoff-Schweizer, P.M. Fedorak, and D.W.S. Westlake.** 1991. Reverse sample genome probing, a new technique for identification of bacteria in environmental samples by DNA hybridization, and its application to the identification of sulfate-reducing bacteria in oil field samples. Appl. Environ. Microbiol. **57:** 3072-3078.
153. **Wang, G.C.Y. and Y. Wang.** 1996. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology **142:** 1107-1114.
154. **Ward, D.M., R. Weller, and M.M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:** 63-65.
155. **Weller, R., and D.M. Ward.** 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. Appl. Environ. Microbiol. **55:** 1818-1822.
156. **Weller, R., J.W. Weller, and D.M. Ward.** 1991. 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. Appl. Environ. Microbiol. **57:** 1146-1151.
157. **White, G.** 1877. The Natural History of Selborne. T. Bell (ed.). Van Voost, London.
158. **Whitman, W.B., Coleman, D.C., and Wiebe, W.J.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. USA **95:** 6578-6583.
159. **Winding, A.** 1994. Fingerprinting bacterial soil communities using Biolog micrtitre plates, pp. 85-94. In K. Ritz, J. Dighton, and K.E. Giller (ed.).Beyond the Biomass. Wiley-Sayce, London.

160. **Winding, A., R. Rønn, and N.B. Hendriksen.** 1997. Bacteria and protozoa in soil microhabitats as affected by earthworms. Biol. Fertil. Soils **24:** 133-140.
161. **Wintzingerode, F. V., U. B. Gobel, E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:** 213-229.
162. **Wise, M. G., J.V. McArthur, and L.J. Shimkets.** 1999. methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. Appl. Environ. Microbiol. **65:** 4887-4897.
163. **Wolter, C. and S. Scheu.** 1999. Changes in bacterial numbers and hyphal lengths during the gut passage through *Lumbricus terrestris*. Pedobiologia **43:** 891-900.
164. **Yeates, G.W.** 1981. Soil nematode populations depressed in the presence of earthworms. Pedobiologia **22:** 191-195.
165. **Zak, J. C., M. R. Willig, D. L. Moorhead, and H. G. Wildman**. 1994. Functional diversity of microbial communities: a quantitative approach. Soil Biol. Biochem. **26:** 1101-1108.
166. **Zarda, B., H. Dittmar, A. Chatzinotas, W. Schönhuber, A. Neef, R.I. Amann, J. Zeyer.** 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch. Microbiol. **168:** 185-192.
167. **Zelles, L., and Q.Y. Bai.** 1994. Fatty acid patterns of phospholipids and lipopolysaccharides in environmental samples. Chemosphere **28:** 391-411.
168. **Zhang, B.G., C. Rouland, C. Lattaud, and P. Lavelle.** 1993. Origin and activity of enzymes found in the gut content of the tropical earthworm *Pontoscolex corethrurus* Muller. Eur. J. Soil Biol. **29:** 7-11.
169. **Zhou, J., M.A. Bruns, and J.M. Tiedje.** 1996. DNA recovery from soil of diverse composition. Appl. Environ. Microbiol. **62:** 316-322.

Figure 1. Flow diagram of the extraction and separation process of lipids and fatty acids [Zelles and Bai, 1994].

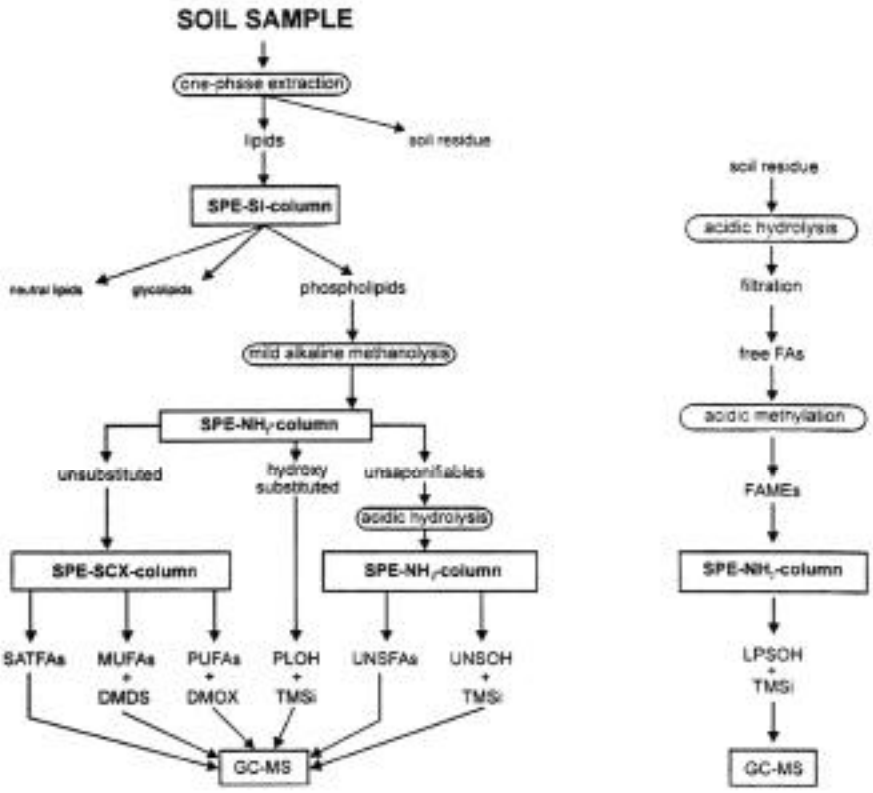

SOIL SAMPLE
one-phase extraction
lipids
soil residue
SPE-Si-column
neutral lipids
glycolipids
phospholipids
mild alkaline methanolysis
SPE-NH2-column
unsubstituted
hydroxy substituted
unsaponifiables
acidic hydrolysis
SPE-SCX-column
SPE-NH2-column
SATFAs
MUFAs + DMDS
PUFAs + DMOX
PLOH + TMSi
UNSFAs
UNSOH + TMSi
GC-MS
soil residue
acidic hydrolysis
filtration
free FAs
acidic methylation
FAMEs
SPE-NH2-column
LPSOH + TMSi
GC-MS

Figure 2. Flow chart showing the different methods used to analyze the 16S rRNA of an environmental sample. RT is reverse transcriptase. [Amann et al., 1995]

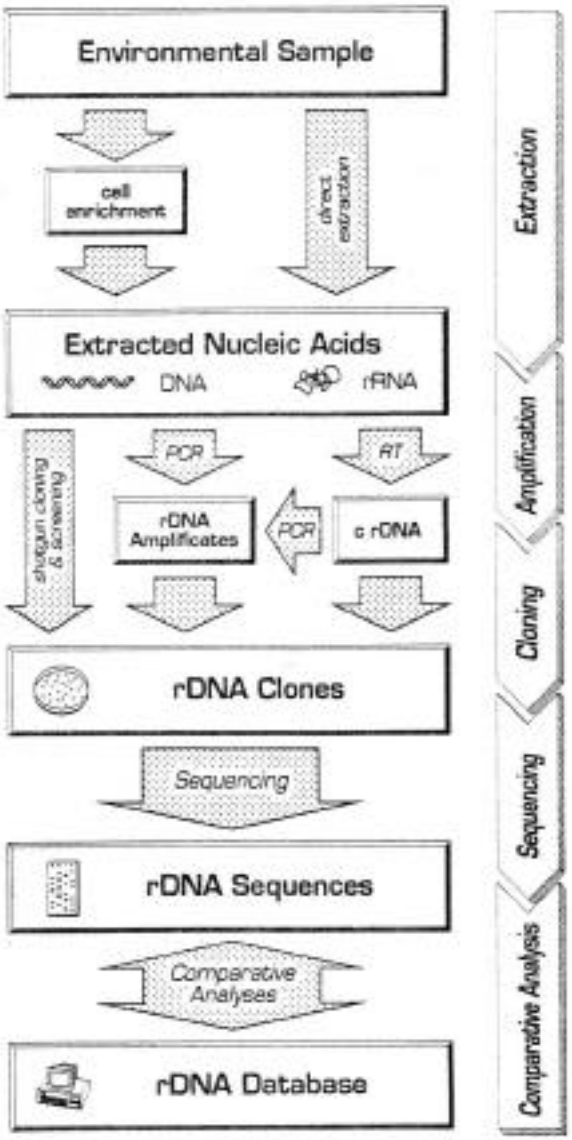

Environmental Sample
cell enrichment
direct extraction
Extracted Nucleic Acids
DNA
rRNA
PCR
RT
shotgun cloning & screening
rDNA Amplificates
PCR
c rDNA
rDNA Clones
Sequencing
rDNA Sequences
Comparative Analyses
rDNA Database
Extraction
Amplification
Cloning
Sequencing
Comparative Analysis

Figure 3. Micro-habitats found in soil [Beare et al., 1995].

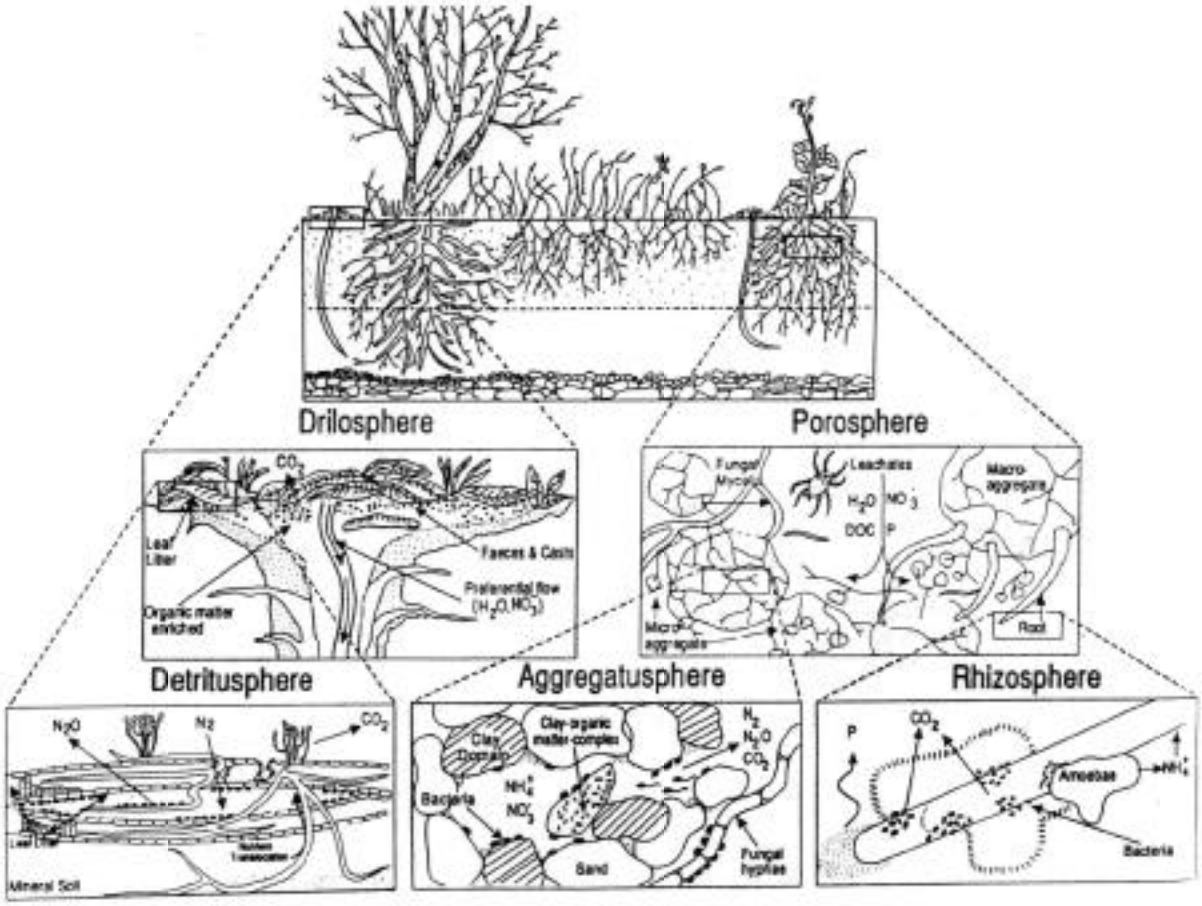

Drilosphere
Porosphere
Detritusphere
Aggregatusphere
Rhizosphere
Leaf Litter
Organic matter enriched
Faeces & Casts
Preferential flow
Fungal Mycelia
Leachates
Macro-aggregate
Micro-aggregate
Root
Clay-organic matter-complex
Clay Domain
Bacteria
Sand
Fungal hyphae
Amoebae
Mineral Soil

Figure 4. Diagram of earthworm digestive tract and nephridia. The structures that are not involved in digestion or excretion are not labeled [Edwards and Bohlen, 1996]

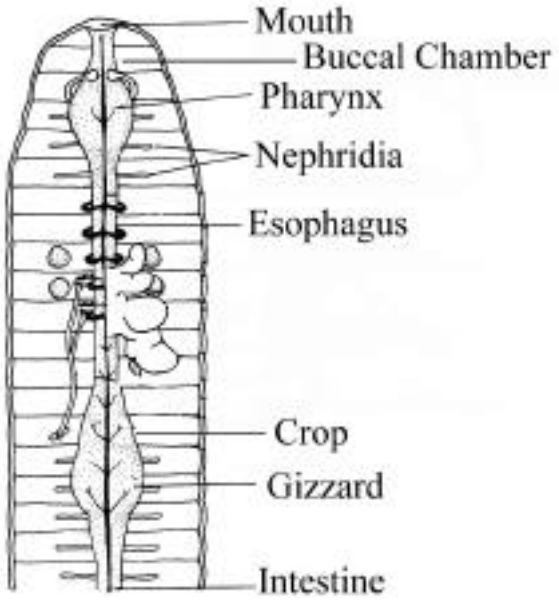
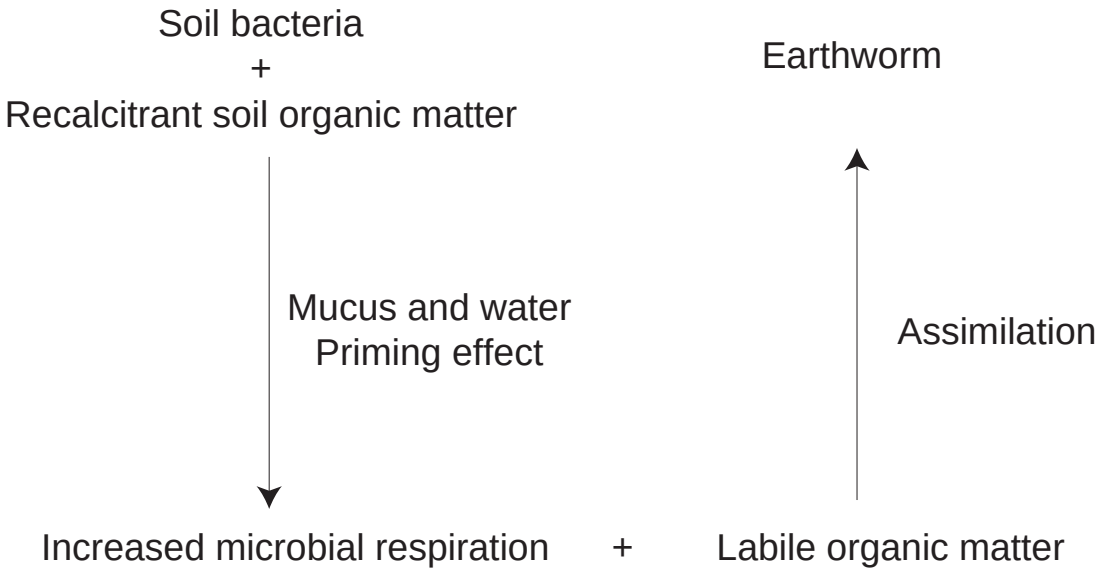

Mouth
Buccal Chamber
Pharynx
Nephridia
Esophagus
Crop
Gizzard
Intestine

Figure 5. Mutualist digestion systems of earthworms. Earthworms benefit from bacteria by assimilating the labile soil organic matter that is derived from recalcitrant organic matter (i.e. chitin and cellulose) that has been broken down by the bacteria (external digestion). Bacteria benefit from the rich organic matter and high water content in mucus secretions in the digestive tract of the earthworms. The stimulation of bacteria, which usually occurs in the foregut, is called the priming effect. [Barois and Lavelle, 1986; Lavelle at al., 1995].


Soil bacteria
+
Recalcitrant soil organic matter
Earthworm
Mucus and water
Priming effect
Assimilation
Increased microbial respiration
+
Labile organic matter

CHAPTER II

PHENOTYPIC COMPARISON OF BACTERIAL ISOLATES FROM FRESH EARTHWORM CASTS, BURROWS AND SURROUNDING SOIL[1]

[1] Furlong, M.A., D.C. Coleman, and W.B. Whitman. 2000. To be submitted.

Abstract

Bacterial isolates were obtained from fresh *Lumbricus rubellus* casts (n=74), earthworm burrows (n=31), and surrounding or bulk soil (n=97) using non-selective sampling and culturing methods in the spring of 1996 and 1997. Approximately 5-7% of the bacterial cells from the bulk soil were grown using these methods. The biotypes of the isolates were characterized using 17 physical and biochemical tests. The biotypes from fresh casts differed from those of the bulk soil and burrows, which were very similar. Unlike the isolates taken from bulk soil and burrow samples, many of the fresh cast isolates reduced nitrate, utilized acetate and Casamino acids, and failed to grow on any of the sugars tested. In contrast, the bulk soil and burrow isolates differed only in cellular morphology and ability to utilize lactose. Further analyses showed that fresh casts contained fewer biotypes and that those biotypes were more similar to each other than were those of the bulk soil or burrow isolates. Analysis of a smaller set of isolates obtained from casts and bulk soil in May of 1999 indicated that the biotypes of the bacteria from bulk soil were fairly constant while the biotypes of the culturable bacteria from casts changed over time.

Introduction

Earthworm activity profoundly affects soil in a number of ways. Earthworm burrowing creates pore spaces in soil, which increases water conductivity and benefits root growth [Edwards et al., 1990; Lee, 1985]. Earthworms break down larger soil particles and leaf litter, which increases the availability of organic matter for microbial degradation [Edwards and Bohlen, 1996]. They also promote macroaggregate formation

by depositing casts rich in glycoproteins, polysaccharides, bacteria and clay [Daniel and Anderson, 1992; Edwards and Bohlen, 1996; Foster, 1988; Tomlin et al., 1995]. Earthworms also affect soil profile formation by mixing litter layers with surface soil layers, which acts to deepen humus layers within soil [Edwards and Bohlen, 1996; Lee, 1985].

Previous observations suggest that the structure of the microbial communities within the drilosphere, i.e. earthworm casts and burrows, is different from that in the surrounding or bulk soil. Numbers of bacteria and microbial activity are generally enhanced in the drilosphere as compared to bulk soil [Barois, 1992; Daniel and Anderson, 1992; Edwards and Fletcher, 1988; Kristufek et al., 1992; Parle, 1963; Pedersen and Hendriksen, 1993; Tiunov et al., 1997; Trigo and Lavelle, 1993]. Also, a higher number of actinomycetes and *Vibrio* spp. are found in earthworm casts as compared to bulk soil [Contreras, 1980; Mariaglieti, 1979]. Karsten and Drake [1995] found more anaerobes and cellobiose-utilizers in earthworm guts than in soil. The differences in microbial activity, bacterial numbers and certain bacterial species between the earthworm gut or drilosphere and bulk soil indirectly support the hypothesis that the bacterial community structures of these habitats are different from that of the soil. This work further examines community differences between the drilosphere and the surrounding soil. An addtional goal of this work is to obtain a collection of isolates typical of the abundant, culturable organisms from fresh earthworm casts, burrows and surrounding soil. Phenotypic characterization of these isolates may then elucidate their roles and comparisons of diversity for this portion of the prokaryotic community.

Materials and Methods

Sampling. All samples were obtained from no-tilled agricultural plots at the Horseshoe Bend Long Term Environmental Research site, which is located in Athens, GA. This site is located on a river floodplain on Hiwassee series soil (fine loamy, siliceous, thermic, Rhodic Kanhapludult). Clover and rye have been the winter cover crops in the plots since 1978 [Barois, 1992; Groffman et al., 1986; Hendrix, 1997; Stinner et al., 1984]. Maize (*Zea mays* L.) was the summer crop in 1996 and 1997. Kenaf (*Hibiscus cannabinus*) and cotton (*Gossypium hirsutum*) were planted during the summer of 1998 and 1999, respectively. In April and May 1996 a shovel was used to expose fifteen soil shelves, approximately one meter in length and 10 cm in depth. Fifty-five soil samples were taken at various depths within the top 5 cm of these shelves. In May 1999 three soil shelves were made, and 15 soil samples were taken in the same manner. Sampling was performed by horizontally inserting a sterile Pasteur pipette 5 mm into the soil. Care was taken to avoid earthworm burrows and roots. These samples were defined as "bulk" soil. Upon removal from the soil, the tip of the pipette containing the soil sample was broken off into a microcentrifuge tube containing 0.5 ml of 0.85% w/v NaCl and crushed with a sterile inoculating stick. In a separate experiment the wet weight of fifty samples was measured, and the average wet weight was 5.2 mg (S.D. = 1.2 mg). Using the same methods, sixteen samples were also taken in March and April of 1996 from the sides of burrows found in the soil shelves or on the soil surface. All bulk soil and burrow samples were taken three or more days following the last rainfall. Samples were also taken from the fresh casts of 29 and 12 *Lumbricus rubellus* worms in April

1997 and May 1999, respectively. These worms were either collected from the soil surface after the application of an electrical current to the soil [Satchell, 1955] or from burrows exposed after excavation of soil shelves. The worms were rinsed with sterile water and placed in sterile petri plates containing a small amount of water. The plates were checked every two hours. When a cast was observed, it was processed as described above .

Bacterial Isolation. Each sample was vortexed for 30 seconds in 0.85% w/v NaCl to disperse bacteria and serially diluted to a factor of 10^{-9} in two types of media. Microscopic examination of soil suspensions after dispersal indicated that 50% of the bacteria were still attached to soil particles (1.09 cells/particle), and the remainder were suspended as single cells. To minimize settling during the serial dilution, each tube was vortexed vigorously before each dilution. The dilution and growth media were MMSA (1 mM K_2HPO_4, 2 mM NH_4NO_3, 1 mM $MgSO_4$, 1% v/v trace minerals [Whitman et al., 1986], 1% v/v iron solution [Whitman et al., 1986], 10% v/v soil extract and 10 mM sodium acetate) at a pH of 7.0 and DNB (50% v/v Difco Nutrient Broth [Difco, 1984]). The soil extract was prepared by the method of Stackebrandt and Prauser [1981], except that it was sterilized by filtration (0.2 µm pore size) rather than by autoclaving. Each bulk soil, burrow, and cast sample taken in 1996 and 1997 was diluted and grown in MMSA and DNB media. The samples taken in May 1999 were diluted and grown only in the MMSA medium. All samples were incubated in the dark at 25 ˚C. After two weeks, the highest dilutions showing growth in each medium were streaked onto solid medium of the same composition (MMSA or DNB with 1.5% w/v agar) for isolation of

pure cultures. A representative of each colony type on each plate was then subcultured in 0.5 ml of Difco Nutrient Broth. In 1999, however, only one colony per sample was selected. After turbidity became visible, 0.5 ml of sterile 15% v/v glycerol was added, and the culture was stored at -70˚ C.

Direct and viable counts. To obtain the viable counts (X), samples were taken from the bulk soil using the same sampling procedure used to obtain the isolates in June and August 1996, February 1997, and February and October 1998. Each sample was serially diluted in medium for preparation of a five-tube MPN. The tubes were incubated in the dark for two weeks at 25 ˚C. The MPN's were scored based on turbidity, according to the tables of de Man [1975]. Direct counts were obtained for the same samples prior to diluting in media for MPN counts. The samples were fixed in 1% w/v of glutaraldehyde and stained for 20 minutes with 4,6-diamidino-2-phenylindole (0.02 mg/ml). Fluorescent cells were counted at 1000X power using an Olympus BHS microscope with a BH2-RFC attachment and a V excitation cube. Free cells (F), single cells attached to particles (As), cells attached in clusters (Ac), and number of clusters (C) were scored. Assuming that 1/2 of the attached cells were not visible on the reverse sides of the particles and clusters, the total number of cells was claculated as $T = F + 2 (As + Ac)$. Then the percent culturability was calculated as $(X / T) \times 100$. The maximum number of culturable units was $V = F + As + C$ and the percent maximum culturable units was $(X / V) \times 100$.

Phenotypic characterization of isolates. Each isolate was cultured from frozen stocks on Difco Nutrient Agar [Difco, 1984] plates, and the colonial morphology was recorded. The presence of catalase was determined by a standard procedure [Smibert and Krieg, 1994]. Each isolate was then subcultured in undiluted Difco Nutrient Broth. The cellular morphology was observed when turbidity became visible. Motility was tested using Difco Motility Test medium and scored according to the Difco procedure [Difco, 1984]. Substrate utilization was tested in 5.0 ml of MMSA minimal medium, prepared without acetate and soil extract. Potential substrates (cellulose, acetate, lactose, Casamino acids, mannose, and cellobiose) were tested at a concentration of 0.2% (w/v). Test media were inoculated with 10 µl of culture and incubated for 14 days at room temperature. If the absorbance at 600 nm increased from 0.00 to ≥ 0.15, the culture was scored as positive for growth. Difco Nitrate reduction medium was inoculated and incubated similarly. It was scored according to the Difco procedure [Difco, 1984]. The solid media that were used included Difco Urea agar, Difco MacConkey medium, Difco Blood agar, Difco Simmon's Citrate, Casein Decomposition agar [Farrar and Reboli, 1981], and Tween Hydrolysis agar [Atlas, 1995]. The isolates were patched onto each plate and incubated at room temperature for 14 days. The media from Difco were scored according to their procedures [Difco, 1984]. The Tween Hydrolysis agar and the Casein Decomposition agar were scored according to standard procedures [Atlas, 1995; Farrar and Reboli, 1981]. For antibiotic resistance, each isolate was spread onto a Nutrient

Agar plate (Difco). Antibiotic discs (Difco) were dispensed onto the plate, and the results were scored according to the Difco procedure [Difco, 1984].

Statistics. The statistical analysis was performed with SAS [Parmacia Biotech, 1996]. A Chi-square test was used to determine if there was a significant difference in phenotypic properties between samples from the three sources. When the p-values were below 0.05, scaled deviance values were analyzed in the SAS genmod procedure to determine if the differences were due to the sample source (fresh cast vs. bulk soil vs. burrow) or the sampling protocol.

Dissimilarity values. Test results were scored as 0 or 1 (negative or positive, respectively) except for cellular morphology and growth on MacConkey's and Blood agar. For morphology, cocci, small rods, medium rods and large rods were scored as -1, 0, 1, and 2, respectively. For MacConkey's agar, no growth, growth without lactose fermentation, and growth with lactose fermentation were scored as 0, 1, and 2, respectively. For Blood agar, no hemolysis, alpha-hemolysis, and beta-hemolysis were scored as 0, 1, and 2, respectively. Dissimilarity values (Di) for every possible pair of isolates were then calculated using the formula $D = |A_i - A_j| + |B_i - B_j| + |C_i - C_j|...+...|Q_i - Q_j|$ (where A through Q refer to the different tests and subscripts i and j refer to each possible pair of isolates). The maximum possible dissimilarity value was 20 for the 17 phenotypic characters. Any two isolates that had a Di = 0 were considered to be of the same biotype.

Calculation of coverage. Coverage was calculated using Good's formula [Good, 1953]. For our purposes coverage = $[1 - (N / n)] \times 100$ (where N = number of biotypes

represented by one isolate and n = the total number of isolates). Coverage was considered to be the estimation of the percentage of all possible biotypes that could be cultured with our isolation procedure.

Lowest D (LDi) analysis. To determine if the biotypes of the May 1999 bulk soil and cast isolates were similar to those of the original bulk soil and cast isolates, LDi analysis was performed. In this analysis the control isolates were those that were collected in 1996 or 1997, and the test isolates were those that were collected in 1999. A matrix of Di values was constructed in Excel comparing the biotypes of the control isolates to those of the test collection. The lowest Di value for each test isolate was then determined. The mean of the lowest Di values for the test isolates in each group (bulk soil and cast) was then calculated. To determine the expectation for the mean, a matrix of Di values was first constructed in Excel comparing the biotypes of each group of control isolates to themselves. After the removal of Di values for comparisons of the isolates to themselves, the lowest Di value for each control isolate was determined. The means of the lowest Di values of 100 random selections equal in number to that of the test isolates were then calculated. The distribution of these means was also determined.

16S ribosomal DNA analysis. The 16S ribosomal DNA of twenty-six isolates from fresh casts were PCR amplified using Ready-To-Go PCR Beads [Pharmacia Biotech, 1996] and primers 27f, 5'-AGA GTT TGA TCM TGG CTC AG-3', and 1392r, 5'-ACG GGC GGT GTG TRC-3' [Lane, 1991]. Isolates were grown on Difco Nutrient Agar plates, and a single colony of each isolate was resuspended and washed two times in 100 µl of sterile distilled water. Each cell suspension was added to a

reaction mixture at a concentration of 8% (v/v) to act as the template DNA, and each reaction mixture was heated for 5 minutes at 94 ˚C. The DNA in the reactions was denatured, extended, and annealed for 1 minute at 94 ˚C, 2 minutes at 72 ˚C and 1 minute at 61 ˚C for 30 cycles on a Thermolyne Thermocycler. The PCR products were purified directly using Prep-A-Gene DNA Purification Systems [Biorad] and sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia. Approximately 500 base pairs of each PCR product were sequenced, and the sequences of rDNA from the isolates with identical biotypes were aligned using Pileup [Genetics Computing Group, 1997].

Results

Isolation of bacteria. To find a medium that allowed growth of a variety of abundant soil bacteria, soil samples were serially diluted in five different types of media (Difco Nutrient Broth, DNB, MMSA, 10% v/v soil extract, MMSA without soil extract), incubated for 10 days, and spread onto solid media. Compared to other media, DNB and MMSA allowed for the growth of the most colonies with different colonial morphologies at the highest dilutions. Since the morphologies of the colonies that appeared on each of these two media were different, both media were used to isolate bacteria from soil.

In designing the sampling protocol, soil was assumed to be a heterogeneous environment composed of a variety of representative microhabitats. Thus, small soil samples (approximately 5 mg wet weight) were taken to take advantage of the possibility that they might contain only a few microhabitats and and with few types of bacteria. To select for abundant organisms, each sample was serially diluted in tubes of liquid media.

To allow slower growing organisms time to appear, the tubes were incubated for two weeks. The highest dilutions yielding growth (the 10^{-6} dilution in most cases) were then plated for isolation of pure cultures. Except for 29 plates from the bulk soil and 11 plates from burrows that were contaminated by ants prior to freezing, isolates were obtained from all of the colony types observed on the original plates. In the end, 74 isolates from 29 fresh cast samples, 31 isolates from 16 burrow samples, and 97 isolates from 55 bulk soil samples were obtained and stored at –70˚ C in 1996 and 1997. During the isolation, the plates contained one, two, or more than two colonial morphotypes 47%, 42%, and 11% of the time, respectively. Regardless of the source of the sample, approximately 76% of the isolates that came from the same sample and were isolated on different media had different colonial morphotypes, even after plating on the same medium, Difco Nutrient Agar. Similarly, only a small percentage of these isolates possessed the same biotype (see below). Therefore, each medium appeared to select for different populations of soil bacteria.

To determine the number of bacteria that were capable of growing using these techniques, MPN values were calculated for the bulk soil samples. The average MPN for bacteria that were grown in MMSA and DNB media were 9.8×10^8 and 6.0×10^8 cells $(\text{gram of wet soil})^{-1}$, respectively. In five other experiments between August 1996 and October 1998, the average percent culturability in MMSA and DNB media was 5% and 2%, respectively. Because many of the cells were attached or in clusters, the percent of the maximum number of culturable units was somewhat higher, 8.5% and 3.2%,

respectively. Because each medium appeared to allow for the isolation of different populations of soil bacteria, > 5% of the bacterial cells present were cultured.

Phenotypic properties of the isolates. To determine the phenotypic properties of the isolates, 20 tests were designed to examine characteristics that could be relevant to the soil habitat or measured conveniently. To ensure reproducibility, each test was replicated 5 times using a subset of 5 randomly chosen isolates. In this screening, the tests for motility and cellulose and citrate utilization proved not to be reproducible and were not used to characterize the isolates.

The phenotypic properties of bulk soil, burrow and fresh cast isolates were different (Table 1). A two-tailed Chi-square analysis identified tests that possessed p-values below 0.05 and might be significantly different between sample sources. These phenotypic tests were further analyzed using scaled deviance to determine if the differences were due to sample source or sampling procedure. In these analyses, the results of 12 of the 17 phenotypic tests varied significantly with sample source. The only tests which were not significantly different were hemolysis, ampicillin and tetracycline resistance, casein hydrolysis, and urea degradation. Pairwise phenotypic differences between fresh cast and burrow isolates, fresh cast and bulk soil isolates, and burrow and bulk soil isolates were also analyzed using a Chi-square analysis. Of the tests that varied significantly with the sample source, the fresh cast isolates were significantly different ($p \leq 0.01$) from the bulk soil and burrow isolates in 11 and 12 tests, respectively. In contrast, only the cellular morphology and lactose utilization were different ($p \leq 0.01$) between the bulk soil and burrow isolates. Thus, the phenotypic differences between the

three groups of isolates were largely due to differences between the habitats of the fresh cast isolates and the bulk soil and burrow isolates.

Phenotypic dissimilarity of the isolates and coverage. As a measure of the diversity within each group of isolates, dissimilarity values (Di) were calculated. Isolates with a Di of 0 had the same responses on all of the phenotypic tests and were defined as possessing the same biotype. The 97 bulk soil isolates possessed 90 different biotypes, the 31 burrow isolates possessed 30 biotypes, and the 74 fresh cast isolates possessed 56 biotypes. This corresponded to a coverage [Good, 1953] of 14.4%, 6.5% and 40.5% for bulk soil, burrow and cast biotypes, respectively, and indicated that the sources contained about 630, 460, and 140 biotypes of culturable bacteria, respectively. Even though the low number of duplicate biotypes and percent coverage suggested that only a small fraction of the total number of biotypes present were in fact isolated, the isolates were typical of the populations of culturable biotypes present. For instance, for both the cast and soil isolates, > 95% coverage was obtained at a Di value of 3 (Figure 1).

Very few biotypes (i.e., Di=0) were found in more than one sample source. Only 4 biotypes were common to both the fresh cast and bulk soil isolates, 3 biotypes were shared by the burrow and bulk soil isolates, and no biotypes were shared between the fresh cast and the burrow isolates. Therefore, any phenotypic similarity between the burrow and bulk soil isolates was not due to a large number of identical biotypes found in both sources. Instead, the similarity was apparently due to different isolates with similar properties.

Within each group of isolates, those from the fresh casts were phenotypically more similar to each other than were the isolates from the bulk soil or burrows. For instance, the mean Di (± standard deviation) for the fresh cast isolates was 5.2 ± 2.4. In contrast, the comparable values for the bulk soil and burrow isolates were 7.2 ± 2.6 and 6.9 ± 2.5, respectively (Figure 2). The lower mean Di for the fresh cast isolates suggested that their biotypes were more similar than the burrow or bulk soil biotypes.

Isolates from the same fresh cast, burrow or bulk soil sample had mean Di (± standard deviation) of 4.7 ± 2.5, 6.1 ± 3.6, and 5.9 ± 3.0, respectively. These values were similar to those found for all the isolates from each of the three sources. Therefore, the phenotypic properties of the isolates were not highly correlated with the individual sample.

If Di also predicted genetic or phylogenetic relationships among the isolates, the distribution of Di would not be expected to be random. To test this possibility, the distribution of Di for the fresh cast and bulk soil isolates were compared to those of models where the phenotypes were distributed randomly among the "isolates". The observed distributions of Di were similar to those expected in the random models (Figure 3). Therefore, Di was probably not a good predictor of phylogenetic relationships.

To confirm this conclusion, about 500 base pairs of the 16S rRNA genes were sequenced from 16 pairs of fresh cast isolates with the same biotypes. The sequences of 7 pairs were >99% similar, 3 pairs were between 98 and 99% similar, and 6 pairs were less than 98% similar. Since only 63% of the pairs with identical biotypes possessed

sequences with greater than 98% similarity, phenotypic similarity, as indicated by low Di values, was not a strong predictor of genetic similarity.

Phenotypic stability. Although the cast and bulk soil isolates were taken during the same season, the bulk soil isolates were taken 1 year prior to the cast isolates. Therefore, it was possible that the observed differences between the bulk soil and the cast isolates were due to sampling time. To address this concern, 14 bulk soil and 12 cast isolates were collected on the same day in May 1999. In this experiment, the isolation was performed on MMSA medium, and only a single colony type was chosen from each enrichment. These isolates were characterized using the phenotypic tests used previously (Table 2).

The sample sizes of the new collections of bulk soil and cast isolates (n=14 and n=12, respectively) were much smaller than the original collections of bulk soil and cast isolates (n=96 and n=74, respectively). Since the mean Di and Chi-square were sensitive to sample size (data not shown), the phenotypic dissimilarity between the different groups could not be compared by these methods. Therefore, the lowest Di (LDi) mean and expected LDi mean were used to describe the level of dissimilarity for each group. The expected LDi mean for bulk soil and cast isolates was calculated from the data on the isolates collected in 1996 and 1997. If the bacterial populations were the same in 1999 as in the preceding years, then the LDi mean of the new collection of isolates should be within the range of the expected values. The expected LDi mean (± standard deviation of the mean) for the bulk soil isolates was 2.1 ± 0.3, and the LDi mean (± standard deviation) for the new bulk soil isolates compared to the original bulk soil isolates was 2.3

± 0.4 (Table 3). This value was within the expected range, indicating that the population of culturable soil bacteria had not changed. In contrast, the expected mean LDi for the cast isolates was 1.4 ± 0.3, which was much less than 3.3 ± 0.4, the value observed. The LD mean for the new cast isolates when compared to the original bulk soil isolates was also high, 2.8 ± 0.4, suggesting that these isolates, were also different from the soil isolates. Therefore, while the phenotypes of the population of culturable bacteria in the soil appeared to be stable, those of the casts population changed over the period examined. A change in crop may have caused the change in the cast communities, since earthworms feed partially on litter. In 1997, maize (*Zea mays* L.) was planted during the growing season, and corn litter was observed in the sample site when the cast samples were taken. In 1998, kenaf (*Hibiscus cannabinus*) was planted during the growing season, and that litter was observed in the sample site in 1999. This change in litter may not have affected the soil community since the litter was not tilled into the soil profile and may not have been available for the soil bacterial communities.

Discussion

A high percentage of the cells present and metabolically active in soil are usually not cultured on standard laboratory media [Roszak and Colwell, 1987]. Thus, the ability to collect typical soil bacteria is limited because the cultured bacteria are not necessarily the most abundant bacteria in the community. However, by using conditions that approximated the natural marine environment, a high percentage, 42-63%, of bacteria in seawater samples were cultured [Button et al., 1993; Schut et al., 1993]. Therefore, a similar approach was attempted to isolate soil bacteria. Since soil is an oligotrophic

environment containing a complex mixture of nutrients, a medium that contained a low carbon content and soil extract was used (MMSA). In soil, turnover times for the bacterial populations are very long. Therefore, long incubation periods were used to isolate slowly growing bacteria. Approximately 5-7% of the total bacteria in these soil samples were cultured using these techniques, which is higher than what is usually reported [Bakken and Olsen, 1987; Liesack et al., 1997; Torsvik et al, 1990] but still only a small fraction of the total number of cells. Although molecular methods may frequently sample a larger fraction of the prokaryotic populations, these methods are not without artifacts, such as PCR biases and incomplete cell lysis. [Von Wintzingerode et al., 1997]. While also limited [Dunbar et al., 1997] culture methods offer an independent estimate of community diversity. In addition, characterization of isolates provides functional information which is absent from most molecular analyses.

Soil bacteria exist either as microcolonies or single cells, which are usually found embedded within or attached to soil aggregates [Foster, 1988]. These soil aggregates, which contain organic matter encrusted with clay and mineral particles, serve as micro-habitats for bacteria. The micro-habitats associated with each aggregate could differ due to variation in the composition, size, and shape of the aggregates. Therefore, it is possible, at least in theory, for each aggregate to contain a distinct bacterial micro-community [Foster, 1988]. Since soil is dominated by the solid phase and is poorly mixed, the soil micro-communities remain physically separated. In this experiment, small soil samples were taken to target these micro-communities. Each of these samples was diluted to extinction, and the bacteria within the highest dilutions were isolated. Thus, the

most abundant bacteria capable of being cultured were isolated from representative micro-habitats with the expectation that greater diversity would be obtained than if the micro-habitats were mixed during sampling. Although the validity of this approach has not been tested, the large number of biotypes found in the bulk soil and burrow isolates attests to their diversity.

The biotypes from the fresh casts were different from those of the burrow and bulk soil in both 1997 and 1999. Presumably, these differences reflect the adaptations of the isolates to their habitats. The bulk soil is expected to be a relatively stable habitat [Mahaffee and Kloepper, 1997], and isolates obtained from both 1997 and 1999 appeared to represent the same populations. *Lumbricus rubellus*, the earthworm source of the fresh cast isolates, is an epigeic earthworm [Bouche, 1977] that feeds on decomposing litter and soil. The prokaryotic community associated with its cast is expected to be partially dependent upon available substrates in the litter. Therefore, the change in the cast communities from 1997 and 1999 is not surprising since there was a change in the litter type available for the worms. Since this is no-till soil, the litter was probably not available for bacteria below the surface of the soil. Therefore, it was not surpising that that the soil community remained stable.

Because cellulose is abundant in litter, many of the fresh cast isolates were expected to degrade cellulose and cellobiose, a common intermediate in cellulose degradation. While it was not possible to test cellulose degradation in a reproducible manner, not many of the fresh cast isolates were able to utilize cellobiose. Moreover, no other sugars tested were utilized by a large proportion of the fresh cast isolates from

1997. Instead they utilized amino acids and acetate. These results suggest that sugars were not a major substrate for the cultured cast flora.

Since nitrate and nitrite are more abundant in casts than surrounding soil [Lee, 1985] and Karsten and Drake [Karsten and Drake, 1997] found N_2O emissions from the gut of *L. rubellus*, bacteria capable of reducing nitrate were expected to be abundant in fresh casts. A high percentage of the fresh cast isolates from 1997, 77%, were capable of reducing nitrate. Similarly, 5 out of 12 cast isolates from 1999 also reduced nitrate. The samples studied were obtained from no-till soil, which contains lower levels of nitrate [Doran, 1980; Dowdell et al, 1975; Moody et al., 1952; Thomas et al., 1973] and more denitrifiers [Doran, 1980] than adjacent tilled soil. Likewise, no-tilled soil contains five times more earthworms than tilled soil [Hendrix et al., 1987]. Therefore, the higher number of denitrifying bacteria in the no-till soil might come from the gut flora of the earthworms.

The phenotypic properties of the isolates from burrows and bulk soil were very similar. Because urea is excreted through the surface of the earthworm body [Edwards and Bohlen, 1996; Lee, 1985], bacteria that degrade urea were expected to be more abundant in the burrow than the bulk soil and fresh casts. However, no differences in the proportion of urea-degrading isolates were found between the sample sources. It is difficult to determine the age of a burrow when sampling, and it is possible that the bacterial communities within the burrows are transient. If this is the case, differences between bulk soil and burrow bacteria may not have been apparent in the experimental design used.

One purpose of this study was to test the hypothesis that the earthworm fresh cast and burrow have different bacterial community structures than bulk soil. Previous literature has already shown that there are greater numbers of total bacteria and some specific bacterial groups and higher microbial activity in guts, casts and burrows as compared to bulk soil [Barois, 1992; , Contreras, 1980; Daniel and Anderson, 1992; Edwards and Fletcher, 1988; Karsten and Drake, 1995; Karsten and Drake, 1997; Kristufek et al., 1992; Lee, 1985; Mariaglieti, 1979; Parle, 1963; Pedersen and Hendriksen, 1993; Tiunov, et al., 1997; Trigo and Lavelle, 1993]. In this study, the fresh cast isolates, which were representative of the gut flora, had different phenotypic properties and a lower level of diversity than the bulk soil isolates. Therefore, the bacterial community that was cultured from the fresh casts of earthworms differed from the community that was cultured from bulk soil.

There are at least three explanations for these differences in community structures. *L. rubellus* feeds partially on decomposing litter. Therefore, if the bacterial community of the litter is different from the soil, then the bacterial community of the earthworm gut as compared to adjacent soil may reflect those differences. In this model, the change in community structure observed in the cast isolates from 1997 and 1999 would be expected due to the change in crop. However, it is also possible that some of the bacteria present in bulk soil are enriched during passage through the earthworm gut. Enrichment could have changed the community structure of the bacterial populations. Lastly, the earthworm gut may contain an active bacterial community attached to the lining of the alimentary canal. Fresh casts could be composed of cells shed from this community.

Although the present study does not distinguish between these possibilities, earthworms are very abundant in no-tilled agricultural soil at Horseshoe Bend, 1,000 worms m^{-2} [Hendrix et al., 1987], and may have a major impact on the bacterial community structure.

References

1. **Atlas, R. M.** 1995. Handbook of Media for Environmental Microbiology. CRC Press, Boca Raton, FL.
1. **Bakken, L.R. and R.A. Olsen**. 1987. The relationship between cell size and viability of soil bacteria. Microb. Ecol. **13**:103-114.
2. **Barois, I.** 1992. Mucus production and microbial activity in the gut of two species of *Amynthas* from cold and warm tropical climates. Soil Biol. Biochem. 24:1507-1510.
3. **Beare, M.H., R.W. Parmelee, P.F. Hendrix, W. Cheng, D.C. Coleman, and D.A. Crossley Jr.** 1992. Microbial and faunal interactions and effects on litter nitrogen and decomposition in agroecosystems. Ecol. Monogr. **62**: 569-591.
4. **Bouché, M. B.** 1977. Soil organisms as components of ecosystems. Ecol. Bull. (Stockholm). **25**:122-132.
5. **Biorad**. 1998. Prep-A-Gene DNA Purification Systems. Hercules, CA.
6. **Button, D. K., F. Schut, P. Quang, R. Martin and B. R. Robertson**. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl. Environ. Microbiol. **59**:881-891.
7. **Contreras, E.** 1980. Studies on the intestinal actinomycete flora of *Eisenia lucens*. Pediobiologia. **20**:411-416.
8. **Daniel, O. and J. M. Anderson**. 1992. Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus.* Soil Biol. Biochem. **24**:465-470.
9. **de Man, J. C.** 1975. Probability of most probable numbers. Eur. J. Appl. Microbiol. **1**:67-78.
10. **Dunbar, J., S. White, and L. Forney.** 1997. Genetic Diveristy through the looking glass: effect of enrichment bias. Appl. Environ. Microbiol. **63**: 1326-1331.
11. **Difco**. 1984. Difco Manual. Difco laboratories, Detroit, Mi.
12. **Doran, J. W.** 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am. J. **44**:765-771.
13. **Dowdell, R. J. and R. Q. Cannell.** 1975. Effect of ploughing and direct drilling on soil nitrate content. J. Soil Sci. **26**:53-61.
14. **Edwards, C. A. and K. E. Fletcher**. 1988. Interactions between earthworms and microorganisms in organic-matter breakdown. Agric. Ecosys. Environ. **24**:235-247.
15. **Edwards, C. A. and P. J. Bohlen**. 1996. Biology of Earthworms. Chapman and Hall, London.
16. **Edwards, W. M., M. J. Shipitalo, L. B. Owens and L. D. Norton.** 1990. Effects of *Lumbricus terrestris* L. burrows on hydrology of continuous no-till corn fields. Geoderma. **46**:73-84.
17. **Farrar, W. E. and A. C. Reboli.** 1981. The Genus *Bacillus* -Medical p. 1763. *In* A Balows, H. Truper, M. Dworkin, W. Harder and K. Schleifer (ed.), The Prokaryotes, 2nd ed. Springer-Verlag, New York.

18. **Foster, R. C.** 1988. Microenvironments of soil microorganisms. Biol. Fertil. Soils. **6**: 189-203.
19. **Genetics Computing Group** (GCG). 1997. Wisconsin Package Version 9.1, GCG. Madison, WI
20. **Good, I. J.** 1953. The population frequencies of species and the estimation of population parameters. Biometrica **40**: 1107-1114.
21. **Groffman, P.M., G.J. House, P.F. Hendrix, D.E. Scott, and D.A. Crossley Jr.** 1986. Nitrogen cycling as affected by interactions of components in a Georgia Piedmont agroecosystem. Ecology **67**: 80-87.
22. **Hendrix, P. F. , D. A. Crossley Jr., D. C. Coleman, R. W. Parmelee, and M. H. Beare.** 1987. Carbon dynamics in soil microbes and fauna in conventional and no-tillage agroecosystems. INTECOL Bull. **15**:59-63.
23. **Hendrix, P.F.** 1997. Long-term patterns of plant production and soil carbon dynamics in a Georgia Piedmont agroecosystem. In: Paul, E.A., Paustian, K., Elliott, E.T., Cole, C.V. (Eds.), Soil Organic Matter in Temperate Agroecosystems: Long-Term Experiments in North America. CRC Press, Boca Raton, pp. 235-245.
24. **Karsten, G. R. and H. L. Drake.** 1995. Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils. Appl. Environ. Microbiol. **61**:1039-1044.
25. **Karsten, G. R. and H. L. Drake.** 1997. Denitrifying bacteria in the earthworm gastrointestinal tract and *in vivo* emission of nitrous oxide (N20) by earthworms. Appl. Environ. Microbiol. **63**:1878-1882.
26. **Kristufek, V., K. Ravasz and V. Pizl.** 1992. Changes in density of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa*. Soil Biol. Biochem. **24**:1499-1500.
27. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Technology in Bacterial Sysematics. John Wiley and Sons, New York.
28. **Lee, K. E.** 1985. Earthworms: Their Ecology and Relationships with Soils and Land Use. Academic Press, Sydney, Australia.
29. **Liesack, W., P.H. Janssen, F.A. Rainey, N. Ward-Rainey, and E. Stackebrandt.** 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, p. 375-439. In J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.). Modern soil microbiology. Marcel Dekker Inc., New York, N.Y.
30. **Mahaffee, W.F. and J.W. Kloepper.** 1997. Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.) . Microbial Ecol. **34**: 210-223.
31. **Mariaglieti, K.** 1979. On the community structure of the gut microbiota of *Eisenia lucens.* Pediobiolgia. **19**:213-220.
32. **Moody, J. E., J. H. Billard, and T. W. Edminster.** 1952. Mulch tillage: some effects on plant and soil properties. Soil Sci. Soc. Am. Proc. **16**:190-194.
33. **Parle, J. N.** 1963. Micro-organisms in the intestine of earthworms. J. Gen. Microbiol. **31**:1-11.

34. **Pederson, J. C. and N. B. Hendriksen.** 1993. Effect of passage through the intestinal tract of detritivore earthworms (*Lumbricus* spp.) on the number of selected Gram-negative and total bacteria. Biol. Fertil. Soils. **16**:227-232.
35. **Pharmacia Biotech.** 1996. Ready-to-go PCR beads. Molecular biological reagents division, Piscataway, New Jersey.
36. **Roszak, D. B. and R. R. Colwell.** 1987. Survival strategies of bacteria in natural environments. Microbiol. Rev. **51**:365-379.
37. **SAS.** 1998. SAS Institute, Cary, NC.
38. **Satchell, J. E.** 1955. An electrical method of sampling earthworm populations, p. 356-364. *In* D. K. McE. Kevan (ed.), Soil Zoology: Proceedings of the University of Nottingham Second Easter School in Agricultural Science. Academic Press, New York.
39. **Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59**:2150-2160.
40. **Smibert, R. and N. Krieg.** 1994. Phenotypic characterization, P. 614. *In* P. Gerhardt, R. G .E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D. C.
41. **Stackebrandt, E. and H. Prauser.** 1981. The family Cellulomonadaceae, p. 1323-1345. *In* A Balows, H. Truper, M. Dworkin, W. Harder and K. Schleifer (ed.), The Prokaryotes, 2nd ed. Springer-Verlag, New York.
42. **Stinner, B.R., D.A. Crossley Jr., E.P. Odum, and R.L. Todd.** 1984. Nutrient budgets and internal cycling of N, P, K, Ca and Mg in conventional tillage. Ecology **65**: 354-369.
43. **Thomas, G. W., R. L. Blevins, R. E. Philips, and M. A. McMahon.** 1973. Effect of a killed soil mulch on nitrate movement and corn yield. Agron. J. **63**:736-739.
44. **Tiunov, A. V., T. G. Dobrovol'skaya and L. M. Polyanskaya.** 1997. Microbial community of the *Lumbricus terrestris* L. earthworm burrow walls. Microbiol. (N.Y.) **66**:349-353.
45. **Tomlin, A. D., M. J. Shipitalo, W. M. Edwards and R. Protz.** 1995. Earthworms and their influence on soil structure and infiltration, p. 159-184. *In* P. F. Hendrix (ed.), Earthworm Ecology and Biogeography. Lewis Publishers, Chelsea, MI.
46. **Torsvik, V., J. Goksỳr and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.
47. **Trigo, D. and P. Lavelle.** 1993. Changes in respiration rate and some physicochemical properties of soil during gut transit through *Allolobophora molleri*. Biol. Fertil. Soils. **15**:185-188.
48. **Von Wintzingerode, F., U.B. Gobel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21**:213-221.

49. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic Methanococci. Sys. Appl. Microbiol. **7**:235-240.

Table 1. Phenotypic characterization of bulk soil, burrow and fresh cast isolates.

Test	Percent occurrence in isolates from: Bulk soil	Burrows	Fresh casts	Chi-square (p-value)[a]	Scaled deviance[a]
cell morphology:	—	—	—	0.024[b]	¾
cocci	9	0	3	¾	16
small rod	13	36	11	¾	21
rod	46	36	58	¾	20
large rod	33	29	28	¾	38
catalase production	90	94	77	0.024	29
hemolysis:	¾	¾	¾	0.480[b]	¾
alpha	30	16	23	¾	¾
beta	7	10	12	¾	¾
hydrolysis:					
casein	22	32	32	0.286	¾
urea	44	58	27	0.007	39[c]
Tween 80	18	13	36	0.006	14
resistance:					
ampicillin	32	29	49	0.043	35[c]
tetracycline	22	0	0	0.339	¾
polymyxin	29	23	7	0.002	27
streptomycin	9	13	0	0.014	32
carbon utilization:					
acetate	45	39	73	0.001	22
lactose	24	52	0	0.001	17
Casamino acids	57	65	84	0.001	29
mannose	35	42	7	0.001	27
cellobiose	23	26	1	0.001	25
MacConkey:	—	—	—	0.001[b]	¾
growth	19	23	91	¾	46
fermentation[d]	10	0	18	¾	23
nitrate reduction:	—	—	—	0.001[b]	¾
partial	28	26	73	¾	37
complete	0	0	4	¾	5

[a] The null hypothesis was: $\mu_{bulk\ soil}=\mu_{drilosphere}=\mu_{cast}$

[b] These tests had more than one response. For the Chi-square analysis, the responses were grouped together to observe overall differences in the test for the three types of isolates. For the Genmod analysis, each response was observed separately.

[c] These d-scale p-values were greater than 0.05.

[d] Fermentation of lactose.

Table 2. Phenotypic characterization of 1999 bulk soil and fresh cast isolates.[a]

Test	Number occurring in isolates from: Bulk soil	Fresh casts
cell morphology:	—	—
cocci	5	3
small rod	2	1
rod	5	3
large rod	2	5
catalase production	11	10
hemolysis:	¾	¾
alpha	2	1
beta	1	1
hydrolysis:		
casein	6	2
urea	3	3
Tween 80	4	6
resistance:		
ampicillin	2	5
tetracycline	0	0
polymyxin	1	3
streptomycin	2	3
carbon utilization:		
acetate	4	5
lactose	1	2
Casamino acids	5	5
mannose	3	3
cellobiose	1	2
MacConkey:	—	—
growth	1	3
Fermentation[b]	1	2
nitrate reduction:	5	5

[a] A total of 14 bulk soil and 12 cast isolates were examined

[b] Fermentation of lactose

Table 3. Expected LDi mean and actual LDi means for isolates compared to different groups.

Isolates being compared	LDi[1] mean ± standard deviation	Expected LDi mean ± standard deviation of the mean[2]
New bulk soil with original bulk soil	2.3±0.4	2.1±0.3
New cast with original cast	3.3±0.4	1.4±0.3
New bulk soil with original cast	3.2±0.4	1.4±0.3
New cast with original bulk soil	2.8±0.4	2.1±0.3

[1]Lowest Di value of an isolate when compared to a group of isolates.

[2]Expected LDi mean ± standard deviation were calculated from the mean LDi of 100 random sub-samples of 14 and 12 original bulk soil and cast samples, respectively.

Figure 1. Coverage [Good, 1953] versus dissimilarity (Di) for fresh cast (●) and bulk soil (▲) isolates. Coverage = [1-N / n] × 100, where N is the number of biotypes with less than or equal the indicated level of dissimilarity to any other biotype and n is the total number of biotypes.

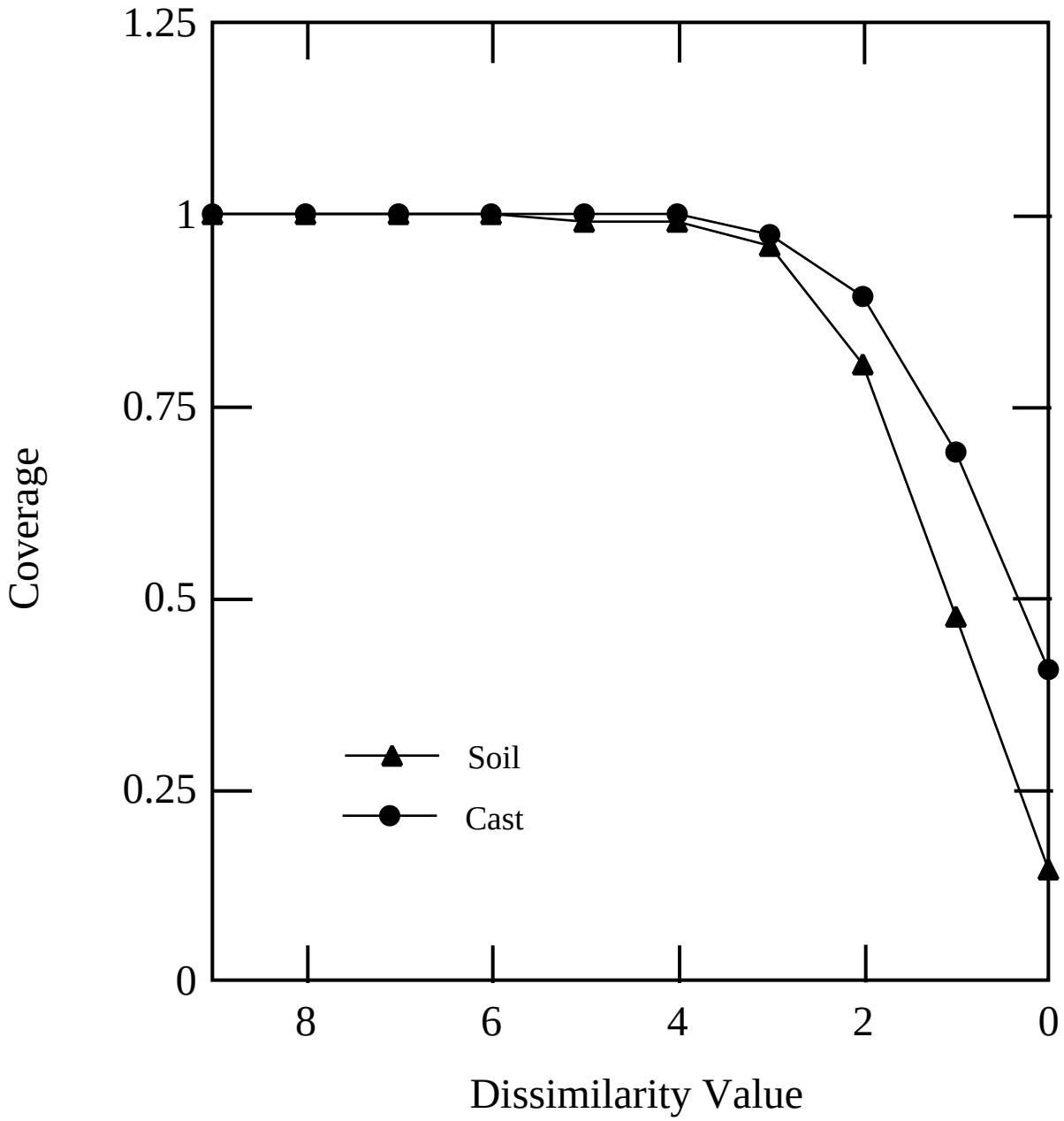

1.25
1
0.75
0.5
0.25
0
Coverage
Soil
Cast
8
6
4
2
0
Dissimilarity Value

Figure 2. Distribution of dissimilarity values (Di) for isolates from the bulk soil (Δ), burrow (□), and fresh cast (○). The percent occurrence is equal to: (number of pairs with specified D) / (total number of pairs within each group) × 100. For the bulk soil, burrows, and fresh casts, the median and mean (± standard deviation) Di values were 7 and 7.2 ± 2.6, 7 and 6.9 ± 2.5, and 5 and 5.2 ± 2.4, respectively.

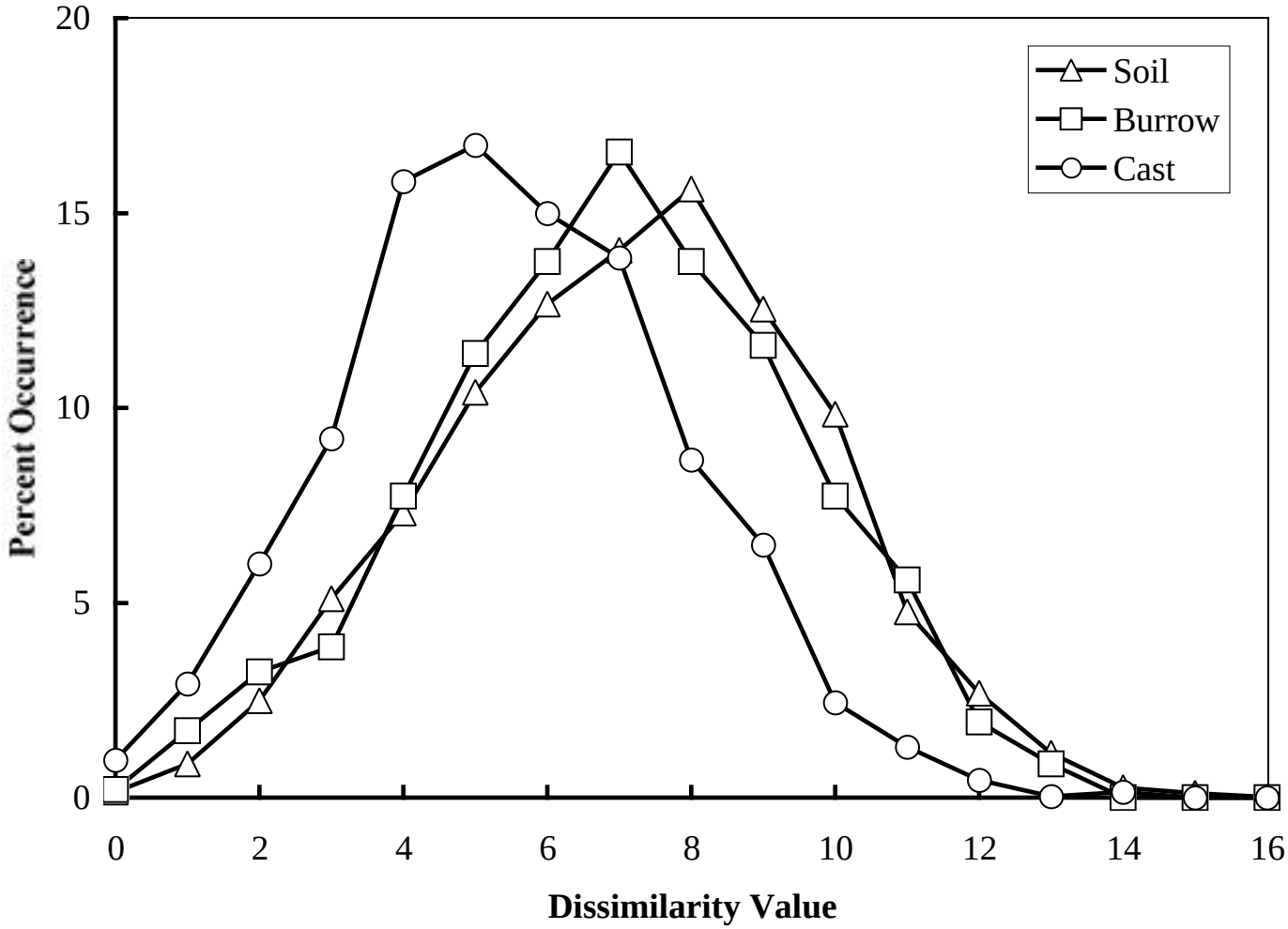
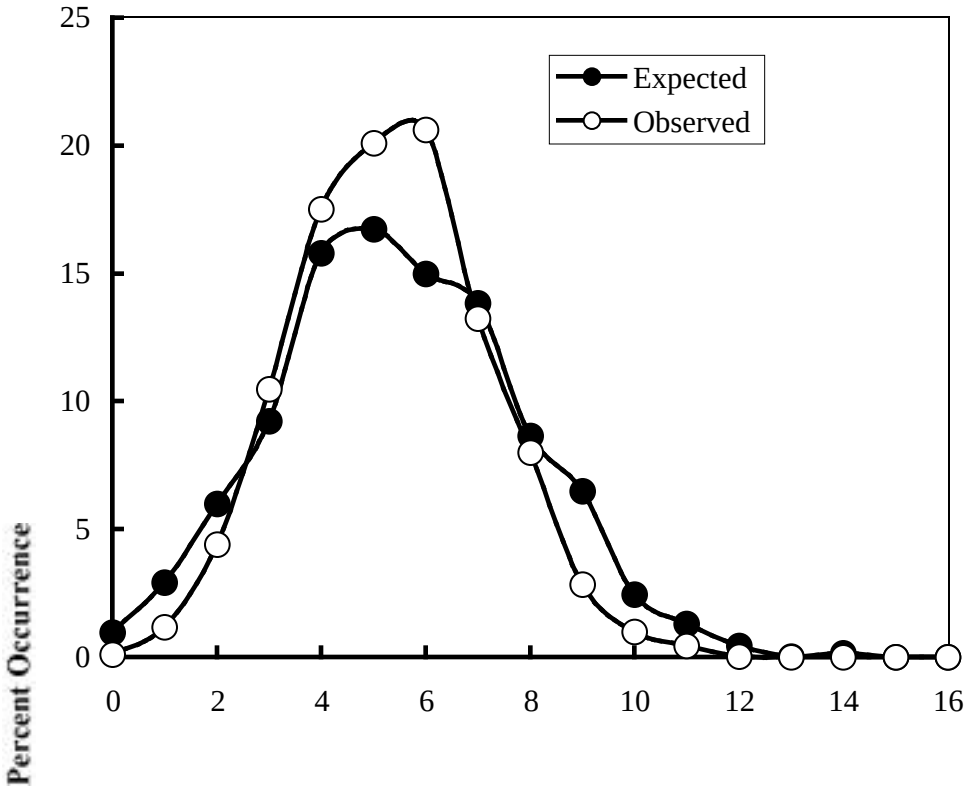
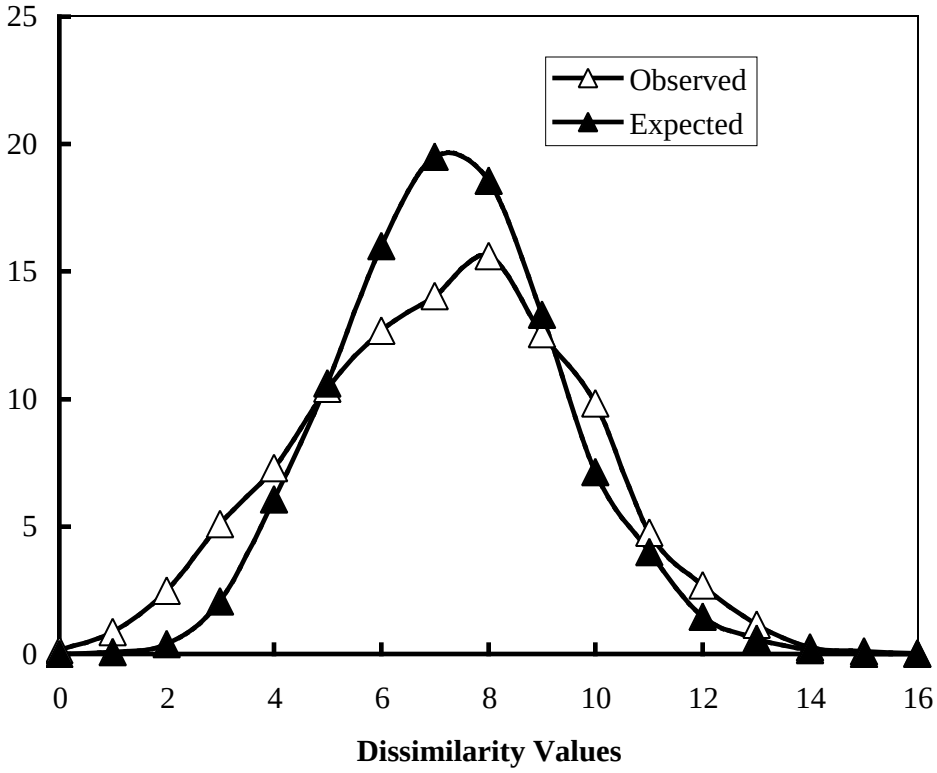

20
15
10
5
0
Percent Occurrence
0
2
4
6
8
10
12
14
16
Dissimilarity Value
Soil
Burrow
Cast

Figure 3. Comparison of the distribution of Di values for fresh cast and bulk soil isolates with random models. The model was generated by randomly assigning test results to the "isolates" using the same ratio of positive to negative results obtained for the original isolates. A new matrix of Di values was then created, and the distribution of Di was determined. Symbols: ○ fresh cast, observed D; ● fresh cast, expected from random model (median = 5, mean ± standard deviation = 5.3 ± 1.9); Δ bulk soil, observed Di; ▲ bulk soil expected from random model (median = 7, mean ± standard deviation = 7.3 ± 2.0).


Expected
Observed
Percent Occurrence
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16


Observed
Expected
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Dissimilarity Values

CHAPTER III

PHYLOGENETIC ANALYSIS AND DIVERSITY OF BACTERIAL ISOLATES FROM FRESH EARTHWORM CASTS, BURROWS AND SURROUNDING SOIL[2]

[2] Furlong, M.A., D.R. Singleton, D.C. Coleman, and W.B. Whitman. 2000. To be submitted.

Abstract

The 16S rRNA genes from bacteria isolated from earthworm casts and burrows and soil were subjected to phylogenetic and diversity analyses. The burrow and soil collections contained isolates that had similar phylogeny and were different from the cast isolates. The majority of the soil and burrow isolates belonged to the high G + C Gram positive bacteria (54% and 48%, respectively). The other isolates were distributed among the low G + C Gram positives and α-, β- and γ- proteobacteria. The majority of the cast isolates were γ-proteobacteria (78%). The remainder of the cast isolates grouped with the high and low G + C Gram positive, α- and β- proteobacteria and cytophaga/flexibacter groups. Soil and burrow isolates were also similarly more diverse than the cast isolates. Soil and burrow isolates each contained twice as many OTUs and had diversity and evenness values that were much greater than the cast isolates. The culture technique used for this experiment targeted some of the novel and culturable organisms found in these environments. Seventy-three percent of all of the isolates may be assigned to new species, and 7% of these may belong to novel genera. Many of these novel organisms grouped well with clones that were previously obtained from environmental samples.

Introduction

Earthworms may be of major importance for crop growth. Some earthworms have been linked to increased crop yields [Doube *et al*., 1995] and soil nitrogen, phosphorus, and potassium content [Tiwari *et al*., 1989]. Although the benefits of earthworms to soil structure are well known [Edwards and Bohlen, 1996], it is uncertain how they contribute to increased chemical fertility. Detritivore earthworms generally increase the microbial activity and biomass of the soil they ingest and process [Barois,

1992; Daniel and Anderson, 1992; Edwards and Fletcher, 1988; Kristufek *et al*., 1992; Parle, 1963; Pederson and Hendriksen, 1993; Tiunov *et al*., 1997; Trigo and Lavelle, 1993], and disburse many of these microbes throughout the soil profile [Hendriksen, 1995; Pederson and Hendriksen, 1993]. Since microorganisms play a pivotal role in nutrient transformations, it is plausible that they contribute to the increased soil fertility associated with earthworm activity. Thus, the key to understanding the role of earthworms in chemical fertility is to understand the role of the microbial communities associated with them.

Since the increased popularity of the molecular approach, the use of the culture approach for studying prokaryotic diversity in environmental samples has dwindled. It has been claimed that the culture approach offers a biased view of natural communities because less than 5% of the prokaryotes in environmental samples are cultured on standard laboratory media [Bakken and Olsen, 1987; Liesack *et al*., 1997; Torsvik *et al*., 1990]. One of the major limitations with the molecular approach, however, is that it provides little functional information about the microbial community unless closely related organisms have already been described, which is often not the case [Wintzingerode, 1997]. This problem is exacerbated by the waning popularity of culture approaches with the acceptance of molecular methods.

The culture approach was used in this study to learn more about the effect of earthworms on the prokaryotic diversity of soil and to identify and describe more of the viable prokaryotes found in agricultural soil. Bacterial isolates were obtained from fresh earthworm casts (n=74), earthworm burrows (n=31), and surrounding or bulk soil (n=99) using non-selective sampling and culturing methods in the spring of 1996 and 1997.

Approximately 5-7% of the bacterial cells from the bulk soil were grown using these methods. Some of the physiological properties of the isolates were examined to characterize and determine the diversity in the three communities. The biotypes of the bacteria from fresh casts differed from those of the soil and burrows, which were very similar. The soil and burrow communities were much more diverse than the cast. And unlike the isolates taken from bulk soil and burrow samples, many of the fresh cast isolates reduced nitrate, utilized acetate and Casamino acids, and failed to grow on any of the sugars tested (Furlong *et al*., submitted). In this paper we analyzed the 16S rDNA of these isolates to describe their phylogeny and compare the diversity of the three communities at the genetic level.

Materials and Methods

Sampling and Isolation. All samples were obtained from no-tilled agricultural plots at the Horseshoe Bend Long Term Research in Environmental Biology (LTREB) site in Athens, GA. The methods of sampling and isolation, designed to yield growth of representative prokaryotes from earthworm casts and burrows and bulk soil, have been described in detail [Furlong *et al*., submitted]. Two non-selective media, MMSA and DNB, were utilized for the original isolates, and only MMSA was used for a second collection of "new" isolates. Small soil samples (approximately 5.3 mg) were taken in order to target microsites. Each sample was vortexed in 0.15% NaCl to disperse bacteria, and serially diluted in the test media to a factor of 10^{-9}. After two weeks, the highest dilutions showing growth were plated for isolation of pure cultures. A representative of each colony type was then subcultured for further classification (only one colony from each plate for the new isolates). Soil and burrow isolates were obtained in the spring of

1996 and cast isolates were obtained in the spring of 1997. Another set of cast and soil isolates were obtained in the spring of 1999, and were referred to as the new isolates. The isolates were named based on their origin (i.e. S for soil, B for burrow, C for cast, NS for new soil and NC for new cast), sample number, the media they were isolated on (i.e. M for MMSA and D for DNB), and the order in which they were picked off the original isolation agar.

PCR amplification and Sequencing. The 16S ribosomal DNA of the 204 isolates were PCR amplified using Ready-To-Go PCR Beads [Parmacia Biotech] and primers 27f, 5'-AGA GTT TGA TCM TGG CTC AG-3', and 1392r, 5'-ACG GGC GGT GTG TRC-3' [Lane, 1991]. Isolates were grown on Difco Nutrient Agar plates, and a single colony of each isolate was resuspended and washed two times in 100 µl of sterile distilled water. Each cell suspension was added to a reaction mixture at a concentration of 8% (v/v) to act as the template DNA, and each reaction mixture was heated for 5 minutes at 94 ºC. The reactions were denatured, extended, and annealed for 1 minute at 94 ºC, 2 minutes at 72 ºC and 1 minute at 61 ºC for 30 cycles on a Thermolyne Thermocycler.

If a PCR product was not obtained using this method, then the DNA from the isolate was extracted first, using the method of Singleton [unpublished]. Briefly, cell cultures (5 ml) were pelleted. Lysis solution [2 ml of 0.15M NaCl, 0.1M NaEDTA (pH 8.0), 1.5 mg / ml of chicken egg white lysozyme (Sigma, 73,000 units/mg protein) and 25 mg/ml PVPP] was added, the tube was mixed briefly, and the suspension was incubated at 37 °C for 1 hour with additional mixing every 15 minutes. Portions of the aqueous suspension, 0.5ml, were transferred to 1.5 ml microfuge tubes, and 400 µl of bead-

beating solution [0.1M NaCl, 0.5M Tris-HCl (pH 8.0), 10% SDS] and 0.1 g of 0.1 mm glass beads were added. The tubes were shaken on a Turbo Mixer (Scientific Industries, Inc., Bohemia) for 3 minutes and centrifuged at maximum speed in a microcentrifuge for 3 minutes. The aqueous layer was transferred to a new tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The aqueous layer was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1, pH 8.0). The DNA was precipitated with a 0.6 volume of isopropanol at -20 °C overnight. The nucleic acid was collected by centrifugation and dried under vacuum. The pellet was suspended in 50 µl distilled and deionized H_2O with 1 µl RNaseA solution [0.5 units/µl DNase-free RNase A from bovine pancreas (Boehringer Mannheim, Indianapolis), 10mM Tris-Cl (pH 7.5) and 15 mM NaCl] and incubated at 37° C for 1 hour. The DNA concentration was estimated after electrophoresis in an agarose gel stained with ethidium bromide (50 µg / ml) and compared with mass standards. Approximately 100 ng of DNA was added to each PCR reaction.

The PCR products were purified directly using Prep-A-Gene DNA Purification Systems [Biorad]. The PCR products were discriminated by polyacrylamide gel electrophoresis in a model 377 automated DNA sequencer (Applied Biosystems Inc.) with Genescan (Applied biosystems Inc.) software package. All PCR products were sequenced from primer 27f [Lane, 1991]. If a suitable, (less than 5 ambiguous bases) 500 base pair sequence, was not obtained by direct sequencing of the PCR product, then it was cloned using pCR 2.0 [Invitrogen] and sequenced from the plasmid using primer 27f [Lane, 1991].

Sequence Analysis. A FastA [Genetics Computing Group (GCG), Wisconsin] search was performed on all sequences to determine which sequences in the GenBank and EMBL databases [Benson *et al*., 1999; Stoesser *et al*., 2000] were most similar. All sequences were compared to each other, and if duplicate sequences occurred (>99% sequence similarity), then they were grouped into an operational taxonomic unit (OTU) and only one sequence was used for further analysis. Sequences from the isolates were aligned with reference organisms using Pileup [GCG, Wisconsin]. The Jukes Cantor [Jukes and Cantor, 1969] algorithm was used to calculate distance and Neighbor-joining [Saitou and Nei, 1987] algorithm was used to construct the phylogenetic trees using Phylip 3.5 software [Felsenstein, 1993]. The data sets were bootstrapped using SEQBOOT (Phylip 3.5). The sequences for the reference organisms and clones were obtained from Genbank. References organisms were chosen if they showed greater than 99% similarity to any of the isolates or if they were useful for distinguishing clades within the tree. The sequences from clones and organisms that have not been described were included in the trees if they showed greater than 97% sequence similarity to any of the isolates.

Diversity Calculations. Rarefaction was used to evaluate OTU richness [Hurlbert, 1971; Krebs, 1989]. Because the original sample sizes differed for the 3 collections, the diversity indices of the OTUs were calculated using normalized sample sizes. The sample size of soil isolates was reduced to 31 and 74 by random selection for the comparison of diversity measurements to the burrow and cast collections, respectively. Evenness was calculated to describe the distribution of abundance of isolates [Pielou, 1977]. Shannon diversity, a general diversity measurement, was used to

evaluate OTU richness and evenness [Shannon and Weaver, 1963]. The distribution of coverage [Good, 1953] at various distance values was calculated for the soil, cast, and burrow isolates. Coverage (C) was determined using the equation, $C = 1 – (n – S) / n$, where S is the is the number of unique sequences at a given evolutionary distance or D value, and n is the total number of sequences in the data set. The distribution of lowest distance (LD) values was also determined for soil and cast isolates (the burrow isolates were not included in this analysis because the coverage was low). A matrix of D values was constructed in Excel comparing the 16S rDNA sequences of the soil and cast isolates. The lowest D (LD) for each soil isolate compared to, or mapped onto, all of the cast isolates, was determined and vice versa.

To determine if the biotypes of the new bulk soil and cast isolates were similar to those of the old bulk soil and cast isolates, a mean LD analysis was performed. In this analysis the control isolates were those that were collected in 1996 and 1997, and the test isolates were those that were collected in 1999. A matrix of D values was constructed in Excel comparing the sequences of the control isolates to those of the test collection. The LD value for each test isolate was then determined. The mean of the LD values for the test isolates in each group (bulk soil and cast) was then calculated. To determine the expectation for the mean, a matrix of D values was first constructed in Excel, comparing the sequences within each group of control isolates. After the removal of D values for comparisons of the isolates to themselves, the lowest D value for each control isolate was determined. For the bulk soil and cast groups, the means of 100 random selections of the lowest D values equal in number to those of the test isolates in that group were calculated, and the distribution of these means was determined.

Results

Phylogeny of the isolates. An initial phylogenetic analysis of the 204 original and 26 new isolates, their closest relatives in the GenBank and EMBL databases and representative of all the major taxonomic groups were used to assign each environmental isolate to a major bacterial division. Only one isolate could not be affiliated with a cultured or uncultured representative of the sequence databases. By this method, it formed a distinct phylogenetic branch and was considered a novel group (data not shown). This isolate, which was only found once, was from an earthworm burrow. Table 1 lists the broad phylogenetic distribution of the remaining soil, cast and burrow isolates. These isolates belonged to previously characterized groups, including the high and low G + C and Gram positives, the cytophaga/flexibacter, and the α-, β-, and γ-proteobacteria. Sequences belonging to the high G + C Gram-positive group were most abundant in the original and new soil (54%) and the earthworm burrow (48%) collections. A Chi-square analysis of the original soil and burrow collections indicated that their phylogenetic distributions were not different ($P > 0.05$). Sequences belonging to the γ-proteobacteria group comprised 78% of the original collection of isolates. A Chi-square analysis of the original cast and soil isolates indicated that the distributions of these collections differed ($P < 0.05$). In contrast, the phylogenetic distribution of the new cast isolates did not contain any representatives of the γ-proteobacteria. Instead, it was dominated by Gram-positive bacteria (Table 1).

Taxon-specific phylogenetic analysis. Five taxon-specific phylogenetic trees (Figures 1 – 5) were constructed containing all of the representative OTUs that grouped

in that taxon, reference sequences from described organisms and from environmental isolates and clones that showed > 97% sequence similarity to any of the OTUs.

All of the high G + C Gram positive (Actinobacteria) isolates grouped with the order *Actinomyceteales*, which included suborders such as, *Corynebacterinaea*, *Streptomycinea*, and *Micrococcinea* (Figure 1). The soil, burrow and cast isolates were evenly distributed throughout these divisions. It is not unusual to find soil bacteria that fall within this group of organisms, since *Actinomycetales* are the most abundant bacteria typically cultured from soil [Paul and Clark, 1996].

In the γ-proteobacteria tree, the isolates grouped with 5 families (*Moraxellaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Aeromonadaceae*, and *Enterobacteriaceae*) (Figure 2). Most of the soil and all burrow γ-proteobacteria isolates (11 and 3 isolates, respectively) were associated with the family *Pseudomonadaceae*. The remaining γ-proteobacteria soil isolates grouped with *Moraxellaceae* (3 isolates), *Xanthomonadaceae* (5 isolates), and *Enterobacteriaceae* (2 isolates). Most of the γ-proteobacteria cast isolates were associated with *Aeromonadaceae* (21 isolates). The remaining cast isolates were found in families that contained soil isolates, including *Pseudomonadaceae* (16 isolates), *Xanthomonadaceae* (1 isolate), and *Enterobacteriaceae* (3 isolates) groups.

The large percentage (29%) of the cast isolates that associated with *Aeromonadaceae* was unexpected. *Aeromonas* spp. are typically associated with fish and aquatic habitats. Although they have never been isolated from soil, they have been previously isolated from earthworm casts [Hendriksen, 1993].

The low G + C Gram positive isolates all grouped within the class *Bacillales* (Figure 3). The majority of the soil, cast, and burrow isolates were associated with the

Bacillus spp. Since *Bacillus* spp. are commonly found in soil, this result was not surprising. Some of the cast and soil isolates grouped with the *Paenibacillus* spp., and some of the soil isolates were associated with the *Brevibacillus* and *Staphylococcus* spp.

The α- and β- proteobacteria were also represented in the soil, cast and burrows. The isolates in the β-proteobacteria group associated with the families in the order *Burkholderiales* including, *Comamonadaceae*, *Burkholderiaceae*, *Oxalobacteraceae*, and *Ralstoniaceae* (Figure 4). Soil and cast isolates grouped with the *Comamonadaceae* and only one soil isolate grouped with *Burkholderiaceae*. The *Ralstoniaceae* and *Oxalobacteraceae* taxa each contained burrow and cast isolates. The isolates belonging to the α-proteobacteria all grouped with the order *Rhizobiales* (Figure 5). All of these taxa are found commonly in soil.

While some (27%) of the isolates obtained from this study were similar to previously described species (16S rDNA > 99.0 % similar), many were not (Table 2). Twenty three percent were possible new species (< 97.5% similarity to described organisms). Seven percent were less than 95.4% similar to a described organism, which means they could be novel genera. One organism was cultured from a burrow that was less than 85% and 92% similar to any described organism or clone, respectively (data not shown). It is believed that the culture technique used for this experiment targeted some of the novel and culturable organisms found in these environments.

Twenty three, 21 and 7 of the cast, soil and burrow isolates, respectively, grouped with clones that were previously obtained from environmental samples, such as soil or seawater (Figures 1 – 4). There were many instances where these isolates grouped more closely with environmental clones than with described organisms. For instance, five cast

OTUs (C27D1, C20D1, C19M1, C21M1, and NC7) of 13 isolates, two burrow OTUs (B4D1 and B33M1) of 3 isolates, and seven soil OTUs (S30D1, NS2, S92D1, S64D1, S78M1, S58M2, and S95M1) of 14 isolates, all grouped with 16S rDNA sequences that were cloned from either soil or the rhizosphere [Hengstmann *et al*., 1999; Marilley and Aragno, 1999; McCaig *et al*., 1999; Shikano and Mitsui, unpublished; Suyama *et al*., 1998] and were never isolated.

A clone library of soil and cast 16S rDNA from this same sample site was obtained in 1999 (March) in a separate experiment. Eleven of the original cast isolates had 16S rDNA that was ≥ 98% similarity to some of these cast clones. Likewise, two of the soil isolates were also ≥ 98% similar to soil clones (data not shown). Overlap between collections of cultivated isolates and environmental clones rarely exists in studies of soil. Nevertheless, these results suggest that in principle, many of the organisms from well recognized groups that have previously been detected only as clones from natural samples can be cultivated by standard methods.

Diversity. Diversity indices were calculated by using sequence data obtained from each original isolate collection. Rarefaction was used to compare the OTU richness within each group of isolates at standardized sample sizes (Figure 6). The soil isolate collection contained about twice as many OTUs as the cast isolate collection at sample sizes greater than 35, and just as many as the burrow collection at all sample sizes. For the diversity calculations (Table 3) the soil isolate collection was reduced to 74 and 31 isolates to normalize the sample size for comparison to the cast and burrow isolates, respectively. The coverage for the burrow and soil OTUs was low, ranging from 32 to 57 %. However, the diversity and evenness for these groups approximated the maximum

possible values. The 80% coverage of the cast isolates indicated that the majority of the cast community culturable with the methods used here was collected. The diversity and evenness values for the cast, which were 2.47 and 1.81, respectively, were lower than those for the soil. The overall diversity for the cast isolates was much lower than the soil and burrow isolates, and this fact was reflected in the OTU richness and evenness.

Coverage vs Distance and LD. Coverage, at different distance values, was also calculated for the original soil, cast and burrow isolates (Figure 7). Coverage for soil isolates was fairly high (>90%) at distance values above 0.05 (less than 95% sequence similarity), suggesting that greater than 90% of the larger taxonomic divisions culturable by the our methods were probably obtained. Coverage for the cast isolates remained high (~ 80%) even at distance values of zero, suggesting that a large portion of the species in the cast, which were culturable by our methods, were obtained. Presumably because of its small sample size, the coverage of the burrow isolates, however, dropped off at distances less than 0.14 (greater than 86% similarity). Because the coverage was so low the LD analysis was not used on the burrow isolates.

The LD distribution showed that more than 50% of the cast isolates had 16S rDNA that was greater than 98% similar (<0.02 distance) to the soil isolate 16S genes (Figure 8). Eighty-one percent of these organisms were included in the γ-proteobacteria group and the other nineteen percent were from the β-proteobacteria, high and low G + C Gram positive groups. These specific relationships can be viewed in the phylogenetic trees (Figures 1 – 5). About 31% of the cast isolates had 16S genes that were less than 87% similar (>0.13 distance) to soil 16S genes (Figure 8). All of these organisms, except two, were related to the *Aeromonas* spp. of the γ-proteobacteria. The LD values for the

soil isolates mapped onto the cast isolates were more evenly distributed, with the majority of the soil isolates being less than 95% similar (≥ 0.06 distance) (Figure 8). Only 21% of the soil isolates were greater than 98% similar to the cast isolates. The isolates were from the γ- and β- proteobacteria and high and low G + C Gram positive groups. While many of the cast isolates were similar to the soil isolates, the casts also contained representatives of a few taxa absent or greatly reduced in soil, which suggested that the cast isolate collection was a subset of the soil collection. In contrast, while a small fraction of the soil isolates were closely related to the cast isolates, the soil contains many taxa not represented in casts.

Community stability. Although the original cast and soil isolates were taken during the same season, the soil isolates were taken 1 year prior to the cast isolates. Therefore, it was possible that the observed differences between the soil and the cast isolates were due to the sampling time. To address this concern, 14 soil and 12 cast isolates were collected on the same day in May 1999. In this experiment, the isolation was performed on MMSA medium, and only a single colony type was chosen from each enrichment. The 16S rDNA of these isolates were sequenced, analyzed, and compared to the original isolates.

The sample sizes of the new collections of soil and cast isolates (n=14 and n=12, respectively) were much smaller than the original collections of isolates. Since the sample size was so small, the diversity could not be measured using the same methods used for the original isolates. In the previous chapter (Phenotypic Comparison of Bacterial Isolates from Soil and Earthworm Casts and Burrow) a new method was introduced that was used to compare the phenotypic dissimilarity between the old and

new isolates. In this method the lowest dissimilarity (LD) mean and expected LD mean were used to describe the level of dissimilarity for each group. That data suggested that the biotypes of the soil community remained stable, while the cast community changed.

Table 1 lists the proportion of new cast and new soil isolates in different phylogenetic groups. The new soil isolates had similar proportions of different phylogenetic groups as the original soil isolates. Also, two of the new soil isolates had 16S rDNA that was greater than 99% similar to original soil isolates. Qualitatively, the distribution of major groups in the new cast isolates appeared different from that of the original cast isolates. This data supported the conclusion made previously; the soil community remained stable, while the cast community changed.

The same mean LD analysis that was used for the phenotypic data was used for the 16S rDNA, except evolutionary distance replaced phenotypic dissimilarity. The expected LD mean for soil and cast isolates was calculated using the data from the isolates collected in 1996 and 1997. If the bacterial populations were the same in 1999 as the preceding years, then the LD mean of the new collection of isolates should be within the range of the expected values. The expected LD mean (± mean standard deviation) for the soil isolates was 0.02 ± 0.05. The LD mean (± standard deviation) for the new soil isolates compared to the original soil isolates was 0.09 ± 0.04 (Table 4) a value expected at a low probability ($p = 0.07$) by chance. Also, the expected mean LD for the cast isolates was 0.02 ± 0.06, and the LD mean for the new cast isolates compared to the original cast isolates was 0.10 ± 0.09. The probability was 0.04 that the observed LD would ever be below the expected range. This analysis suggested that the soil and cast bacteria isolated in 1999 were different than the original collection. Although the

biotypes and the distribution of major phylogenetic groups of the culturable bacteria in the soil appeared to be stable, the distribution of genera and species of bacteria in soil may have changed over the period examined. This analysis also supported the conclusion made previously that the cast community changed between the original and new sampling times.

A possible explanation for these differences may be due to the sampling technique. During the isolation, the samples were serially diluted in growth media and the highest dilutions containing growth were plated. In earlier isolation, all colonies with different colonial morphotypes were isolated from the plates. In the new isolation, however, only the most abundant colony was taken from each plate. This selection may have made the communities look different.

Correlation analysis. The analysis of the 16S rDNA of the original soil, burrow and cast isolates agreed well with the conclusion made from the phenotypic data, which suggested that the burrow and soil communities were very similar and were much more diverse than the cast community [Furlong *et al.*, submitted]. The analyses of the phenotypic data involved comparing dissimilarity (Di) of the isolates from each habitat. Di was calculated by adding the number of phenotypic characteristics in which each pair of isolates differed. An analysis was performed to see if the dissimilarity (Di) between the phenotypes of the cast isolates was correlated with their 16S rDNA genetic distance (D). Although r was small ($r = 0.49$), this correlation was significant, because of the large number of comparisons ($n = 2,775$). Thus, as shown previously [Furlong *et al.*, submitted], the phenotypic dissimilarity (Di) was a poor predictor of phylogenetic relationships (D).

Discussion

The main focus of this study was to determine how *Lumbricus rubellus* activity affected the prokaryotic diversity of soil. It was shown that the prokaryotic community in soil was different and much more diverse than that of earthworm casts. There are at least four explanations for these differences. First, the conditions in the alimentary canal of the worms, i.e. higher moisture, organic carbon and total nitrogen content [Karsten and Drake, 1995; Daniel and Anderson, 1992] may have selected for a certain portion of the soil prokaryotic community. This model was partially supported by the LD analysis, which showed that while greater than 50% of the cast community was very similar to members of the soil community, a small portion of the cast isolates were not abundant in the soil collection. Second, there may have been an active prokaryotic community associated with the alimentary canal, and casts may have contained some of those cells shed from that community. The large numbers of *Aeromonas* spp. detected exclusively in the cast supports this model. *Aeromonas* spp. have been isolated from *L. rubellus* previously [Hendriksen, 1995], but have never been isolated from soil even though they grow well on rich media [Furlong *et al*., submitted; Hendriksen, 1995]. Their presence in the casts and absence from soil suggests that they may be associated with the earthworm. Third, since *L. rubellus* is an epigeic earthworm, it feeds on leaf litter as well as soil. The differences in the community structures of soil and casts were possibly due to the presence of the litter community in the casts. This possibility was not tested in this experiment. Fourth, the earthworms could be selectively digesting some of the bacteria. This explanation is not probable since it has been shown that numbers of bacteria are much higher (2- to 15- fold) in fresh casts of *L. rubellus* as compared to ingested soil

[Daniel and Anderson, 1992; Karsten and Drake, 1995; Kristufek *et al*., 1992]. This increase would not be expected if they were digesting the bacteria. It is quite possible that the community observed in the fresh casts resulted partially from an indigenous gut flora, partially from an enrichment of specific soil and litter bacteria during gut passage, and partially from soil and litter bacteria that passed through unchanged.

There are two explanations for the change in biotypes and community composition of the cast from 1997 and 1999. One explanation may be the difference in food source for the earthworm. Corn litter was available on the surface of the no-tilled plot during sampling in 1997 and kenaf litter was present in 1999. Since *L. rubellus* feeds partially on litter, the litter type may have influenced the gut microbial communities. Another explanation may be that the organisms found in the original cast samples were still present in the casts in 1999 and were unculturable at the time of sampling. For instance, it was surprising to find that none of the new cast isolates grouped with the γ-proteobacteria when they made up 78% of the original community. In a separate experiment by Singleton *et al*. [unpublished], a 16S rDNA clone library was constructed from DNA extracted from fresh casts that were collected in 1999. Twenty percent of the bacteria identified in this clone library were related to the γ-proteobacteria and 8 of those clones were identical to isolates collected from casts in 1997. Therefore, even though the γ-proteobacteria were not cultured in the new collection, they were abundant in the community.

When natural prokaryotic communities are examined using molecular retrieval methods, the communities look much more diverse than when culture methods are used. Typically, the large numbers of bacteria observed by molecular methods have never been

cultured or described. This observation has frequently been interpreted to mean that most microorganisms in environmental samples are unculturable [Pace, 1996]. However, an alternative explanation is that the diversity of soil microorganisms is so large that previous collections of cultures and clones have not fully sampled it, resulting in the lack of overlap between clones and cultures from the same environment.

The inability to identify and describe environmental clones originates from the fact that there are only 5,000 species of described bacteria [Bull *et al*., 1992; Wintzingerode *et al*., 1997]. The isolation and characterization of more novel soil bacteria may alleviate this discrepancy. One of the goals of this study was to isolate more of the uncultured bacteria by using culture techniques that approximated the natural environment [Furlong *et al*., submitted]. It was described previously that approximately 5-7% of the total bacteria in these soil samples were cultured using these techniques [Furlong *et al*., submitted], which is higher than what is usually reported [Torsvik *et al*., 1990]. In this study it was shown that greater than 23% of the isolates had less than 97% 16S rDNA sequence similarity to any described organism, suggesting that some novel species may have been isolated. These isolates may now be described more completely, which will help to increase the pool of described species of bacteria and our knowledge of function of bacteria in natural ecosystems. Importantly, only one isolate was found that resembled any of the deep clades of uncultured bacteria frequently detected in soil. Therefore, alternative approaches may have to be employed to describe these organisms.

References

1. **Bakken, L.R. and R.A. Olsen**. 1987. The relationship between cell size and viability of soil bacteria. Microb. Ecol. **13**:103-114.
2. **Barois, I.** 1992. Mucus production and microbial activity in the gut of two species of *Amynthas* from cold and warm tropical climates. Soil Biol. Biochem. **24**: 1507-1510.
3. **Benson DA, Boguski MS, Lipman DJ, Ostell J, Ouellette BF, Rapp BA, Wheeler DL**. 1999. Genbank. Nucleic Acids Res. **27**: 12-17.
4. **Bull, A.T., M. Goodfellow, and J.H. Slater.** 1992. Biodiversity as a source in innovation in biotechnology. Annu. Rev. Microbiol. **46**: 219-252.
5. **Daniel, O., and J.M. Anderson**. 1992. Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus* Hoffmeister. Soil Biol. Biochem. **24**:465-470.
6. **Doube, B.M., P.M. Williams, and P. Willmott**. 1995. The effect of two species of earthworm (Aporrectodea trapezoides and A. rosea) on growth of wheat, barley, and faber beans in three soil types in the greenhouse. Soil Biol. Biochem. **29**: 503-509.
7. **Edwards, C.A. and P.J. Bohlen**. 1996. Biology of Earthworms. Chapman and Hall, London.
8. **Felsenstein, J.** 1993. PHYLIP: phylogeny inference package. University of Washington, Seattle.
9. **Furlong, M.A., D.C. Coleman, and W.B. Whitman.** 2000. Comparison of bacterial isolates from fresh earthworm casts, burrows and surrounding soil. Appl. Environ. Microbiol. Submitted.
10. **Good, I. J.** 1953. The population frequencies of species and the estimation of population parameters. Biometrica **40**: 1107-1114.
11. **Hendriksen, N.B.** 1995. Effects of detritivore earthworms on dispersal and survival of the bacterium *Aeromonas hydrophila*. *Acta Zool.* Fennica **196** :115-119.
12. **Hendrix, P. F. , D. A. Crossley Jr., D. C. Coleman, R. W. Parmelee, and M. H. Beare**. 1987. Carbon dynamics in soil microbes and fauna in conventional and no-tillage agroecosystems. INTECOL Bull. **15**:59-63.
13. **Hengstmann,U., K.J.Chin, P.H Janssen,. and W Liesack.** 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. **65**:5050-5058.
14. **Hurlbert, S.H.** 1971. The nonconcept diversity: a critique and alternative parameters. Ecology. **52**:577-586.
15. **Jukes, T.H., and C.R. Cantor**. 1969. Evolution of protein molecules, pp. 21-132. *In* H.N. Munro (ed.), Mammalian protein metabolism. Academic Press Inc., New York.
16. **Karsten, G.R., and H.L. Drake.** 1995. Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soil. Appl. Environ. Microbiol. **61**: 1039-1044.
17. **Krebs, C.J.** 1989. Ecological Methodology. Harper and Row, New York.
18. **Kristufek, V., K. Ravasz and V. Pizl.** 1992. Changes in density of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa*. Soil Biol. Biochem. **24**:1499-1500.

19. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Technology in Bacterial Sysematics. John Wiley and Sons, New York.
20. **Liesack, W., P.H. Janssen, F.A. Rainey, N. Ward-Rainey, and E. Stackebrandt.** 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, p. 375-439. In J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.). Modern soil microbiology. Marcel Dekker Inc., New York, N.Y.
21. **Marilley, L. and M. Aragno**. 1999. Phylogenetic diversity of bacterial communities from different proximity to *Lolium perenne* and *Trifolium repens* roots. Appl. Soil Ecol. **13**: 127-136.
22. **McCaig,A.E., L.A Glover,. and J.I Prosser**. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. **65**:1721-1730.
23. **Pace, N**. 1996. New perspective on the natural microbial world: molecular microbial ecology. ASM News **62**:463-470.
24. **Parle, J.N**. 1963. Micro-organisms in the intestines of earthworms. J. Gen. Microbiol. **31**: 1-11.
25. **Paul, E.A., and F.E. Clark.** 1996. Soil Microbiology and Biochemistry. 2nd edn. Academic Press, San Diego.
26. **Pederson, J.C. and N.B. Hendriksen**. 1993. Effect of passage through the intestinal tract of detritivore earthworms (*Lumbricus* spp.) on the number of selected Gram-negative and total bacteria. Biol. Fertil. Soils. **16**: 227-232.
27. **Pielou, E**.C. 1977. Mathematical Ecology. Wiley, New York.p. 385.
28. **Saitou, N. and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406-425.
29. **Shannon, C.E., and W. Weaver.** 1963. The mathematical theory of communication. Univ. Illinois Press, Urbana. P.117.
30. **Stoesser, G., W. Baker, A.E. van den Broek, E. Camon, P. Hingamp, P. Sterk, and M.A. Tuli.** 2000. The EMBL nucleotide sequence database. Nucleic Acids Res. **28**: 19-23.
31. **Suyama,T., Y. Tokiwa, P. Ouichanpagdee,T. Kanagawa, and Y. Kamagata.** 1998. Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. Appl. Environ. Microbiol. **64**: 5008-5011.
32. **Tiunov, A.V., T.G. Dobrovol'skaya and L.M. Polyanskaya**. 1997. Microbial community of the *Lumbricus terrestris* L. earthworm burrow walls. Microbiol. (N.Y.) **66**: 349-353.
33. **Tiwari, S.C., B.K. Tiwari and R.R. Mishra**. 1989. Micrbial populations, enzyme activities and nitrogen-phosphorus-potassium enrichment in earthworm casts and in the surrounding soil of a pineapple plantation. Biol. Fertil. Soils. **8**: 178-182.
34. **Torsvik, V., J. Goks_yr and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.
35. **Trigo, D. and P. Lavelle**. 1993. Changes in respiration rate and some physicochemical properties of soil during gut transit through *Allolobophora molleri* Biol. Fertil. Soils. **15**: 185-188.

36. **Wintzingerode, F.V., U.B. Gbel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21**: 213-229.

Table 1. Proportion of soil, cast, and burrow isolates in different phylogenetic groups.

Phylogenetic Groups Represented	Original isolates[a, b] Soil (n = 99)	Cast (n = 74)	Burrow (n = 31)	New isolates[c] Soil (n = 14)	Cast (n = 12)
High G+C Gram Positive	0.54	0.12	0.48	0.57	0.75
Low G+C Gram Positive	0.15	0.04	0.19	0.21	0.17
α-Protoeobacteria	0.05	0.01	0.07	0.07	0.00
β-Proteobacteria	0.05	0.04	0.10	0.07	0.08
γ-Proteobacteria	0.21	0.78	0.13	0.07	0.00
Cytophaga/Flexibacter	0.00	0.01	0.00	0.00	0.00
Unknown Taxon	0.00	0.00	0.03	0.00	0.00

[a]A Chi-square analysis indicated that the composition of the soil community was significantly different from that of the cast, but not different from that of the burrow at the 0.05 significance level.
[b]Original cast isolates were collected in 1997 and original soil isolates were collected in 1996.
[C]New cast and soil isolates were collected in 1999.

Table2. Similarity of 16S rDNA of isolates to previously described organisms

% similarity	Number of Isolates		
	Soil	Cast	Burrow
> 99.0	27	30	6
99.0 – 97.5	52	41	22
97.4 – 96.5	24	11	2
≤ 95.4	10	4	1

Table 3. Comparison of diversity indices for standardized samples of the original soil isolates with cast and burrow isolates.

Diversity index[b]	Sample of 74 isolates[a]				Sample of 31 isolates[a]			
	Cast	Soil	Index Minimum[c]	Index Maximum[c]	Burrow	Soil	Index Minimum[c]	Index Maximum[c]
% Coverage	0.80	0.57	0.00	1.00	0.32	0.32	0.00	1.00
Diversity	2.47	3.65	0.00	4.30	3.21	3.21	0.00	3.43
Evenness	1.81	2.18	0.00	2.30	2.27	2.27	0.00	2.30

[a]For all indices, the numbers of isolates in individual collections were reduced to 74 and 31 by randomly removing sequences from larger collections. This standardized the data sets so that they could be compared accurately.
[b]Coverage, diversity, and evenness were calculated using standard formulas [Good, 1953; Pielou, 1977; Shannon and Weaver, 1963].
[C]Values are the minimum and maximum values which could be obtained for isolates containing n sequences at minimum diversity and maximum diversity.

Table 4. Lowest Distance (LD) analysis for new isolates.

Collections being compared	LD mean ± standard deviation	Expected LD mean ± standard deviation of the mean[2]	P-value[4]
New soil with original soil	0.09±0.04	0.02±0.05	0.07
New cast with original cast	0.10±0.09	0.02±0.06	0.04
New soil with original cast	0.13±0.10	0.02±0.06	0.04
New cast with original soil	0.09±0.04	0.02±0.05	0.07

[1]Lowest distance value of an isolate when compared to a group of isolates.
[2]Expected LD mean ± standard deviation were calculated from the mean LD of 100 random sub-samples of 14 and 12 original soil and cast isolates, respectively. Only isolates from MMSA media were used.
[3]The probability that the expected LD would exceed the observed LD was estimated from the distribution of the expected mean values in 100 random samples.

Figure 1. Neighbor joining tree showing the relationship of isolates to reference members of the high G + C Gram positive organisms based on analysis of 388 bases of aligned 16S rDNA sequences. Soil, cast, burrow, new soil and new cast OTUs are all in bold and begin with the prefix S, C, B, NS, or NC, respectively. The number in parentheses after each OTU designation refers the number of isolates within that OTU. The number in parentheses after each reference sequence refers to the accession number. Bootstrap values that are greater than 60% are indicated at the nodes. The scale bar represents Jukes Cantor distance. This tree was rooted with *Escherichia coli*.

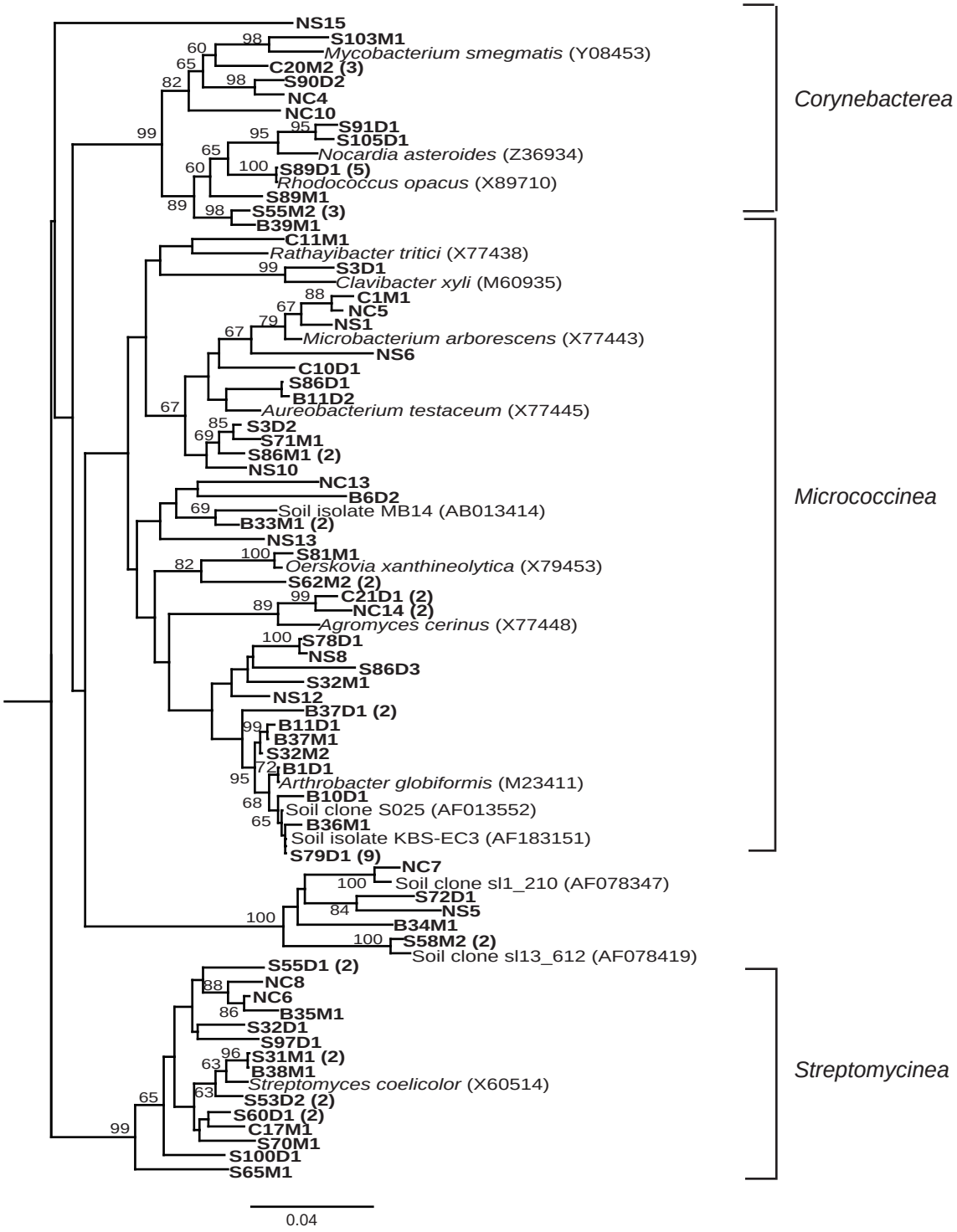

NS15
S103M1
Mycobacterium smegmatis (Y08453)
C20M2 (3)
S90D2
NC4
NC10
S91D1
S105D1
Nocardia asteroides (Z36934)
S89D1 (5)
Rhodococcus opacus (X89710)
S89M1
S55M2 (3)
B39M1
Corynebacterea
C11M1
Rathayibacter tritici (X77438)
S3D1
Clavibacter xyli (M60935)
C1M1
NC5
NS1
Microbacterium arborescens (X77443)
NS6
C10D1
S86D1
B11D2
Aureobacterium testaceum (X77445)
S3D2
S71M1
S86M1 (2)
NS10
NC13
B6D2
Soil isolate MB14 (AB013414)
B33M1 (2)
NS13
S81M1
Oerskovia xanthineolytica (X79453)
S62M2 (2)
C21D1 (2)
NC14 (2)
Agromyces cerinus (X77448)
S78D1
NS8
S86D3
S32M1
NS12
B37D1 (2)
B11D1
B37M1
S32M2
B1D1
Arthrobacter globiformis (M23411)
B10D1
Soil clone S025 (AF013552)
B36M1
Soil isolate KBS-EC3 (AF183151)
S79D1 (9)
Micrococcinea
NC7
Soil clone sl1_210 (AF078347)
S72D1
NS5
B34M1
S58M2 (2)
Soil clone sl13_612 (AF078419)
S55D1 (2)
NC8
NC6
B35M1
S32D1
S97D1
S31M1 (2)
B38M1
Streptomyces coelicolor (X60514)
S53D2 (2)
S60D1 (2)
C17M1
S70M1
S100D1
S65M1
Streptomycinea
0.04

Figure 2. Neighbor joining tree showing the relationship of isolates to reference members of the γ-proteobacteria based on analysis of 384 bases of aligned 16S rDNA sequences. Soil, new soil, cast, new cast and burrow OTUs are all in bold and begin with the prefix S, NS, C, NC, or B, respectively. The number in parentheses after each OTU designation refers the number of isolates within that OTU if there are more than one. The number in parentheses after each reference sequence refers to the accession number. Bootstrap values that are greater than 60% are indicated at the nodes. The scale bar represents Jukes Cantor distance. This tree was rooted with *Clostridium innocuum*.

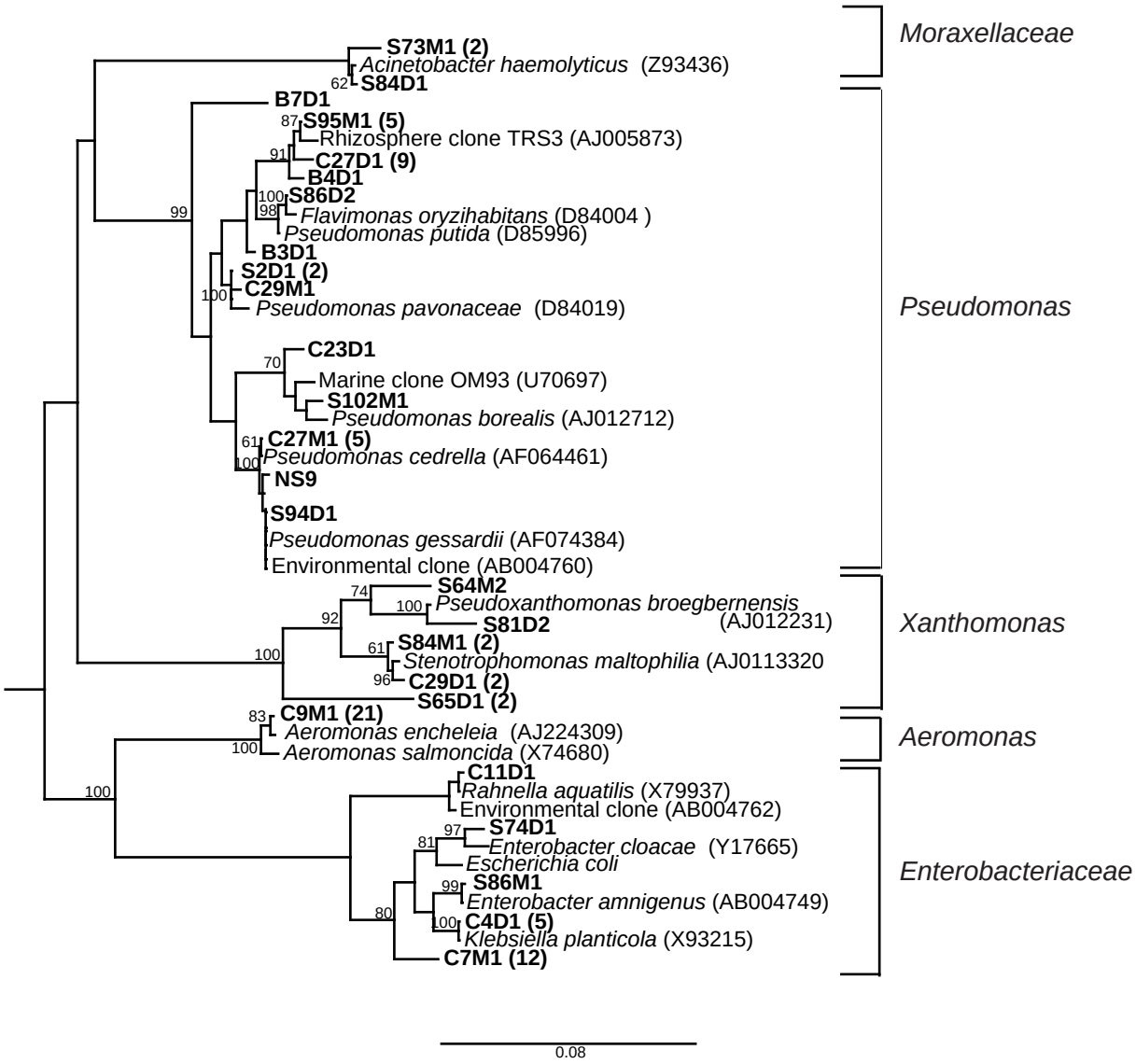

S73M1 (2)
Acinetobacter haemolyticus (Z93436)
S84D1
B7D1
S95M1 (5)
Rhizosphere clone TRS3 (AJ005873)
C27D1 (9)
B4D1
S86D2
Flavimonas oryzihabitans (D84004)
Pseudomonas putida (D85996)
B3D1
S2D1 (2)
C29M1
Pseudomonas pavonaceae (D84019)
C23D1
Marine clone OM93 (U70697)
S102M1
Pseudomonas borealis (AJ012712)
C27M1 (5)
Pseudomonas cedrella (AF064461)
NS9
S94D1
Pseudomonas gessardii (AF074384)
Environmental clone (AB004760)
S64M2
Pseudoxanthomonas broegbernensis
(AJ012231)
S81D2
S84M1 (2)
Stenotrophomonas maltophilia (AJ0113320
C29D1 (2)
S65D1 (2)
C9M1 (21)
Aeromonas encheleia (AJ224309)
Aeromonas salmoncida (X74680)
C11D1
Rahnella aquatilis (X79937)
Environmental clone (AB004762)
S74D1
Enterobacter cloacae (Y17665)
Escherichia coli
S86M1
Enterobacter amnigenus (AB004749)
C4D1 (5)
Klebsiella planticola (X93215)
C7M1 (12)
Moraxellaceae
Pseudomonas
Xanthomonas
Aeromonas
Enterobacteriaceae
0.08

Figure 3. Neighbor joining tree showing the relationship of isolates to reference members of the low G + C Gram positive organisms based on analysis of 392 bases of aligned 16S rDNA sequences. Soil, cast, burrow, new cast, or new soil OTUs are all in bold and begin with the prefix S, C, B, NC, or NS, respectively. The number in parentheses after each OTU designation refers the number of isolates within that OTU. The number in parentheses after each reference sequence refers to the accession number. Bootstrap values that are greater than 60% are indicated at the nodes. The scale bar represents Jukes Cantor distance. This tree was rooted with *Escherichia coli*.

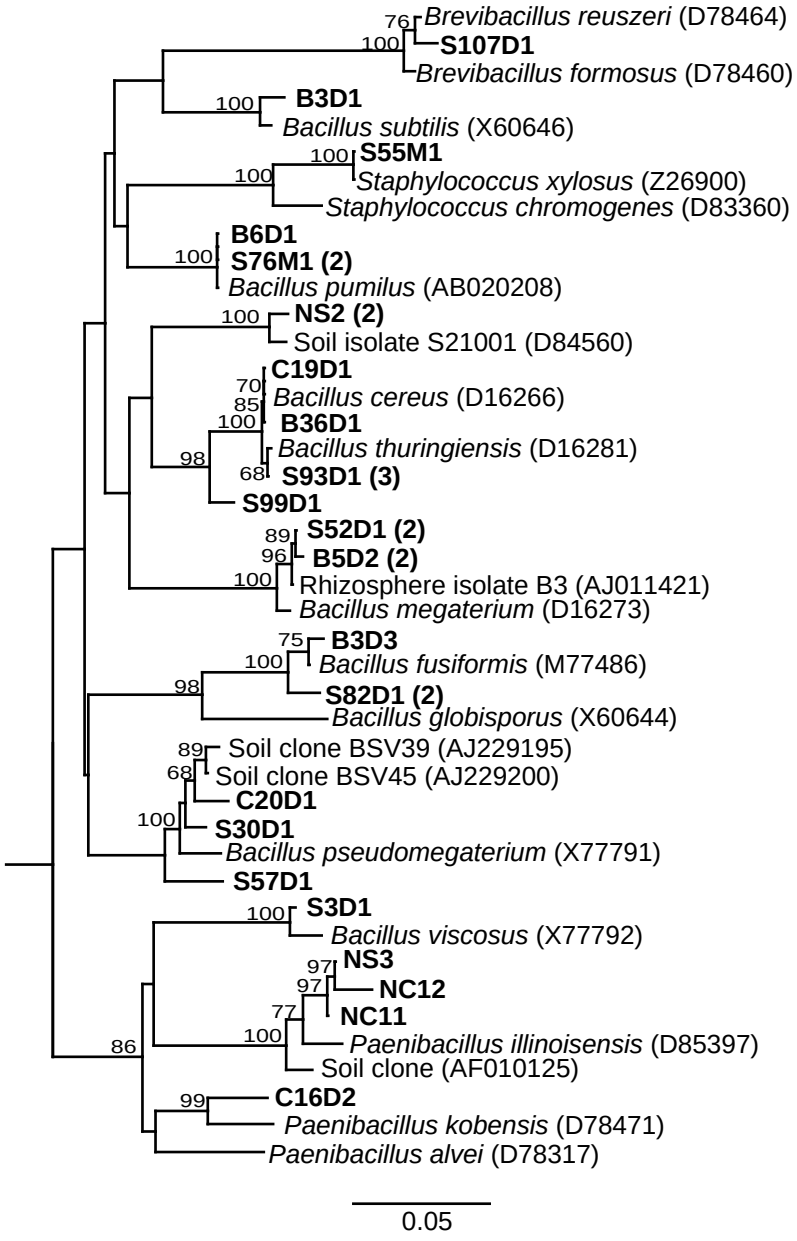

76
Brevibacillus reuszeri (D78464)
100
S107D1
Brevibacillus formosus (D78460)
100
B3D1
Bacillus subtilis (X60646)
100
S55M1
100
Staphylococcus xylosus (Z26900)
Staphylococcus chromogenes (D83360)
B6D1
100
S76M1 (2)
Bacillus pumilus (AB020208)
100
NS2 (2)
Soil isolate S21001 (D84560)
C19D1
70
Bacillus cereus (D16266)
85
100
B36D1
Bacillus thuringiensis (D16281)
98
68
S93D1 (3)
S99D1
89
S52D1 (2)
96
B5D2 (2)
100
Rhizosphere isolate B3 (AJ011421)
Bacillus megaterium (D16273)
75
B3D3
100
Bacillus fusiformis (M77486)
98
S82D1 (2)
Bacillus globisporus (X60644)
89
Soil clone BSV39 (AJ229195)
68
Soil clone BSV45 (AJ229200)
C20D1
100
S30D1
Bacillus pseudomegaterium (X77791)
S57D1
100
S3D1
Bacillus viscosus (X77792)
97
NS3
97
NC12
77
NC11
100
Paenibacillus illinoisensis (D85397)
86
Soil clone (AF010125)
99
C16D2
Paenibacillus kobensis (D78471)
Paenibacillus alvei (D78317)
0.05

Figure 4. Neighbor joining tree showing the relationship of isolates to reference members of the β-proteobacteria based on analysis of 391 bases of aligned 16S rDNA sequences. Soil, cast, burrow, new cast and new soil OTUs are all in bold and begin with the prefix S, C, B, NC and NS, respectively. The number in parentheses after each OTU designation refers the number of isolates within that OTU. The number in parentheses after each reference sequence refers to the accession number. Bootstrap values that are greater than 60% are indicated at the nodes. The scale bar represents Jukes Cantor distance. This tree was rooted with *Clostridium innocuum*.

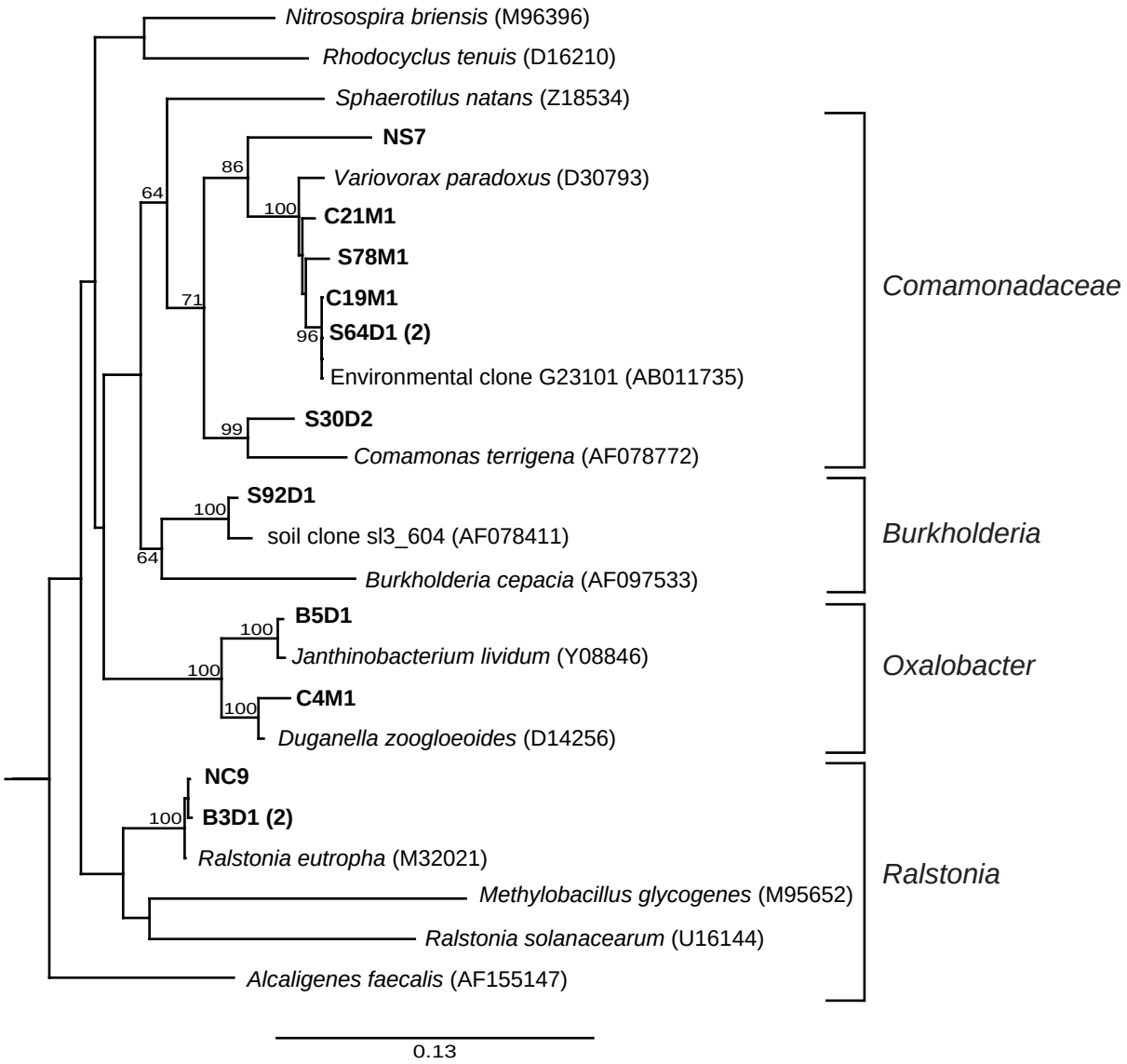

Nitrosospira briensis (M96396)
Rhodocyclus tenuis (D16210)
Sphaerotilus natans (Z18534)
NS7
Variovorax paradoxus (D30793)
C21M1
S78M1
C19M1
S64D1 (2)
Environmental clone G23101 (AB011735)
S30D2
Comamonas terrigena (AF078772)
S92D1
soil clone sl3_604 (AF078411)
Burkholderia cepacia (AF097533)
B5D1
Janthinobacterium lividum (Y08846)
C4M1
Duganella zoogloeoides (D14256)
NC9
B3D1 (2)
Ralstonia eutropha (M32021)
Methylobacillus glycogenes (M95652)
Ralstonia solanacearum (U16144)
Alcaligenes faecalis (AF155147)
86
64
100
71
96
99
100
64
100
100
100
100
Comamonadaceae
Burkholderia
Oxalobacter
Ralstonia
0.13

Figure 5. Neighbor joining tree showing the relationship of isolates to reference members of the α-proteobacteria based on analysis of 379 bases of aligned 16S rDNA sequences. Soil, cast, burrow, new cast and new soil OTUs are all in bold and begin with the prefix S, C, B, NC, or NS, respectively. The number in parentheses after each OTU designation refers the number of isolates within that OTU. The number in parentheses after each reference sequence refers to the accession number. Bootstrap values that are greater than 60% are indicated at the nodes. The scale bar represents Jukes Cantor distance. This tree was rooted with *Clostridium innocuum*.

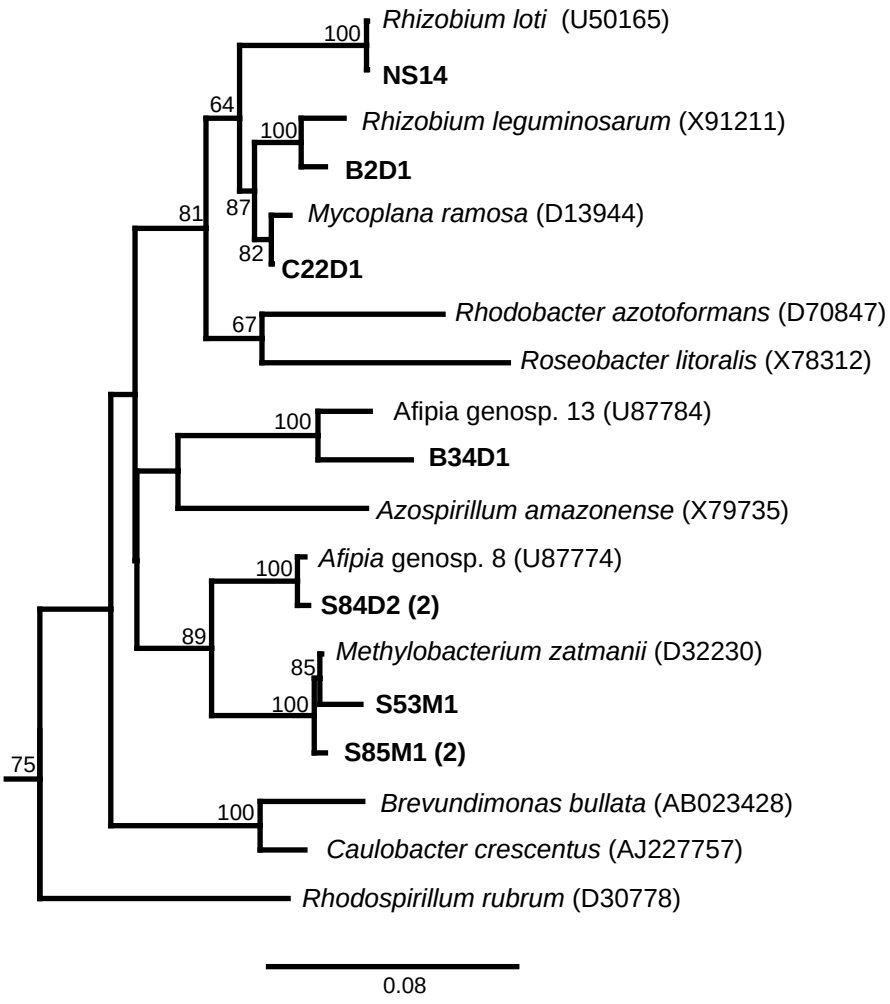

100
Rhizobium loti (U50165)
NS14
64
100
Rhizobium leguminosarum (X91211)
B2D1
81
87
Mycoplana ramosa (D13944)
82
C22D1
67
Rhodobacter azotoformans (D70847)
Roseobacter litoralis (X78312)
100
Afipia genosp. 13 (U87784)
B34D1
Azospirillum amazonense (X79735)
100
Afipia genosp. 8 (U87774)
S84D2 (2)
89
Methylobacterium zatmanii (D32230)
85
100
S53M1
S85M1 (2)
75
100
Brevundimonas bullata (AB023428)
Caulobacter crescentus (AJ227757)
Rhodospirillum rubrum (D30778)
0.08

Figure 6. Phylogenetic richness curves. The expected number of OTUs found in different sample sizes of the soil (●), cast (◆) and burrow (Δ) isolates was calculated by rarefaction (Hurlbert, 1971; Krebs, 1989).

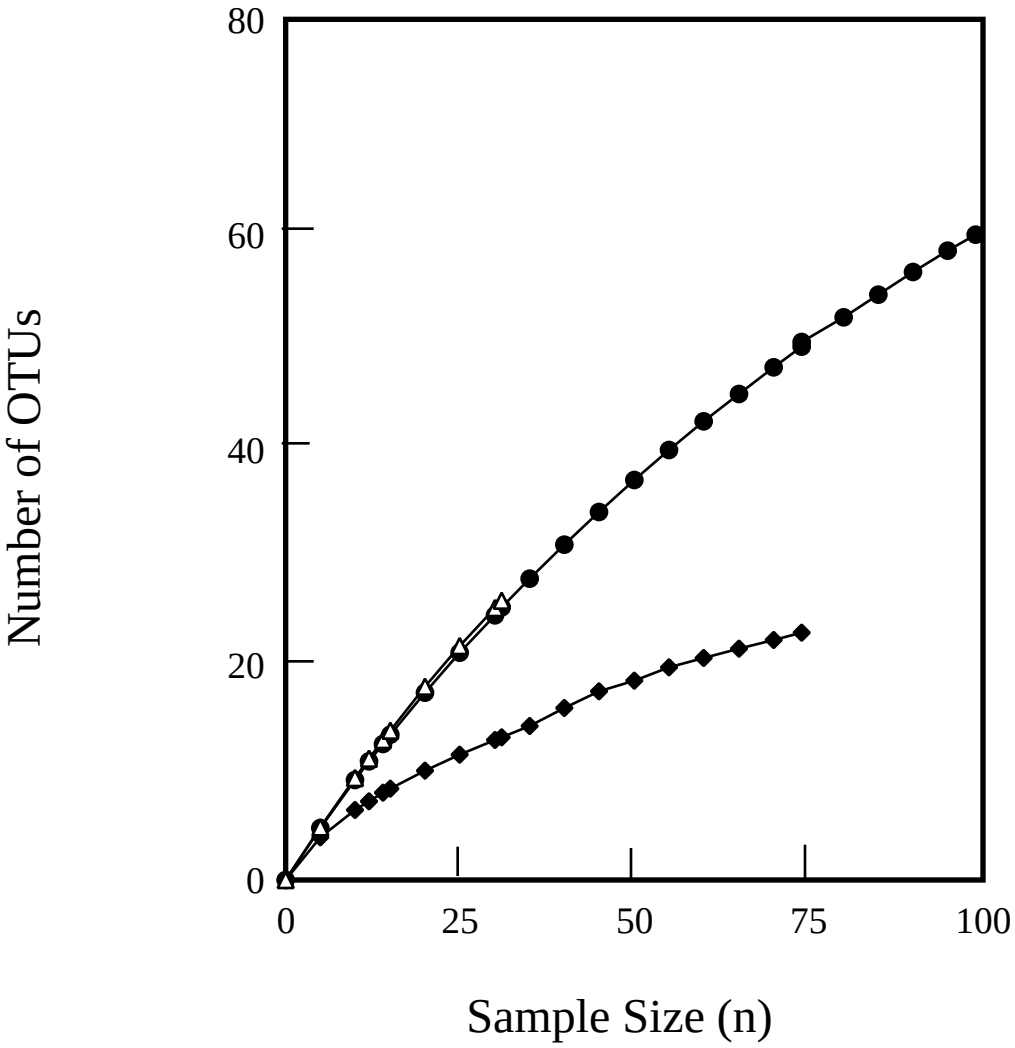

80
60
40
20
0
0
25
50
75
100
Number of OTUs
Sample Size (n)

Figure 7. Coverage of the culturable prokaryotes in soil (Δ), casts (■), and burrows (❍) at different distance (D) values. Coverage (C) was calculated by modifying Good's formula (Good, 1953) so that C = 1-(n-S) / n, where n = number of isolates and S = number of OTUs. OTU was defined using the various values of D.

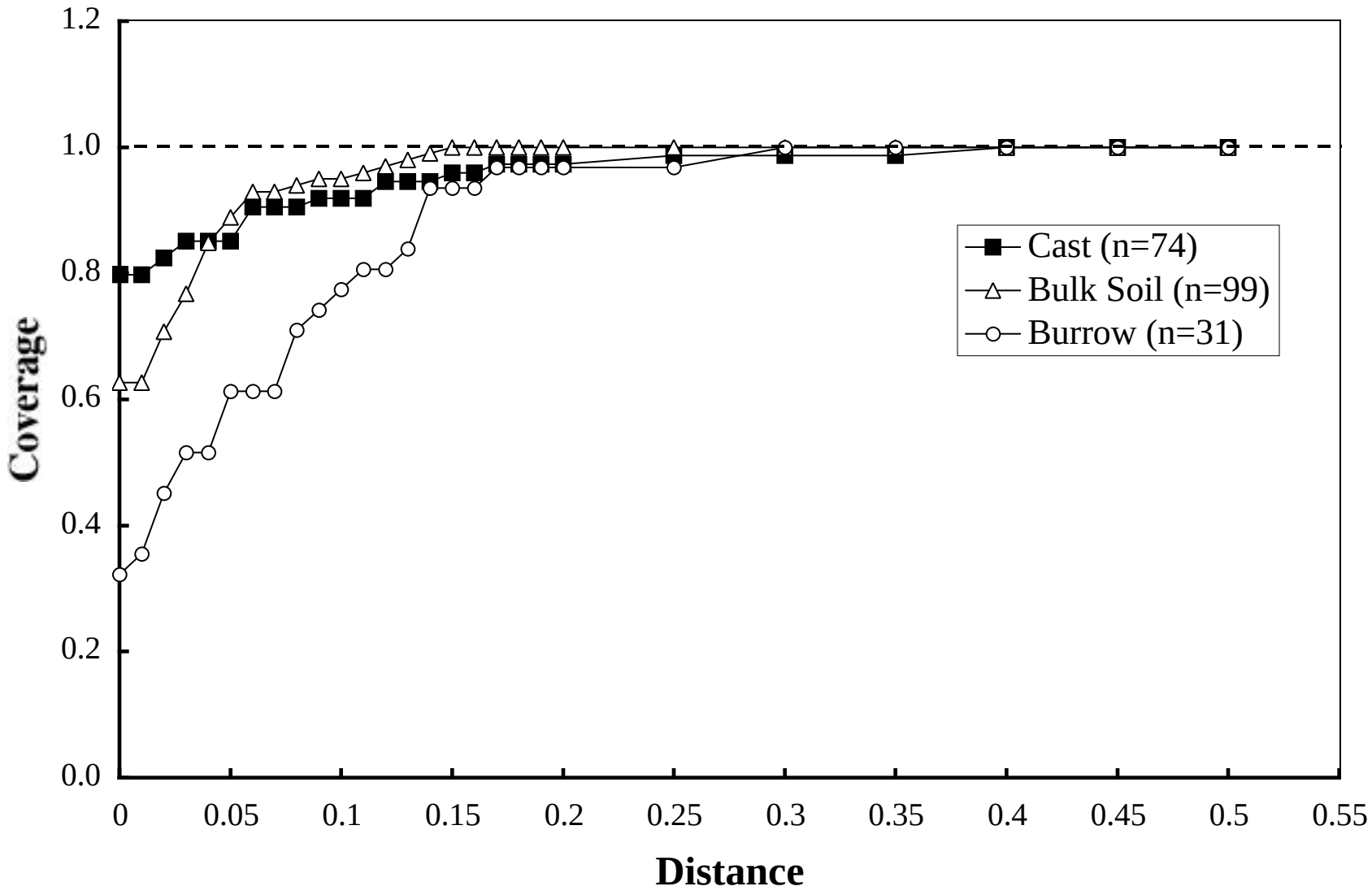
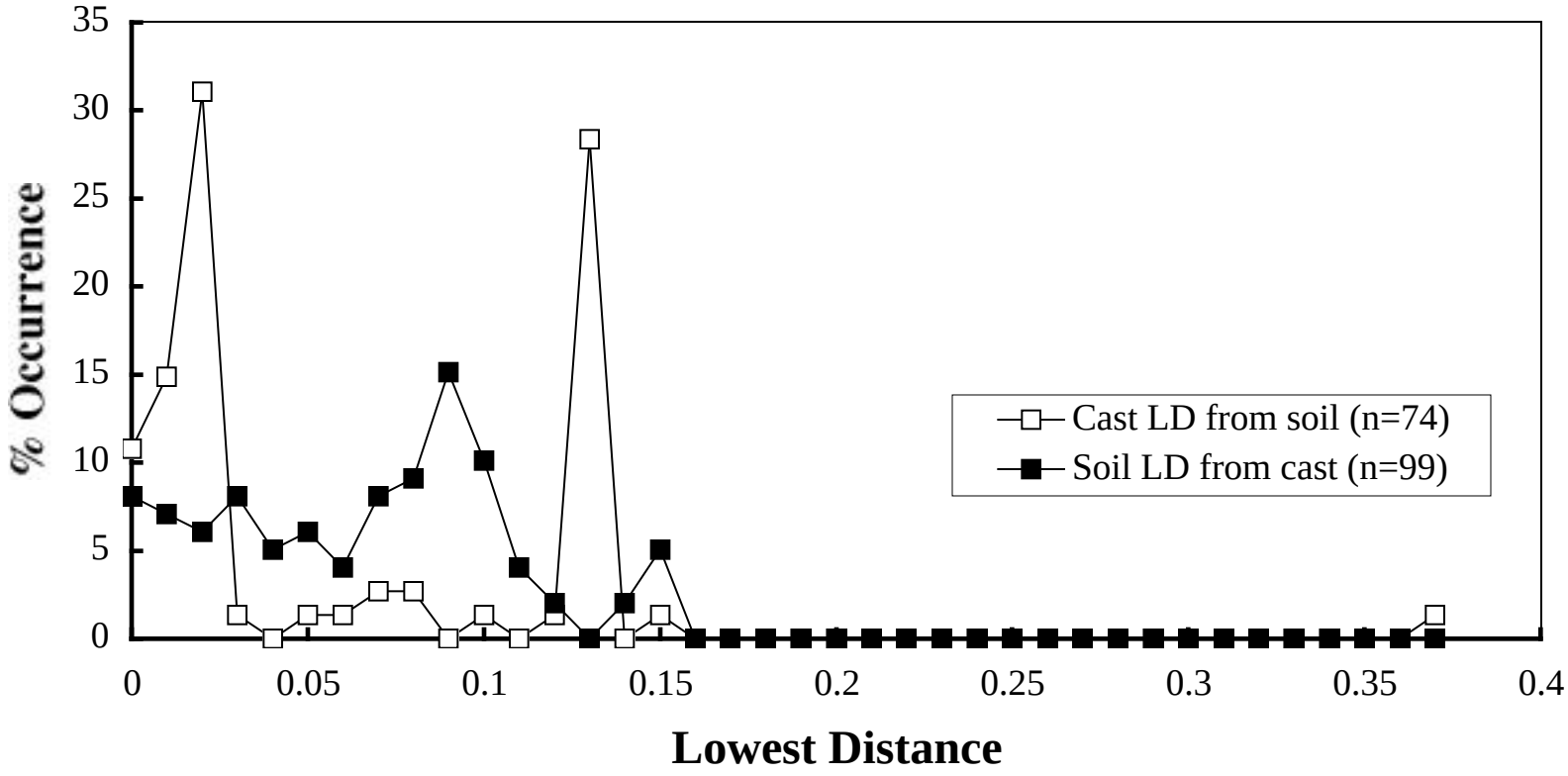

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Coverage
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0.55
Distance
Cast (n=74)
Bulk Soil (n=99)
Burrow (n=31)

Figure 8. Lowest distance (LD) between cast (□) and soil (■) isolates mapped onto each other. LD values were calculated from a matrix of Jukes Cantor distance values occurring between soil and cast isolates.


35
30
25
20
15
10
5
0
% Occurrence
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
Lowest Distance
Cast LD from soil (n=74)
Soil LD from cast (n=99)

CHAPTER IV

COMPARISON OF BACTERIAL AND ARCHAEAL 16S RIBOSOMAL DNA FROM TCE CONTAMINATED AND UNCONTAMINATED GROUNDWATER[3]

[3] Furlong, M.A., R.L. Brigmon, and W.B. Whitman. 2000. To be submitted.

Abstract

DNA was extracted from two groundwater samples. One sample was from a well within a region in an aquifer that had been contaminated with trichloroethylene (TCE) containing industrial waste (well 62D) and the other was from an uncontaminated region in the same aquifer (well 43B). Two 16S rDNA clone libraries were constructed from the bacterial DNA. Eight and forty-one clones from 43B and 62D, respectively, were sequenced. The 16S rDNA sequences from all of the 43B clones were >99% similar to *Pseudomonas gessardi*. The DNA from eighteen unsequenced clones from 43B were digested with *Eco* R1 and all showed the same DNA banding pattern as the other clones. Therefore, 1 ribotype represented all 26 clones from 43B. Eighteen ribotypes resulted from the forty-one clones from 62D and one was a chimera. Sixty-five, thirty-three, and three percent of the 62D clones were related to the β- and γ- proteobacteria and an unknown group, respectively. The majority of the γ- proteobacteria (85%) grouped with the methanotrophic Methylococcaceae family and the remainder grouped with the pseudomonads. Forty-six percent of the β-proteobacteria grouped with methylotrophs and the remainder grouped with various genera in the order Burkholderiales. Archaeal DNA was detected in 63D and not 43B. One ribotype represented the fifteen archaeal clones that were sequenced. This ribotype grouped with the crenarchaeota.

Introduction

Trichloroethylene (TCE) contamination of groundwater has become a major problem in industrial nations. This compound is widely used in industrial processes and

is the most frequently found chemical contaminant of groundwater. This contamination has become a serious threat because groundwater is an important source for drinking water and agricultural irrigation and TCE is toxic to animals.

Cleanup of TCE contaminated groundwater to mandated levels (below 5µg/l) [Federal Register, 1989] using intrinsic bioremediation is less expensive and time consuming than air sparging or vacuum extraction methods [Kao and Prosser, 1999; Reddy and Adams, 1998; Travis and Rosenberg, 1997]. One intrinsic bioremediation method involves stimulating the growth of methanotrophic bacteria by injecting methane and other nutrients, which these organisms require for growth, into the contaminated aquifer. Methanotrophic bacteria use the enzyme methane monooxygenase to cometabolize methane with other organic compounds, including TCE [Dalton, 1992]. Since these bacteria are ubiquitous in natural environments [Hanson and Wattenberg, 1991] and grow well in the presence of methane and TCE, it seems likely that their growth could be stimulated in a TCE contaminated environment by adding methane.

Intrinsic bioremediation technology was demonstrated on a TCE contaminated aquifer at the Department of Energy's Savannah River site (SRS) in 1992 and 1993 [Travis and Rosenberg, 1997; Brockman, *et al.*, 1995]. Methanotrophs were targeted for enrichment using air, methane, nitrous oxide and triethyl phosphate injections. Evidence for an increase in TCE degrading methanotrophic bacteria and TCE mineralization was found following these injections [Travis and Rosenberg, 1997; Brockman, *et al.*, 1995; Bowman, *et al.*, 1993].

Although methanotrophs are important to the oxidation of TCE in these experiments, other heterotrophic prokaryotes are important for the complete mineralization of TCE to CO_2 [Uchiyama, *et al.*, 1992]. Little is known about the community structure of TCE-contaminated groundwater and less is known about the effect of the nutrient injections on the prokaryotic community of groundwater. Information about the groundwater prokaryotic community may be helpful in designing more efficient TCE biodegradation methods at contaminated sites.

Other TCE-contaminated aquifers at the SRS are being studied to learn more about the effect of TCE and methane injections on the prokaryotic community of groundwater. The long term goals of the project are to determine the differences in the prokaryotic community structure within and outside the zone of TCE contamination before and after nutrient injections. In this portion of the project the prokaryotic community in TCE contaminated groundwater was compared to that from uncontaminated groundwater in the same aquifer by analyzing the 16S rDNA of the indigenous prokaryotes.

Materials and Methods

Sample collection. An 8 liter water sample was taken from a monitoring well (43B) adjacent to the landfill and outside of the plume of TCE and other chlorinated hydrocarbon contaminants. Likewise, a 10 liter water sample was taken from a well (62D) adjacent to the same landfill and inside of the plume of contaminants. Each sample was filtered sequentially through filters with pore sizes of 2.7µm, 1.0µm, 0.7µmm, and 0.2µm, respectively. No cells were visible on the 2.7µm pore size filters. Less than 3

cells/field were visible on the 0.7 and 1.0 µm pore size filters. The 0.2µm pore size filters contained the most cells on their surface (>100 cells/field), therefore these filters were used to examine the microbial communities in the groundwater.

DNA extraction. The filters were transported on ice and stored at –70 °C for 1 week before processing. Filters were crushed with a sterile spatula in a sterile centrifuge tube. Lysis solution [2 ml of 0.15M NaCl, 0.1M NaEDTA (pH 8.0), 1.5 mg / ml of chicken egg white lysozyme (Sigma, 73,000 units/mg protein) and 25 mg/ml PVPP] was added, the tube was mixed briefly, and the suspension was incubated at 37 °C for 1 hour with additional mixing every 15 minutes. A 0.5ml portion of the aqueous suspension was transferred to a 1.5 ml microfuge tube and 400 µl of bead-beating solution [0.1M NaCl, 0.5M Tris-HCl (pH 8.0), 10% SDS] and 0.1 g of 0.1 mm glass beads were added. The tubes were shaken on a Turbo Mixer (Scientific Industries, Inc., Bohemia) for 3 minutes and centrifuged at maximum speed in a microcentrifuge for 3 minutes. The aqueous layer was transferred to a new tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The aqueous layer was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1, pH 8.0). The DNA was precipitated with a 0.6 volume of isopropanol at -20 °C overnight. The nucleic acid was collected by centrifugation and dried under vacuum. The pellet was suspended in 50µl distilled and deionized H_2O with 1µl RNAse A solution [0.5 units/µl DNAse-free RNAse A from bovine pancreas (Boehringer Mannheim, Indianapolis), 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl] and incubated at 37° C for 1 hour. A Wizard DNA Cleanup

kit (Promega, Madison) was used to further purify the DNA before the PCR. Each filter yielded approximately 0.5 µg of DNA in 50 µl.

PCR and Cloning. The DNA from wells 62D and 43B were each subjected to two PCR amplification reactions. The 16S ribosomal DNA was PCR amplified using Ready-To-Go PCR Beads (Pharmacia Biotech, Piscataway). In the first reaction, the bacterial primer 27f, 5'-AGA GTT TGA TCM TGG CTC AG-3' [Lane, 1991] and the universal primer 1392r, 5'-ACG GGC GGT GTG TRC-3' [Lane, 1991] were used. In the second reaction, the archaeal primer 21f, 5'-TTC CGG TTG ATC CYG CCG GA-3' and the universal primer 1392r [Lane, 1991] were used. Template DNA was added to a reaction mixture at a volume of 2 µl (0.02 µg), and each primer was added at a concentration of 0.8 µM. The reaction mixtures were heated for 5 minutes at 94 °C. The denaturation, elongation, and annealing conditions were 1 minute at 94 °C, 2 minutes at 72 °C and 1 minute at 61 °C. The PCR products were electrophoresed on a 0.8% agarose gel and purified from gel slices using Prep-A-Gene DNA Purification Systems (Biorad, Hercules). The amplified DNA was ligated into vector pCR 2.0 (Invitrogen, Carlsbad) and transformed into *E. coli Top 10* cells. The PCR inserts in the resulting clones were sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia. Primers 27f and 21f were used to sequence the bacterial and archaeal clones, respectively. Approximately 600 base pairs from each clone were sequenced. The clone SRS62DBA50 was further sequenced with the bacterial primer 327f (5'-ACC GCT TGT GCG GGC CC-3') [DeLong *et al.*, 1988] and the universal primer 1392r to yield a

sequence with 1,298 nucleotides. The clone SRS62DAR03 was further sequenced with the universal primers 21f, 1392r, 519r (5'-GWA TTA CCG CGG CKG CTG-3'), 907r (5'-CCG TCA ATT CMT TTR AGT TT-3'), and 926f (5'-AAA CTY AAA KGA ATT GAC GG-3') [Lane, 1991] to yield a sequence with 1,346 nucleotides.

Phylogenetic Analysis. A FastA search was performed on all sequences to determine which sequences in the GenBank and EMBL databases [Benson *et al.*, 1999; Stoesser *et al.*, 2000] were most similar. All sequences were compared to each other, and if duplicate sequences occurred (>99.6% sequence similarity), then only one of each type were used for further analysis. The program Check Chimera, from the Ribosomal Database Project [Maidak *et al.*, 1999], was used to determine if any of these sequences were chimeric. Phylip 3.5 [Felsenstein, 1993] was used to construct phylogenetic trees on all remaining sequences. The Jukes Cantor formula was used to calculate distances, and the Neighbor joining, Parsimony, and Fitch-Margoliash algorithms were used to calculate the phylogenetic relationships.

Results

Uncontaminated Groundwater Community from Well 43B. Approximately 130 colonies containing PCR-amplified bacterial 16S rDNA were obtained from the sample from the uncontaminated well, 43B. Eight of these clones were sequenced, and they were virtually identical. Four of the sequences had exactly the same sequence, and four contained one or two different nucleotides out of 550 positions examined. This level of difference is within the range expected from Taq DNA polymerase error [Takai and Horikoshi, 1999]**.** Since the sequences were greater than 99.6% similar to each other, only

one was chosen for subsequent analyses (SRS43BBA18). Plasmids representing 18 additional clones from well 43B were digested with *Eco* R1, and the clones were found to contain a DNA banding pattern identical to that of the sequenced clones. Therefore, all 26 clones examined from well 43B examined by either sequencing or restriction endonuclease pattern appeared to be from closely related organisms (Table 1).

The sequence of one representative clone of this group, SRS43BBA18, was 99.5% similar to that of *Pseudomonas gessardii*. Phylogenetic analysis placed SRS43BBA18 with *P. gessardii* and *Pseudomonas libaniensis* in the *Pseudomonas* group of the gamma proteobacteria (Figure 1). *P. gessardii* and *P. libaniensis* were first isolated from spring water from France and Lebanon, respectively [Verhille *et al.*, 1999; Dabboussi *et al.*, 1999].

No PCR products were found after amplification of the sample from well 43B with the archaeal primer, even after increasing the template DNA amount to 0.05 µg. Therefore, it was assumed that well 43B contained very few archaea.

TCE-Contaminated Groundwater Community fromWell 62D.

Approximately 150 colonies containing PCR-amplified bacterial 16S rDNA were obtained from the sample from well 62D, and forty-one of these clones were sequenced. One sequence was found to be chimeric and was eliminated from further analysis. When the remaining sequences were compared to each other, seventeen ribotypes were found (Table 1). Sequences SRS62DBA43, -11, -07, -03, -19, -09, -21, and -05 were related to the beta proteobacteria, and sequences SRS62DBA39, -44, -37, -10, -32, -12, -24, and -01 were related to the gamma proteobacteria**.** The sequences with the highest similarity to clone

SRS62DBA50 were from 4 uncultured and unclassified eubacterial clones found in a hydrocarbon- and chlorinated-solvent-contaminated aquifer, which possessed only 87-90% sequence similarity. Sequence SRS62DBA50 had less than 75% sequence similarity to the sequences of any cultured organism in the database.

To determine the most closely related sequences, the clones from the gamma and beta proteobacterial groups and clone SRS62DBA50 were analyzed separately. Within the gamma proteobacteria (Figure 1), SRS62DBA01, -24, -12, -32, -10, and -37 grouped consistently with the Methylococcaceae family. In 93% of the replicates SRS62DBA1, -24 grouped with a cluster of *Methylomonas* species. SRS62DBA12, -32, -10, and -37 always grouped together with *Methylobacter psychrophilus* and isolate BB5.1, but the branching order within this clade varied. Clones SRS62DBA39 and -44 consistently clustered with the *Pseudomonas* species. Although the branching order within these two clades differed sometimes, SRS62DBA39 was always found clustered with *P. lundensis, P. fluorescens, P. brassicacearum* and *P. syringae,* and SRS62DBA44 was always found grouped with *P. libaniensis* and *P. gessardii*. Very similar results were also obtained by parsimony and the Fitch-Margoliash analyses. Using these algorithms, the SRS clones grouped with the same organisms and clones as found with the neighbor-joining analysis, and differences occurred only within the deepest branches of the trees.

Within the beta proteobacteria (Figure 2), SRS62DBA21 and -05 clustered with the methylotrophs in the beta proteobacteria group 85% of the time; otherwise it grouped with the *Thiobacillus* group. SRS62DBA43, -11, -07 and -03 clustered consistently with *Psuedomonas mephitica* and *Janthinobacterium lividum*. However, the branching order

of SRS62DBA07 and -03 varied, which caused some low bootstrap values within this clade. SRS62DBA19 and -09 clustered with *Pseudomonas lemoignei*, but the branching order within this group changed. Similar results were also obtained by parsimony and the Fitch-Margoliash analyses. In these analyses, SRS62DBA09 and –19 remained associated with the clade including *Herbaspirillum*, *Janthinobacterium*, and *Duganella.* However, -09 and –19 did not always associate with *P. lemoignei* and branched deeper within the clade instead. Moreover, within the clade that contained SRS62DBA03 and -07 the branching order varied.

The 500 base pair sequence that resulted from clone SRS62DBA50 possessed only low similarity to other sequences in the Genbank and EMBL databases (Benson *et al.*, 1999; Stoesser *et al.*, 2000), and 798 more positions of this clone were sequenced. A phylogenetic tree was constructed with the almost complete 16S rDNA sequence of SRS62DBA50 and sequences from a diverse collection of cultured prokaryotes (data not shown). SRS63DBA50 did not group consistently with any group of organisms. It showed a very weak association with members of the Gram positive group when the tree was constructed with the neighbor joining or Fitch-Margoliash criterion, but the bootstrap value for this association was below 50. This association was not observed in the tree constructed by parsimony.

The program Check Chimera indicated that the SRS62DBA50 sequence was not a chimera, but other analyses were done to support this finding. FastA analyses were used on several different fragments of the sequence and each fragment had relatively the same % sequence similarity to the same sequences in the Genbank and EMBL databases

[Benson *et al.,* 1999; Stoesser *et al.,* 2000]. A program called mfold 3.0 [Zuker *et al.,* 1999 and Mathews *et al.,* 1999] was used to predict the secondary structure of the sequence. This secondary structure contained many of the same features as an *E. coli* 16S rRNA secondary structure. Since the sequence folded into a recognizable 16S rRNA molecule and did not have small regions within it that had high sequence similarity to other sequences, SRS62DBA50 was not a chimera.

Approximately 160 clones containing PCR-amplified archaeal 16S rDNA was found in the sample from well 62D. Fifteen clones of archaeal 16S rDNA from amplification of DNA from well 62D were sequenced (Table 1). Sequences of 10 clones had 100% sequence similarity, and the remaining 5 each contained 1 independent substitution out of 450 positions. Only one clone (SRS62DAR03) was chosen for further analysis. This clone was fully sequenced (1346 nucleotides) for the phylogenetic analysis. Within the GenBank and EMBL databases (Benson *et al.,* 1999; Stoesser *et al.,* 2000), the sequence with the highest similarity (89%) to that of SRS62DAR03 was a crenarchaeote clone from a marine sponge, *Cenarchaeum symbiosum* (Figure 3). SRS62DAR03 clustered with a group of unidentified marine archaeal clones that group with other crenarchaeota in phylogenetic trees constructed using neighbor-joining, Fitch-Margoliash, and parsimony analyses. The bootstrap values associated with this grouping were high, indicating a stable relationship in the neighbor-joining tree.

Discussion

A phospholipid fatty acid (PLFA) analysis was performed by Microbial Insights Inc. for this project. The samples for this analysis were taken from well 43B and 62D at

the same time as these molecular analyses. The PLFA profile of the sample from well 43B supported the hypothesis that the microbial community in well 43B was dominated by *Pseudomonas gessardi* or a closely related organism. Because only 4 types of phospholipids were detected, the community in this groundwater appeared to be simple. Sixty-six percent of the phospholipids detected were 18:1ω7c, which is common in many pseudomonads. The remaining phospholipids included normal saturated fatty acids, 14:0, 16:0 and 18:0, which are common in many bacterial groups.

Seventeen phospholipids were detected in well 62D [Microbial Insights, 1999], suggesting that it contained a community more diverse than 43B. The 16S rDNA data indicated that the community contained at least 7 different genera. Six and one half percent of the phospholipids found in 62D were the monoenoic biomarker 16:1ω8c, which is found in *Methylomonas* [Microbial Insights, 1999]. Similarly, 7.5% of the bacterial clones sequenced from 62D grouped with *Methylomonas.* The PLFA profile also suggested that the community in 62D contained many Gram negative bacteria since 77% of the phospholipids were monoenoics, which are common in Gram negative bacteria or proteobacteria (Microbial Insights, 1999). With the exception of SRS62DBA50, all of the bacterial clones sequenced from 62D grouped with the proteobacteria.

The PLFA profile from well 62D also contained low levels of the biomarkers for *Desulfovibrio* (i17:1ω7c) at 0.4%, *Methylosinus* (18:1ω8c) at 5.3%, *Desulfobacter* (10me16:0) at 1.1%, and the Gram positive or sulfate-reducing bacteria (terminally branched saturated phospholipids) at 2.0% [Microbial Insights, 1999]. The clones from

the 16S rDNA experiments did not group phylogenetically with any *Methylosinus* (a member of the alpha proteobacteria), sulfate-reducing or Gram positive bacteria. Since these biomarkers represented a fairly low percentage of all the phospholipids present, it is possible that these organisms were present in the well, but their 16S rDNA was not detected because of their low abundance. It is also possible that the markers are not unique, and are found in one of the groups of organisms detected by 16D rDNA sequencing.

Well 62D is located within a plume of chlorinated hydrocarbon contamination. These contaminants are not at toxic levels and probably act as major sources of carbon and energy for the microbial communities in the aquifer. The presence of these extra nutrients may explain why there is a more diverse community in this well as compared to well 43B.

Trichloroethylene (TCE) is one of the contaminants found within well 62D. The methane monooxygenase of some methane-oxidizing bacteria (methanotrophs), such as *Methylomonas*, *Methylobacter*, *Methylosinus*, *Methylococcus*, and *Methylomicrobium*, cometabolize TCE [Rosenzweig *et al.*, 1993]. Therefore, an environment with methane and TCE may enrich for these methane oxidizers. The 16S rDNA data showed that 28% of the bacterial clones sequenced clustered with the type I methanotrophs. It seems possible that the methane-oxidizing members in this community may be enriched further by pumping methane and oxygen into this community. The enrichment of these organisms may expedite the degradation of the contaminating plume.

Thirty percent of the bacterial clones sequenced from well 62D clustered with the *Methylophilus* and *Methylobacillus* members of the β proteobacteria. *Methylophilus* and *Methylobacillus* are methanol oxidizers. Methanol is one of the byproducts of methane oxidation [Anthony, 1986], and it is likely that these bacteria are utilizing the methanol produced by the type I methanotrophs. Methanol oxidizers are commonly found in association with methane-oxidizing bacteria for this reason [Hanson, 1980].

The *Pseudomonas* and *Burkholderia* groups contain metabolically diverse bacteria. Some *Pseudomonas* and *Burkholderia*, such as *P. putida* and *B. cepacia*, can oxidize TCE [Li *et al.*, 1992; Newman *et al.*, 1995; Whittaker *et al.*, 1999]. 35% and 5% of the bacterial clones sequenced grouped with the *Burkholderia* and *Pseudomonas* groups, respectively. With the exception of a close relative of *P. gessardii* these bacteria were not detected in the uncontaminated well (43B), and it is likely that the contaminating compounds have enriched for these organisms and act as a source for their carbon and energy. Therefore, these organisms could contribute to the breakdown of TCE and other contaminants found at the site.

Since the bacterial and archaeal clones SRS62DBA50 and SRS62DAR03 did not cluster with any cultured and/or characterized prokaryote in the Genbank and EMBL databases, little can be said about their presence in the aquifer. Both seem to be novel organisms. An attempt to culture these organisms would be helpful to determine the significance of their presence in the well.

References

1. **Anthony, C**. 1986. Bacterial oxidation of methane and methanol. Advances in Microbial Physiol. **27**:113-210.
2. **Benson DA, Boguski MS, Lipman DJ, Ostell J, Ouellette BF, Rapp BA, Wheeler DL**. 1999. Genbank. Nucleic Acids Res. **27**: 12-17.
3. **Bowman, J. P., L. Jiménez, I. Rosario, T. C. Hazen, and G. S. Sayler.** 1993. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol. **59**:2380-2387.
4. **Brockman, F. J., W. Payne, D. J. Workman, A. Soong, S. Manley, and T. C. Hazen**. 1995. Effect of gaseous nitrogen and phosphorus injections on in situ bioremediation of a trichloroethylene-contaminated site. J. Hazard. Mater. **41**:287-298.
5. **Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D., and Leclerc, H.** 1999. Pseudomonas libanensis sp. Nov., a new species isolated from Lebanese spring waters. Int. J. Syst. Bacteriol. **49**: 1091-1101.
6. **Dalton, H**.. 1992. Methane oxidation by methanotrophs: physiological and mechanistic implications. *In* Methane and Methanol Utilizers. J. C. Murrell and H. Dalton. Plenum, New York.
7. **DeLong, E. F., Olsen, G. J., Pace, N. R.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriology **170**:720-726.
8. **Felsenstein, J**. Phylip ver. 3.5, 1993. University of Washington, Seatle, WA.
9. **Hanson, R. S. and E. V. Wattenberg**. 1991. Ecology of methylotrophic bacteria. In Biology of Methylotrophs. I. Goldberg and J. S. Rokem. Butterworth-Heinemann, Boston.
10. **Kao, C. M. and J. Prosser**. 1999. Intrinsic bioremediation of trichloroethylene and chlorobenzene: field and laboratory studies. J. Hazard. Mater. **69**:67-69.
11. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Technology in Bacterial Systematics. John Wiley and Sons, New York.
12. **Li, S., Wackett, L. P.** 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. and Biophys. Res. Commun. **185**:443-451.
13. **Maidak, B. L., Cole, J. R., Parker, C. T., Jr, Garrity, G. M., Larsen, N., Li, B., Lilburn, T. G., McCaughey, M. J., Olsen, G. J., Overbeek, R., Pramanik, S., Schmidt, T. M., Tiedje, J. M. and Woese, C. R.** A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. **27**:171-173 (1999)
14. **Mathews, D.H., Sabina, J., Zuker, M. and Turner, D. H.** 1999 Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. J. Mol. Biol. 288: 911-940.
15. **Microbial Insights**, Inc. 1999. Rockford, TN.

16. **Newman, L. M. and Wackett, L. P.** 1995. Purification and characterization of toluene 2-monoxygenase from *Burkholderia cepacia*. Biochemistry **34**:14066-14076.
17. **Reddy, K. R. and J. A. Adams.** 1998. System effects of benzene removal from saturated soils and ground water using air sparging. J. Environ. Eng. **124**:288-299.
18. **Rosenzweig, A. C., Frederick, C. A., Lippard S. J., and Nordlund, P**. 1993. Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. Nature **366**:537-543.
19. **Stoesser, G., Baker, W., van den Broek, A. E., Camon, E., Hingamp, P., Sterk, P., and Tuli, M. A.** 2000. The EMBL nucleotide sequence database. Nucleic Acids Res. **28**: 19-23.
20. **Takai, K. and Horikoshi, K.** Genetic diversity of archaea in deep-sea hydrothermal vent environments. Genetics **152**:1285-1297.
21. **Travis, B. J. and N. D. Rosenberg**. 1997. Modeling in situ bioremediation of TCE at Savannah River: effects of product toxicity and microbial interactions on TCE degradation. Eviron. Sci. Technol. **31**:3093-3102.
22. **Uchiyama, H. T., T. Nakajima, O. Yagi, T. Nakahara**. 1992. Role of heterotrohic bacteria in complete mineralization of trichloroethylene by *Methylocystis* sp. strain M. Appl. Environ. Microbiol. **58**:3067-3071.
23. **Verhille, S., Baida, N., Daboussi, F., Hamze, M., Izard, D., and Leclerc, H.** 1999. *Pseudomonas gessardii* sp. Nov. and *Pseudomonas migulae* sp. Nov., two new species isolated from natural mineral waters. Int. J. Syst. Bacteriol. **49**: 1559-1572.
24. **Whittaker, M., Monroe, D. and Oh, D. J.** 1999. University of Minnesota. http://www.labmed.umn.edu/umbbd/tce/tce_map.html
25. **Zuker, M., Mathews, D. H. and Turner, D. H.** 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. *In* RNA Biochemistry and Biotechnology. J. Barciszewski & B.F.C. Clark (eds.) NATO ASI Series, Kluwer Academic Publishers.

Table 1. 16S rDNA sequences from well 62D and 43B amplified with bacterial primers and archaeal primers

Representative Clones	**Number of clones found with this ribotype[a]**	**Closest Relative[b]**	**Percent similarity[c]**
SRS43BBA18	26	*Pseudomonas gessardii*	99.5
SRS62DBA05	7	beta proteobacterium TBW3	90.8
SRS62DBA21	5	beta proteobacterium TBW3	90.3
SRS62DBA03	1	*Zoogloea ramigera*	96.8
SRS62DBA07	1	*Zoogloea ramigera*	97.8
SRS62DBA11	1	*Zoogloea ramigera*	97.2
SRS62DBA19	1	*Zoogloea ramigera*	96.2
SRS62DBA43	1	*Pseudomonas mephitica*	98.0
SRS62DBA09	9	*Pseudomonas lemoignei*	94.2
SRS62DBA12	3	*Methylobacter psychrophilus*	97.9
SRS62DBA32	1	*Methylobacter psychrophilus*	97.5
SRS62DBA10	1	*Methylomonas sp.* T20	97.4
SRS62DBA37	3	*Methylomonas sp.* T20	98.2
SRS62DBA01	2	*Methylomonas sp.* KSPIII	96.2
SRS62DBA24	1	*Methylomonas sp.* KSPIII	95.9
SRS62DBA39	1	*Pseudomonas brassicacearum*	98.9
SRS62DBA44	1	*Pseudomonas gessardii*	99.8
SRS62DBA50	1	*Acetobacterium wieringae*	71.6
SRS62DAR03	15	*Cenarchaeum symbiosum*	89.3

[a]Ribotype includes all sequences with ≥99.6% sequence similarity.

[b]Refers to the sequence in the Genbank or EMBL databases (Benson *et al.*, 1999; Stoesser *et al.*, 2000) that possessed the highest percent similarity.

[c]Percent similarity = 100 X (number of identical bases) / (total number of positions compared)

Figure 1. Phylogenetic tree generated by the neighbor-joining method from an alignment of 510 nucleotide positions showing the relationships between well 43B and 62D clones and bacteria and clones related to the γ-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. This tree was rooted using *Bacillus subtilis, Clostridium kluyveri*, *Clostridium innocuum,* and *L*actobacillus *acidophilus* 16S rDNA sequences. Bootstrap values greater than 50 are reported adjacent to each node.

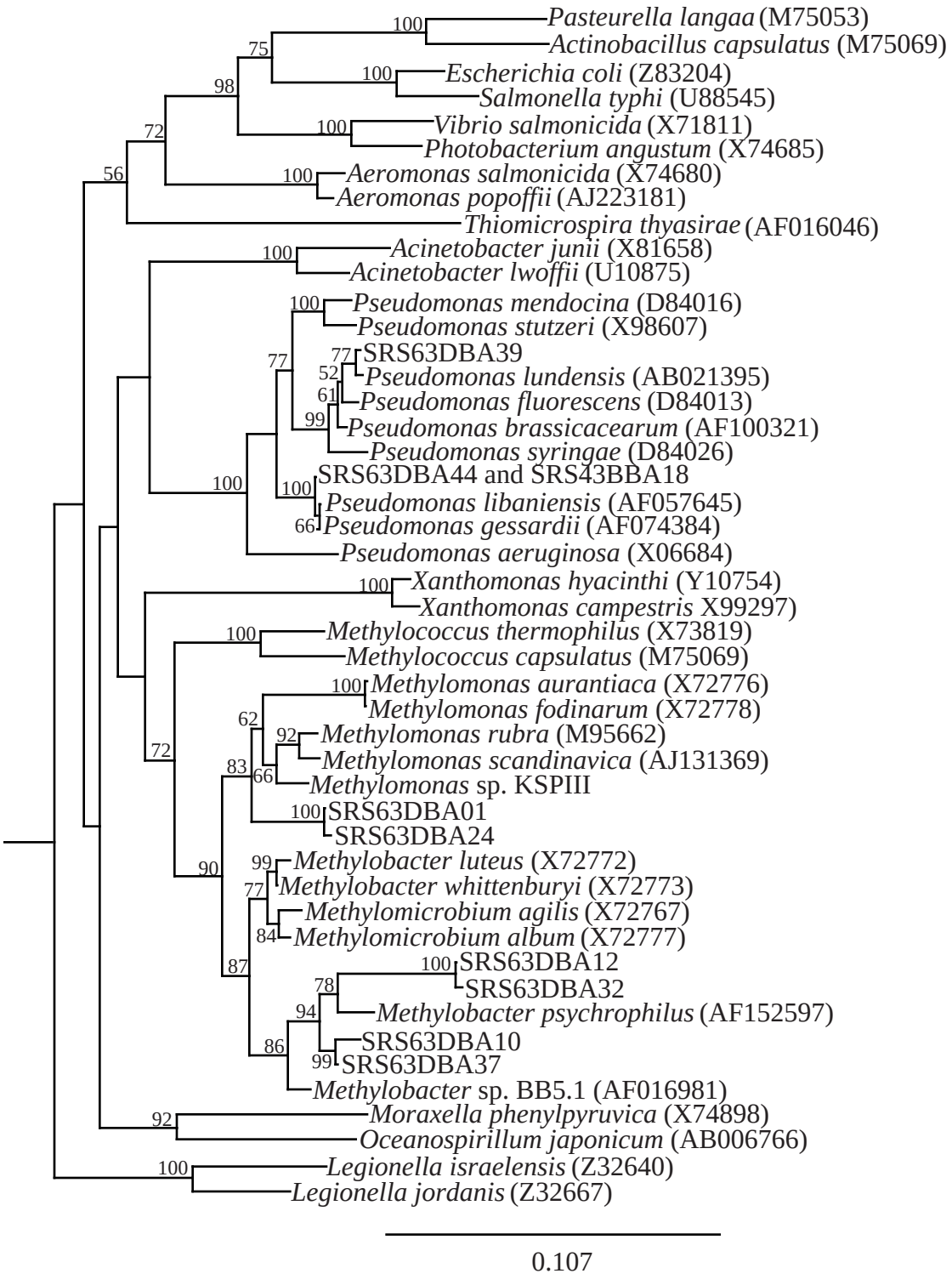

Pasteurella langaa (M75053)
Actinobacillus capsulatus (M75069)
Escherichia coli (Z83204)
Salmonella typhi (U88545)
Vibrio salmonicida (X71811)
Photobacterium angustum (X74685)
Aeromonas salmonicida (X74680)
Aeromonas popoffii (AJ223181)
Thiomicrospira thyasirae (AF016046)
Acinetobacter junii (X81658)
Acinetobacter lwoffii (U10875)
Pseudomonas mendocina (D84016)
Pseudomonas stutzeri (X98607)
SRS63DBA39
Pseudomonas lundensis (AB021395)
Pseudomonas fluorescens (D84013)
Pseudomonas brassicacearum (AF100321)
Pseudomonas syringae (D84026)
SRS63DBA44 and SRS43BBA18
Pseudomonas libaniensis (AF057645)
Pseudomonas gessardii (AF074384)
Pseudomonas aeruginosa (X06684)
Xanthomonas hyacinthi (Y10754)
Xanthomonas campestris X99297)
Methylococcus thermophilus (X73819)
Methylococcus capsulatus (M75069)
Methylomonas aurantiaca (X72776)
Methylomonas fodinarum (X72778)
Methylomonas rubra (M95662)
Methylomonas scandinavica (AJ131369)
Methylomonas sp. KSPIII
SRS63DBA01
SRS63DBA24
Methylobacter luteus (X72772)
Methylobacter whittenburyi (X72773)
Methylomicrobium agilis (X72767)
Methylomicrobium album (X72777)
SRS63DBA12
SRS63DBA32
Methylobacter psychrophilus (AF152597)
SRS63DBA10
SRS63DBA37
Methylobacter sp. BB5.1 (AF016981)
Moraxella phenylpyruvica (X74898)
Oceanospirillum japonicum (AB006766)
Legionella israelensis (Z32640)
Legionella jordanis (Z32667)
0.107

Figure 2. Phylogenetic tree generated by the neighbor-joining method from an alignment of 500 nucleotide positions showing the relationships between well 62D clones and bacteria and clones related to the β-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. This tree was rooted using *B. subtilis, C. kluyveri*, *C. innocuum,* and *L. acidophilus* 16S rDNA sequences. Bootstrap values greater than 50 are reported adjacent to each node.

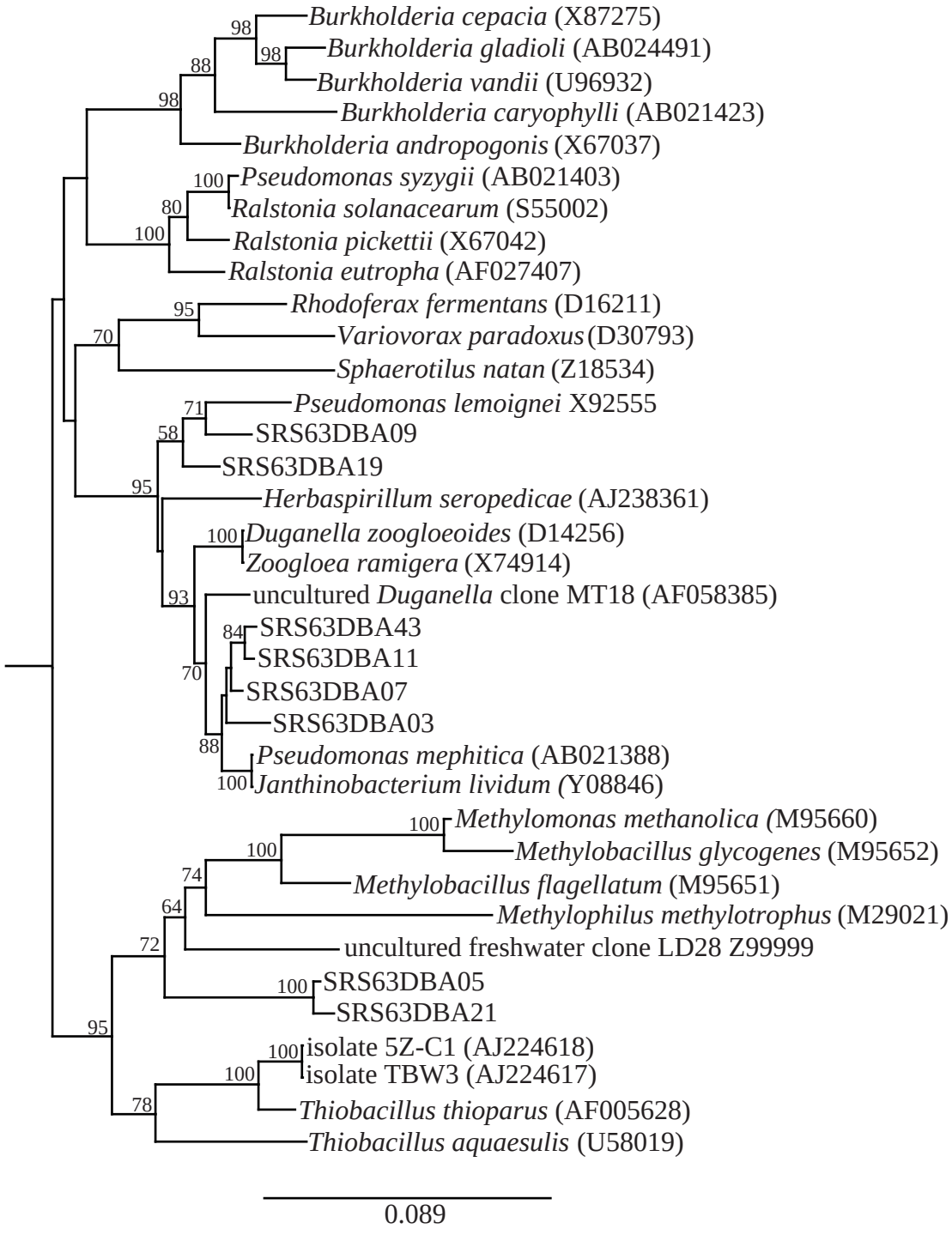
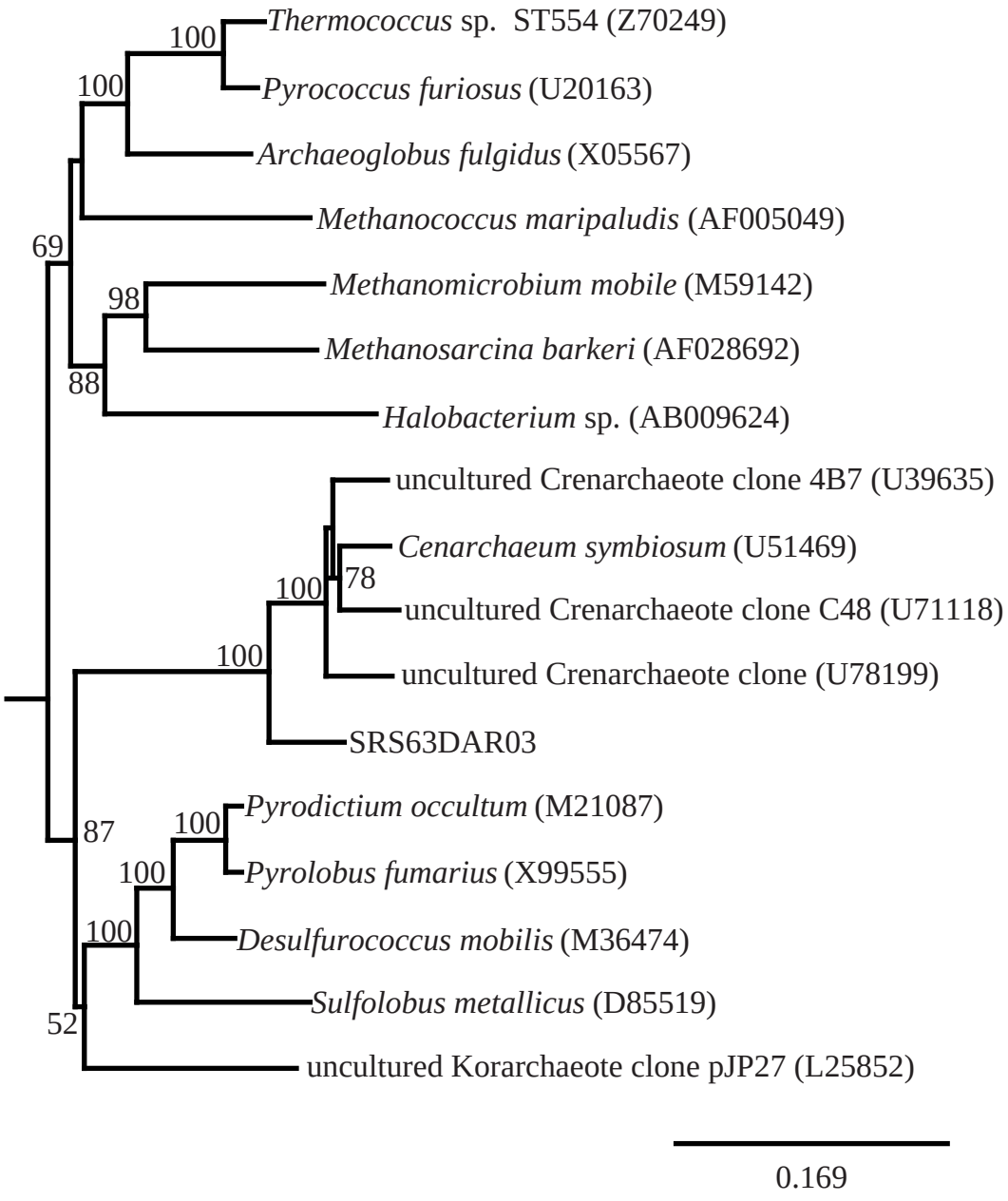

Burkholderia cepacia (X87275)
Burkholderia gladioli (AB024491)
Burkholderia vandii (U96932)
Burkholderia caryophylli (AB021423)
Burkholderia andropogonis (X67037)
Pseudomonas syzygii (AB021403)
Ralstonia solanacearum (S55002)
Ralstonia pickettii (X67042)
Ralstonia eutropha (AF027407)
Rhodoferax fermentans (D16211)
Variovorax paradoxus (D30793)
Sphaerotilus natan (Z18534)
Pseudomonas lemoignei X92555
SRS63DBA09
SRS63DBA19
Herbaspirillum seropedicae (AJ238361)
Duganella zoogloeoides (D14256)
Zoogloea ramigera (X74914)
uncultured Duganella clone MT18 (AF058385)
SRS63DBA43
SRS63DBA11
SRS63DBA07
SRS63DBA03
Pseudomonas mephitica (AB021388)
Janthinobacterium lividum (Y08846)
Methylomonas methanolica (M95660)
Methylobacillus glycogenes (M95652)
Methylobacillus flagellatum (M95651)
Methylophilus methylotrophus (M29021)
uncultured freshwater clone LD28 Z99999
SRS63DBA05
SRS63DBA21
isolate 5Z-C1 (AJ224618)
isolate TBW3 (AJ224617)
Thiobacillus thioparus (AF005628)
Thiobacillus aquaesulis (U58019)
98
98
88
98
100
80
100
95
70
71
58
95
100
93
84
70
88
100
100
100
74
64
72
100
95
100
100
78
0.089

Figure 3. Phylogenetic tree generated by the neighbor-joining method from an alignment of 1,346 nucleotide positions showing the relationships between well 62D clones and bacteria and clones related to the Archaea. The scale bar indicates Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. This tree was rooted using an *Escherichia coli* 16S rDNA sequence. Bootstrap values greater than 50 are reported adjacent to each node.


Thermococcus sp. ST554 (Z70249)
100
Pyrococcus furiosus (U20163)
100
Archaeoglobus fulgidus (X05567)
Methanococcus maripaludis (AF005049)
69
Methanomicrobium mobile (M59142)
98
Methanosarcina barkeri (AF028692)
88
Halobacterium sp. (AB009624)
uncultured Crenarchaeote clone 4B7 (U39635)
Cenarchaeum symbiosum (U51469)
78
100
uncultured Crenarchaeote clone C48 (U71118)
100
uncultured Crenarchaeote clone (U78199)
SRS63DAR03
Pyrodictium occultum (M21087)
87
100
100
Pyrolobus fumarius (X99555)
100
Desulfurococcus mobilis (M36474)
Sulfolobus metallicus (D85519)
52
uncultured Korarchaeote clone pJP27 (L25852)
0.169

CHAPTER V

CONCLUSION

The research presented in the preceding chapters dealt with the microbial diversity of two different ecosystems and the effects of different processes on the diversity. The research discussed in this study focused on three main issues. One important aspect was the use of different methods (molecular retrieval versus culture) to examine microbial diversity in environmental samples. The second important issue examined was the stability of microbial communities over time. The third and most important issue examined was the sensitivity of microbial diversity to processes that change their habitat. Specifically, microbial populations in soil were sensitive to the effects of earthworms, and microbial populations in groundwater were sensitive to TCE contamination.

Two different approaches were used to examine the microbial diversity of soil and groundwater ecosystems. Molecular retrieval methods were used for groundwater and culture methods were used for the soil. While both approaches were equally useful for showing differences in microbial diversity within different habitats in these ecosystems, they each had specific strengths and weaknesses. For instance, the molecular retrieval approach offered a less biased picture of the microbial community in TCE contaminated and uncontaminated groundwater and was performed in a short period of time. The problem with this technique was that no functions could be assigned to many of the organisms detected in the TCE-contaminated water because they were closely related to physiologically diverse prokaryotes. In contrast, physiological functions could be assigned to all organisms found in bulk and earthworm associated soils, because they have been isolated and preserved. However, only a small percentage of the bacteria in soil were examined because most of them were not cultured. These studies were useful

for illustrating the strengths and weaknesses of two methods commonly used to examine microbial diversity in natural ecosystems.

The studies on microbial diversity in soil revealed that natural microbial communities are not always stable. The taxonomy and physiology of the bacteria that were isolated from the earthworm digestive tract changed over time. The physiology of the soil bacteria did not change over time. While the soil bacteria were sensitive to temporal change at the species level, they did not change at the order level. The reason for these changes was unclear, but they are important to consider when planning methods to study microbial diversity.

In these studies, fresh casts collected from earthworms contained bacterial communities that were physiologically and phenotypically different and less diverse than those from surrounding soil. These communities contained many more nitrate-reducing and Casamino acid- and acetate- utilizing organisms and gamma proteobacteria than soil. These differences were probably due to three factors. The gut environment may cause a select group of organisms in soil to be more abundant and culturable after passage. Since the earthworms that were used in these experiments fed partially on leaf litter, then the differences in bacterial communities may have also been due to the presence of these organisms in the leaf litter. Lastly, earthworm guts may contain an indigenous microbial flora that is shed in their casts. At present, we do know how the earthworm feeding and/or digestive processes affect soil microbial communities, and more research is needed to elucidate these differences.

Contamination of groundwater from adjacent landfills caused the taxonomy of microbial populations in groundwater to change and to become more diverse. There was

an increase in the numbers of taxa in contaminated water as compared to uncontaminated water. The presence of some of the bacteria in the contaminated well may have been due to their ability to metabolize the contaminants. For instance, many potential TCE-degrading bacteria were detected, and TCE was one of the major contaminants identified in the water. Since normal groundwater contains a low concentration of nutrients, it is a stressful environment for most microorganisms. Stressful environments can be characterized as having low biodiversity. It is possible that the contamination in the groundwater acted as a nutrient source, making the habitat less stressful for a larger group of organisms. Therefore, the increase in diversity was not surprising.

Advances made in understanding microbial diversity studies, like these, are important for the understanding of soil and water quality. For example, if earthworms do have a significant impact on the nitrogen cycle, then understanding this relationship may be helpful for developing methods of increasing soil fertility. Also, knowing the effects of chemical contamination on microbial diversity can be helpful for detecting chemical contaminants in natural ecosystems and possibly reducing them by bioremediation. There are endless applications for microbial diversity studies, and there is much more left to discover.