PROKARYOTIC COMMUNITIES ASSOCIATED WITH THE EARTHWORM *Lumbricus rubellus* AND THE AGRICULTURAL SOIL IT INHABITS

by

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ABSTRACT

16S ribosomal RNA (rRNA) gene clone libraries were constructed from the casts of the earthworm *Lumbricus rubellus* and the agricultural soil it inhabits. Both samples were very diverse and contained a large number of bacterial taxa. However, increased numbers of sequences belonging to the Actinobacteria, Firmicutes, and *Pseudomonas* taxa, in addition to decreased numbers of one phylogenetically deep unknown taxon were found in the cast library. To examine if these differences were statistically significant, a novel method of comparing 16S rRNA clone libraries was developed (LIBSHUFF). This analysis showed that the cast sample was significantly different from the soil sample, and that the differences in abundance of all four of the previously named taxa were responsible for the difference. The analysis also suggested that the cast bacterial population was derived from the soil population. Archaeal diversity was low in both the soil and cast samples and consisted of sequences from a common soil archaeal lineage.

To examine the possibility of an indigenous intestinal community, earthworms were collected, the intestines dissected and washed, and 16S rRNA clone libraries

constructed from DNA extracted from the intestinal material. A significant number of prokaryotes remained attached to the intestine after washing. At least three taxa, belonging to the Acidobacteria, Firmicutes, and one deep *Mycoplasma*-associated lineage were found in high numbers in the intestine libraries, and were not found in clone libraries made from the cast material or the surrounding soil. An additional taxon, belonging to the β-proteobacteria was also detected in signficant numbers in the intestine of earthworms as well as cast material. With the exception of the β-proteobacteria, none of the taxa were found in all of the earthworms screened, suggesting that while these sequences may represent intestine-associated organisms, they are not necessarily stable in the *L. rubellus* population.

An organism isolated from the burrow of an earthworm, which represents the type strain in a novel genus (*Solirubrobacter*) was characterized. This organism represents only the third cultured species in the *Rubrobacteridae* subclass of the Actinobacteria phylum and bears similarity to a number of clones recovered from soils around the world.

INDEX WORDS: *Lumbricus rubellus*, Earthworm cast, Earthworm intestine, Soil, 16S rRNA, LIBSHUFF, *Solirubrobacter*

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DEDICATION

To Amy, for her love and patience.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. Introduction - Soil prokaryotes

An estimated 4-6 x 10^{30} prokaryotes inhabit the earth, and a large percentage of these (2.6 x 10^{29}) are found in soil (Whitman et al., 1998). Direct counts of bacteria in soil samples are typically 10^{9}-10^{10} cells per gram (dry weight) of soil (e.g. Skinner et al., 1952; Øvreås and Torsvik, 1998). Using DNA reassociation curves of genomic DNA extracted from soil, Torsvik et al. (1991) estimated 4000 unique genomes in a soil sample. Later tests on a variety of soils using similar techniques revealed as few as 1000 genomes in a sandy agricultural soil and as many as 6000 genomes in a forest soil (Torsvik et al., 1996). Using the definition of a bacterial species as two genomes with less than 70% DNA reassociation (Wayne et al., 1987), Dykhuizen (1998) projected as many as 10^{9} – 10^{12} different species of bacteria may exist on the planet. Given these estimates, soil microorganisms not only represent a sizable percentage of life in the biosphere, but the diversity of these prokaryotic communities is enormous.

2. Molecular microbial ecology

The study of soil bacterial and archaeal communities requires a means by which the majority of the population can be observed. When studies were limited to traditional culture-based methods, the cultivated fraction of the bacterial community was often less than 1% of the total number of cells (e.g. Skinner et al., 1952). The advent of molecular techniques has allowed researchers to bypass the requirement for a pure culture in the laboratory by permitting direct examination of an organism's DNA. In particular, the relative ease of polymerase chain reaction (PCR) amplification and sequencing of

specific genes has resulted in the development of a variety of molecular techniques to study prokaryotic populations, including as-of-yet uncultured organisms.

Most of the molecular techniques commonly used involve the analysis of the ribosomal RNA (rRNA) genes. In bacteria and archaea, the 16S rRNA gene is most commonly used, although sequence data for the 23S and 5S rRNA genes of many organisms are also available. The 16S rRNA gene was first identified as a molecular chronometer (reviewed in Woese 1987) and was used to help define the now familiar three domain theory of phylogeny (bacteria, archaea, and eucarya). Researchers have since applied the gene to resolve the phylogeny of existing taxonomic groups as well as to identify uncultivated organisms by their 16S rRNA gene sequence similarity to known organisms. An especially beneficial property of the 16S rRNA was the presence of multiple conserved regions found in the genes of nearly all prokaryotes, which allowed the development of PCR primers and specific oligonucleotide probes, that are now the basis for many molecular microbial ecology techniques.

Although molecular studies allow examination of uncultured organisms, a number of factors must be taken into account that may influence the representation of various organisms in the analysis. One concern is the extraction of DNA from soil microorganisms and whether or not all of the different cell types present in soil are lysed equally. Typically, DNA is recovered from a soil sample by lysis of the organisms in the soil followed by recovery of the nucleic acids, although the removal of cells from the soil matrix prior to DNA recovery has also been examined. A variety of chemical (e.g. phenol, sodium dodecyl sulfate [SDS]), enzymatic (e.g. lysozyme incubation), and physical treatments (e.g. freeze-thawing, bead-beating, sonication) have been developed

for DNA extraction from environmental samples and compared (Frostegård et al., 1999; Martin-Laurent et al., 2001; Miller et al., 1999). Of special interest in DNA extraction are endospores and other resilient cell types, which may be present in fairly high numbers in soils and are highly resistant to environmental stress. Physical disruption by a process such as bead-beating has been found to be the most effective in recovering DNA from these cell types (Moré et al., 1994). Failure to recover DNA from one or more groups of organisms can lead to an underestimation of community diversity and non-detection of potentially abundant or physiologically important groups of organisms.

2.1 PCR amplification of 16S rRNA genes for molecular ecology studies

The application of the PCR to microbial ecology studies has thus far revealed an astounding amount of uncultured prokaryotic diversity and provided a means to obtain the large number of specific genes required for many common molecular protocols. However, it is doubtful that the ratios of the recovered amplicons are truly representative of the distribution of organisms in the environmental sample. An excellent review of these concerns can be found in von Wintzingerode et al. (1997). Briefly, these problems include inhibition of the PCR reaction by substances such as humic acids (which are commonly co-extracted with nucleic acids from soil samples), preferential amplification of sequences by differential primer specificity or gene copy number, and formation of PCR artifacts such as chimeras and mutants. While these problems cannot be completely controlled, steps such as reducing the number of cycles in the PCR program can help minimize the introduced bias, and computer analyses have been developed to help detect chimeric molecules (Robinson-Cox et al., 1995). Even still, there is currently no way to

eliminate these problems, and these factors must be taken into account during data analyses.

2.2 Qualitative analyses of microbial communities relying on amplified 16S rDNA

Once a collection of 16S rRNA genes have been obtained, a number of procedures can then be performed. For example, 16S rRNA clone libraries are commonly constructed to examine prokaryotic communities in soil systems (see below). In these studies, a portion or the near-entirety of the 16S rRNA gene are cloned into a vector and transformed into a lab strain, typically *Escherichia coli*. Colonies are selected that each contain a single 16S rRNA gene. These 16S rDNA inserts can be sequenced (either partially or completely) providing phylogenetic information on the total community. Alternatively, a procedure such as a restriction fragment length polymorphism (RFLP; also known as amplified ribosomal DNA restriction analysis [ARDRA]) analysis can be performed to screen the library for different ribotypes (e.g. Cho and Kim, 2000; Smit et al., 1997). In RFLP analyses, individual 16S rRNA genes are digested with one or more endonucleases, and the digestion pattern is examined after electrophoresis in an agarose gel. Matching digestion patterns can be indicative of related or identical sequences, and the number of patterns representative of the community complexity.

A large number of other techniques relying on PCR amplified 16S rRNA genes have also been developed. For instance, in terminal RFLP (t-RFLP), a fluorescent dye is attached to one of the primers in the PCR reaction, the resulting amplicons are digested with one or more restriction endonucleases, and the entire mixture is run on a capillary

electrophoresis instrument (or similar apparatus) to separate the fluorescently labeled 16S rDNA fragments and produce a profile of the bacterial community (e.g. Dunbar et al., 2000; Liu et al., 1997). Fragments of similar sizes generally correspond to a specific taxonomic group of organisms, and the intensity of the resulting peaks corresponds to relative abundance of amplicons in the PCR mixture. In length heterogeneity analysis by PCR (LH-PCR), phylogenetic groups are distinguished by the natural variation in sequence length between two conserved regions of the 16 rRNA gene rather than the occurrence of restriction sites, but a profile similar to that of t-RFLP is produced (Suzuki et al., 1998). Similarly to t-RFLP and LH-PCR, density gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) can be used to create a community profile by separating PCR fragments of similar length but differing sequence on a gel with a gradient created by either temperature or chemical denaturants (for a review, see Muyzer and Smalla, 1998). A method that relies on the secondary structure of single-stranded PCR products (single-strand-conformation polymorphism [SSCP]), produces similar results and has been used (for example) to compare the differences in populations between the rhizosphere communities of two species of plants (Schweiger and Tebbe, 1998), the populations in a decomposing agricultural substrate (Peters et al., 2000), and the community of a PCP contaminated soil (Beaulieu et al., 2000). While techniques that produce a profile for the entire community have their advantages (t-RFLP, DGGE, TGGE, SSCP), they are limited in that subsequent steps must be often be taken to identify the particular groups (peaks in the case of t-RFLP, bands in the case of DGGE, TGGE or SSCP) within the profile. One advantage of clone sequence libraries is that information concerning the phylogeny of the clones is immediately available. In

fact, the construction of a clone library prior to the use of one of these other described techniques may give valuable information about the generated profile.

2.3 Quantifying microbial abundance in environmental samples

The largest limitation of the previously described molecular techniques is their inability to determine the actual abundances of specific organisms in an environmental sample. Because they all rely on an initial PCR step that may introduce bias in the amplification of some rRNA genes, the observed abundances of genes after the PCR may not be representative of the initial amounts. To address this concern, other procedures have been developed that attempt to quantify the initial abundance of organisms.

Advances in PCR technology have allowed the development of quantitative PCR techniques (e.g. Suzuki et al., 2000; Becker et al., 2000). This promising technology has been used to quantify the numbers of *Pseudomonas* organisms in soils (Johnson et al., 1999) and the abundances of uncultured marine bacteria and archaea (Suzuki et al., 2000). In both of these studies, the numbers obtained by quantitative PCR correlated well with other methods.

Other quantitative procedures rely on the utilization of oligonucleotide probes that are used to hybridize to DNA or RNA extracted from an environmental sample. Because there is no PCR amplification, the calculated results should be representative of the number of target genes in the extracted nucleic acid sample. In some cases, the extracted DNA or RNA is immobilized on a solid support, and fluorescent, radioactive, or enzyme labeled probes are hybridized to the sample (e.g. Edgcomb et al., 1999; Linne von Berg and Bothe, 1992; Stahl et al., 1988). In alternative protocols, target sequences are

immobilized on a solid support, and the labeled genomic DNA is hybridized (reverse sample genome probing [RGSP]; Shen et al., 1988; Voordouw et al., 1993). The amount of bound probe is then compared to standards, and the number of target sites in the sample is calculated. Hybridization with fluorescently labeled oligonucleotide probes that can penetrate whole cells and bind target sequences intracellularly (fluorescent in situ hybridization [FISH]; DeLong et al., 1989) has become quite popular in quantifying the abundance of dominant members of prokaryotic communities (e.g. Zimmermann et al., 2001; Rosselló-Mora et al., 1999; Llobet-Brossa et al., 1998; Santo Domingo et al., 1998; Snadir et al., 1997). This protocol allows the visual examination of targeted cells by fluorescence microscopy and can give information about the abundance, distribution, and cellular morphology of the cells in their natural environment. The limitation of many of these quantitative protocols is that the abundance of organisms in the entire community cannot be determined without multiple applications of the procedure for each organism tested. In a complex environment such as soil, the analysis of hundreds or thousands of different organisms would be nearly impossible. Therefore, the newest techniques such as DNA microarrays are an attempt to quantitatively describe entire populations, although an initial screen must often be performed to determine the organisms that are present. The technology is currently limited by the expense of the equipment and a lack of understanding of the standard conditions necessary to achieve optimal hybridization for all of the taxa tested, although promising results in at least one instance have been reported (Small et al., 2001).

3. The prokaryotic communities of soils – a molecular perspective

Soils in a variety of locations around the world have been examined using several of the culture independent techniques described in the previous section. Hugenholtz et al. (1998a) published a mini-review summarizing some of the new discoveries based on 16S rRNA gene clone libraries. This section will explain the trends among soil clone libraries and highlight some of the more prominent studies.

The first published 16S rRNA gene clone library from a soil system was derived from a randomly chosen Australian soil and only 30 clones were sequenced (Liesack and Stackebrandt, 1992) compared to the hundreds of clones which may appear in contemporary libraries. As might be expected given what is currently known about the uncultured diversity of soil bacteria, a fair number of clones did not associate with known divisions, and the authors were particularly surprised by the presence of sequences related to the *Planctomycetales* in their sample (Liesack and Stackebrandt, 1992). Today, it is not uncommon to observe this group in soil clone libraries (e.g. Kuske et al., 1997; Felske et al., 1997).

Sequences from groups defined entirely, or primarily, by clonal sequences appear frequently in soil clone libraries. Perhaps the most prevalent group of not-yet-cultured organisms are the Acidobacteria (also called the "*Acidobacterium*/*Holophaga* phylum"), which appear in most soil clone libraries (Barns et al., 1999; Hugenholtz et al., 1998a), although they were not always identified as such (Borneman et al., 1996; Kuske et al., 1997). The phylogenetic positioning of the organism *Acidobacterium capsulatum* as a new lineage was proposed in 1995 (Hiraishi et al., 1995), and a more thorough analysis of a number of bacterial sequences led to the development of the Acidobacteria as a novel

phylum (Ludwig et al., 1997). This taxon contains only two other cultured representatives besides *A. capsulatum*; *Holophaga foetida* and *Geothrix fermentans*. The remainder of the division remains almost entirely described by clonal sequences. However, one research group appears to have isolated a member of the division in a mixed culture (McCaig et al., 2001). If a mixed culture is a requirement for the growth of organisms in this taxon, or the associated organisms provide a key growth factor missing in standard laboratory media, it may help to explain the low representation of the division by isolates.

The division Verrucomicrobia, similarly to the Acidobacteria, is found in most soil clone libraries (91% of the libraries examined; Buckley and Schmidt, 2001) and contains only a few cultured members. The organism for which the taxon is named, *Verrucomicrobium spinosum*, was a freshwater isolate (Schlesner, 1987), although other strains have been recovered from rice paddy soil (Janssen et al., 1997). Of interest, the three new isolates from the rice paddy soil were what have been called "ultramicrobacteria" due to their extremely small size (cell volume < 0.1 μm^3) and bore little similarity morphologically to the sole *Verrucomicrobium* isolate. However, these soil isolates provided some idea of the phenotypic capabilities of the taxa, including the fact that they grew anaerobically, which may help explain their poor cultivation since many isolation studies focus on aerobic organisms. Since 1997, no new isolates appear to have been recovered, although large numbers of *Verrucomicrobium*-related sequences are commonly retrieved from environmental samples (Hugenholtz et al., 1998a) and represent a wide amount of sequence variation within the group (O'Farrell and Janssen, 1999).

Other divisions with some isolate representation are occassionally encountered in soil clone libraries. These include the *Planctomycetes* (Liesack and Stackebrandt, 1992), Green Non-Sulfur bacteria (*Chloroflexi*), and *Nitrospira* (Bruns et al., 1999; Stephen et al., 1998; Hiorns et al., 1995) groups. Other divisions are based entirely on clonal sequences and are often refered to as "candidate divisions". One example of this is candidate division TM7, named after a sequence recovered from a peat bog (Rheims et al. 1996). Similarly to the Acidobacteria and Verrucomicrobia, members of candidate division TM7 are present in various environments (e.g. Borneman and Triplett, 1997; Bond et al., 1995). Unlike other divisions defined entirely by sequence data, some work has been done to elucidate information concerning the organisms in the taxa without cultivation. Using molecular techniques such as FISH and transmission electron microscopy (TEM), one study concluded that division TM7 represented a third Gram-type positive lineage (in addition to the Firmicutes and Actinobacteria), and that some members may have a sheathed filament morphology (Hugenholtz et al., 2001). Aside from their environment, we know very little of the organisms in other candidate divisions without cultured representatives. For example, a study of bacterial sequences recovered from Obsidian Pool, a hot spring in Yellowstone National Park, revealed a number of novel lineages in the Bacteria domain (Hugenholtz et al., 1998b). Some of these divisions, such as OP11 (named for the clone which defined the lineage), may appear infrequently in soil clone libraries (Hugenholtz et al., 1998a).

Sequences belonging to phyla with broad isolate representation are also commonly encountered in soil clone libraries and in many cases make up a large percentage of the total library. Actinobacteria (high G+C Gram positives) have

traditionally been a major component of isolate libraries from soil, and this apparent abundance has not been challenged by clone libraries (Hugenholtz et al., 1998a). However, while the Actinobacteria phylum is well represented by isolates, various taxa within the phylum appear widespread in soils with poor culture representation. A good example of this is the Rubrobacter subdivision, which has been found in clone libraries from a variety of soils (Holmes et al., 2000). Other phylogenetically deep groups in the Actinobacteria have also been reported (Rheims et al., 1999; Ludemann and Conrad, 2000). Other phyla with good isolate representation are found in large numbers in clone libraries. These taxa include the Proteobacteria (primarily α-, also β-, γ-, and δ-, but not including ε-), Firmicutes (low G+C Gram positives), and Cytophaga-Flavobacteria-Bacteroides (CFB bacteria) group.

The number of bacterial phyla has doubled since the introduction of clone libraries to the study of environmental communities (Woese, 1997; Hugenholtz et al., 1998a; see Figure 1.1). Nevertheless, not all environmental sequences group within a described lineage. Many soil clone libraries describe a number of sequences phylogenetically unaffiliated with a group. For example, McCaig et al. (1999) reported eight "Sourhope groups" which were not classified into other phyla. These sequences represented 9.3% of their total libraries. Eighteen percent of a bacterial library from Amazonian soils were not affiliated with a described lineage (Bornemann and Triplett, 1997). Approximately 6% of clones affiliated with the rhizosphere soil of two plants belonged to unique lineages (Marilley and Aragno, 1999). In some cases, sequences which were not identified at the time of publication were later placed into a described division. An example of this is the study of Kuske et al. (1997) in which a number of

sequences of "uncertain affiliation" were suspected to be, and later confirmed, as members of the Acidobacteria. Undoubtedly, while some of these sequences may be undetected PCR artifacts and do not truly represent novel organisms, a large percentage of them will most likely help define one or more new lineages in the future.

3.1. Comparing soil clone libraries

While comparisons of soil clone libraries can be made by contrasting the presence and abundance of various taxa, the problems associated with placing sequences in known divisions (see above), and indeed, getting all researchers to recognize the same phylogenetic groupings limits this type of comparison. Additionally, different researchers utilize different PCR primers and conditions, as well as different DNA extraction protocols, which may affect the composition of clone libraries.

In addition to these potential problems, not all studies examine the same portion of the 16S rRNA gene. Optimally, the nearly complete 16S rRNA would be obtained for all clones in a study. However due to time or financial constraints, this may not always be feasible, especially when large numbers of sequences are examined. Thus, many researchers analyze partial sequences. One problem that arises is the issue what portion of the gene is sequenced. For example, Stackebrandt et al. (1993) analyzed the 5' end of the 16S rRNA gene while Ueda et al. (1995) analyzed the 3' end of the gene. Other studies sequence clones from near the middle of the gene (McCaig et al., 1999), and still others have obtained sequences using primers from the vector. In one instance, this resulted in a library in which half of the sequences described the 5' end, and the other half the 3' end (Borneman et al., 1996). This lack of standardization, combined with

potentially short sequences which may only partially overlap the information from clones in another study, makes it difficult to make direct comparisons between many studies.

4. Soil archaeal populations

When Woese first proposed his three domain model of life (1987), the archaea were represented almost entirely by organisms that grew in extreme environments, and included two major lineages; the Crenarchaeota (thermophiles) and Euryarchaeota (methanogens, halophiles, and sulfur-metabolizing thermophiles). Since then, various archaeal sequences have been retrieved from environments including soils (e.g. Bintrim et al., 1997), freshwater and marine sediments (Schleper et al., 1997; Munson et al., 1997), marine systems (Fuhrman and Davis, 1997), sludges (Sekiguchi et al., 1998), subsurface (Chandler et al., 1998), and hot springs (Takai and Sako, 1999). Many of these sequences bear little sequence similarity to previously cultured organisms.

Unlike the bacteria, an explosion of new archaeal divisions based on clone sequences has not occurred, and most recovered soil archaeal sequences group phylogenetically within the Crenarchaeota. An exception to this are two sequences from Obsidian Pool in Yellowstone National Park, which were so different from both the Crenarchaeota and the Euryarchaeota that a new division within the Archaea was proposed, the Korarchaeota (Barns et al., 1996). Studies of a forest soil from Finland (Jurgens and Saano, 1999), agricultural soils from Wisconsin and Japan (Bintrim et al., 1997; Ueda et al., 1995), and a tropical soil in the Amazon basin (Borneman and Triplett, 1997) have all revealed archaeal sequences that group within a single lineage in the Crenarchaeota. These studies provide evidence for a widespread crenarchaeotic

population in soils that does not share the thermophilic phenotype associated with the isolates from the division. Unfortunately, because no cultured representatives of this lineage are available, nothing about their physiology or role in terrestrial systems is known.

5. Soil prokaryotic communities – a molecular perspective – summary

Soil prokaryotic communities are a diverse mixture of phylogenetically dissimilar organisms. A "typical" soil clone library of at least 100 clones, if one truly existed, would most likely contain at least ten phyla. Clones from the Actinobacteria and Acidobacteria lineages would probably be quite abundant. Members of the Firmicutes, CFB group, Verrucomicrobia, and α-Proteobacteria would be well represented, as well as the other subgroups of the Proteobacteria (except for the epsilon subgroup). Sequences from phyla such as *Chloroflexi* and *Nitrospira* may detected in small numbers, as well as some phyla with uncultured members such as candidate division TM7. Depending on the sample and environmental conditions, various groups, such as γ-Proteobacteria may be recovered more often than anticipated. A small number of sequences may be found that cannot currently be placed into a recognized division. The archaeal diversity would probably be much lower, with a handful of ribotypes branching deeply within the Crenarchaeota.

6. Prokaryotic associations with earthworms

Earthworms are soil invertebrates that are capable of influencing the structure and chemical composition of the soil around them. Earthworm burrowing can increase soil

porosity (Edwards and Shipitalo, 1998; Lee, 1985), contribute to the breakdown of litter (Edwards and Bohlen, 1996), increase soil aggregation (Edwards and Shipitalo, 1998; Edwards and Bohlen, 1996; Tomlin et al., 1995), and is generally thought to promote soil health. For instance, a study incorporating earthworm worked pig-manure (vermicompost) into soil showed that increased amounts of the vermicompost could significantly increase the size of tomato plants (Atiyeh, et al., 2000). The factors that influence the growth of plants must undoubtedly affect soil microbial populations as well.

Earthworms can affect soil prokaryotes in several ways. Perhaps the most obvious interaction occurs when the earthworm feeds on soil or litter containing microorganisms. Several studies have examined the effects of passage through the intestinal tract of earthworms on bacterial communities (Figure 1.2). In general, an increase in bacterial numbers has been observed in the earthworm cast (feces) as compared to the surrounding soil (Schönholzer et al., 1999; Wolter and Scheu, 1999; Pedersen and Henricksen, 1993; Barois, 1992; Daniel and Anderson, 1992; Kristufek et al., 1992; Edwards and Fletcher, 1988; Dkhar and Mishra, 1986; Parle, 1963a) This may be due to the contrast in environmental conditions of the intestine. While soils generally contain few nutrient sources, the intestines of earthworms can have increased amounts of ammonia (Tillinghast et al., 2001), organic carbon (Barois and Lavelle, 1986), moisture (Barois, 1992), and a different pH (Wallwork, 1983). The (fairly) long transit time of ingested material in earthworms such as *Lumbricus rubellus* (6-8 hours; Daniel and Anderson, 1992) and *Lumbricus terrestris* (8-20 hours; Hartenstein and Amico, 1983), may allow for several doublings of some soil bacteria. This phenomenon would help to explain the increased numbers of culturable bacteria in earthworm casts.

Having passed through the earthworm intestine, the cast material is of a different composition than the surrounding soil, including a higher moisture content, more organic-C (Daniel and Anderson, 1992), and higher nitrogen content (Elliott et al., 1991). Fluorescent staining of the casts of *L. terrestris* has elucidated the structural changes in the soil caused by intestinal passage (Altemüller and Joschko, 1992). In most soil types, the cast material was found to be more homogenous than the surrounding soil implying that most of the ingested soil was mixed by passage through the earthworm. Bacterial "streaks" were also apparent in areas where the soil was not as disturbed. The authors hypothesized that these "streaks" of high bacterial numbers may have been due to the production of a polysaccharide slime layer. The polysaccharide produced by the higher number of bacteria in the casts may promote a higher aggregate stability in the cast than the surrounding soil. However, in a contrasting study, Parle (1963b) did not find a correlation between cast stability and polysaccharide concentration, even though the casts contained more polysaccharide material than the surrounding soil. In another study, Hindell et al. (1997) found that casts of the earthworm *Aporrectodea rosea* were less stable than soil initially, but became more stable after air drying. This was most likely due to the increased moisture content of the fresh cast. Thus, while earthworms disrupt and mix the soil and promote soil aggregation, the role of bacteria in this process is not entirely understood.

The microbial populations in casts of *L. terrestris* were studied temporally to examine the length of time that the cast may influence soil microbial populations (Tiunov and Scheu, 2000). While initial microbial respiration rates were high, within 10 days after deposition the rates had dropped. Microbial biomass also dropped during the same

time interval. It was assumed that the easily metabolized C-sources enhanced in the cast were utilized during this time period. Thus, while a fresh cast stimulated that portion of the soil community that responds to the available nutrients, the effect was temporary. Therefore, the cast represented a transient micro-environment of mixed soil that provided a temporary nutrient source in the oligotrophic soil.

Microbial populations in the drilosphere (the soil, 1-2 mm, lining earthworm burrows) may also be different than those in surrounding soil. Most studies on the microbial populations of the drilosphere have been performed with *L. terrestris*. Earthworm secretions enhance the amount of NO_3^-, NH_4^+, and organic carbon in the drilosphere as compared to bulk soil (Parkin and Berry, 1999; Devliegher and Verstraete, 1997). Higher numbers of bacteria have been observed in the drilosphere (Polyanskaya and Tiunov, 1996; Tiunov et al., 1997; Devliegher and Verstraete, 1997). In particular, one study found higher numbers of siderophore-producing bacteria and fluorescent pseudomonads in the drilosphere found in the upper 0-5 cm of the soil profile as compared to non-drilosphere soils (Devliegher and Verstraete, 1997). Parkin and Berry (1999) also found higher populations of nitrifying and denitrifying bacteria in the drilosphere. Within 2 mm of the burrow wall, Tiunov et al. (1997) found higher numbers of bacteria belonging to the *Aquaspirillum* and *Cytophaga* genera. In contrast to the bacterial populations, fungi did not appear enhanced in the drilosphere (Polyanskaya and Tiunov, 1996). Thus, the conditions in the drilosphere appear to enhance the growth of specific bacterial taxa.

6.1. Microorganisms associated with earthworms

Various genera and specific organisms have been associated with earthworms. For instance, Márialigeti (1979) isolated 473 bacteria from the intestine of *Eisenia lucens*, and 73% of them were characterized as *Vibrio* spp. Given the results of Toyota and Kimura (2000), in which the dominant isolate (> 90%) recovered from starved *Eisenia foetida* worms was identified as *Aeromonas hydrophila*, it appears likely that the *Vibrio* spp. recovered from *E. lucens* may actually have been *Aeromonas* spp. Other studies have investigated the survival of *Aeromonas hydrophila* in earthworm worked soil (Hendriksen, 1995) and during passage through the intestine (Pedersen and Hendricksen, 1993). They found that this particular organism seems well adapted to survive in the presence of earthworms.

Members of the Actinobacteria also appear abundant in earthworm intestines. Parle (1963a) found more actinomycetes in the guts of *L. terrestris*, *Allolobophora caliginosa*, and *A. longa* than in the surrounding soil. The bacterium *Streptomyces olivocinereus*, when fed to the earthworm *Eisenia foetida*, while partially digested in the foregut, showed recovery and growth in the hindgut and cast of the earthworm (Polyanskaya et al., 1996). Contreras (1980) isolated *Streptomyces lipmanii*, as well as *Nocardia* and *Micromonospora* spp., in large numbers from the intestine of *Eisenia lucens*. The intestines of *Lumbricus rubellus* and *Octolasion montanum* also contained significant number of actinomycetes, and *Streptomyces* spp. (notably *S. diastatochromogenes* and *S. nogalater*) were particularly abundant (Kristufek et al., 1993). In addition, *L. rubellus* contained a large number of *Micromonospora* organisms, which were not present in *O. montanum* (Kristufek et al., 1993), suggesting that

microbial populations were not consistent between earthworm species, even in the same soils.

Whether or not earthworms contain an indigenous microbial population is not clear. The organisms that were found in high numbers in the studies described in this section were also present in the ingested material. For instance, *E. foetida* feeds readily on animal dung, where *Aeromonas* bacteria are found in high numbers. Past comparisons of isolates from both the soil and intestine of earthworm have found no difference in the soil and intestine populations (Parle, 1963a; Bassalik 1913) and led one researcher to state that "…earthworms possess no indigenous gut microflora" (Satchell, 1983). Thus, while certain taxa appear to thrive in association with earthworms, culture-based techniques have not distinguished any organisms uniquely associated with the animals.

7. Molecular studies of prokaryotes associated with earthworms

While molecular techniques have been applied to study the microbial populations in soil systems (see above) only a handful of studies have used these techniques to examine earthworms. Fischer et al. (1995) used DAPI stain direct counts and FISH with a general bacterial probe and α-, β-, and γ-proteobacterial specific probes to examine the abundances of these groups in the fore-, mid-, and hindgut of *L. terrestris*. While all proteobacterial subgroups increased in abundance from the foregut to the hindgut and cast, one of the most striking observations was that γ-Proteobacteria increased nearly 28-fold from the foregut to the hindgut. The α-Proteobacteria also increased 4-fold between the hindgut and cast. The same group later examined the intestines of *L. terrestris* using a wider suite of probes (Schönholzer et al., 2002). They did not reproduce their findings

that Proteobacteria organisms increased in abundance by passage through the earthworm intestine. However, they did document a considerable increase in δ-Proteobacteria and Cytophagales organisms in the earthworm casts. Unfortunately, oligonucleotide probes can only examine a specific portion of the entire community, so the abundance of non-targeted organisms could not be tracked. Nevertheless, these studies gave new information about the affect of the earthworm intestine on particular bacterial groups.

Perhaps the best support for an indigenous earthworm intestinal community came from the studies of Jolly et al. (1993) and Vinceslas-Akpa and Loquet (1995), who used electron microscopy to examine the walls of the intestine. Both studies, one in *L. terrestris* and *Octolasion cyaneum* and the other in *Eiseni fetida andrei*, visualized filamentous bacteria attached to the intestinal wall in the hindgut of the worms. Each considered these organisms potential indigenous organisms for earthworms, although the identity of the bacteria could not be established using their methods. To date, no further studies have examined the intestinal community using other molecular methods, so the question of what bacteria, if any, are associated with earthworms remains unresolved.

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Figure 1.1. Phylogenetic tree showing diversity of phyla and candidate divisions as determined by isolates and 16S rRNA clone sequences (from Hugenholtz et al., 1998a).

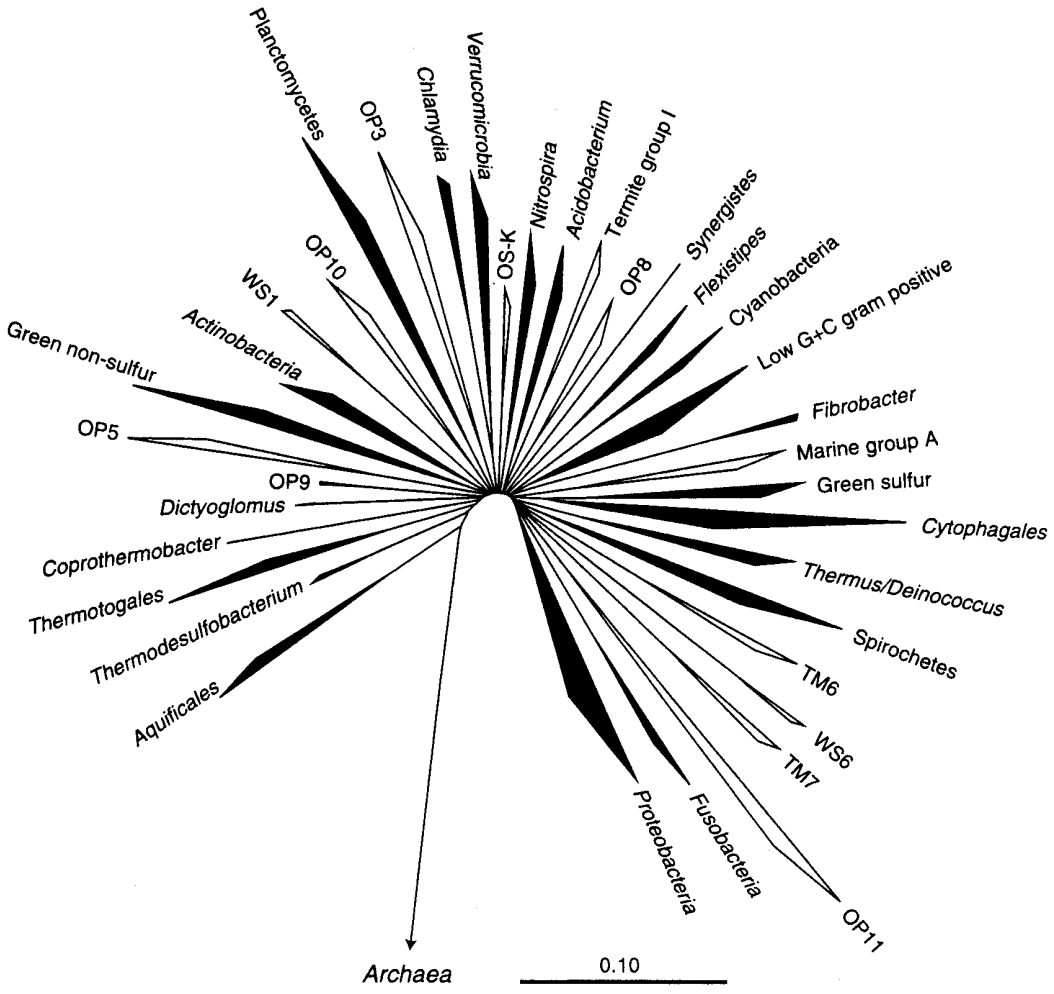
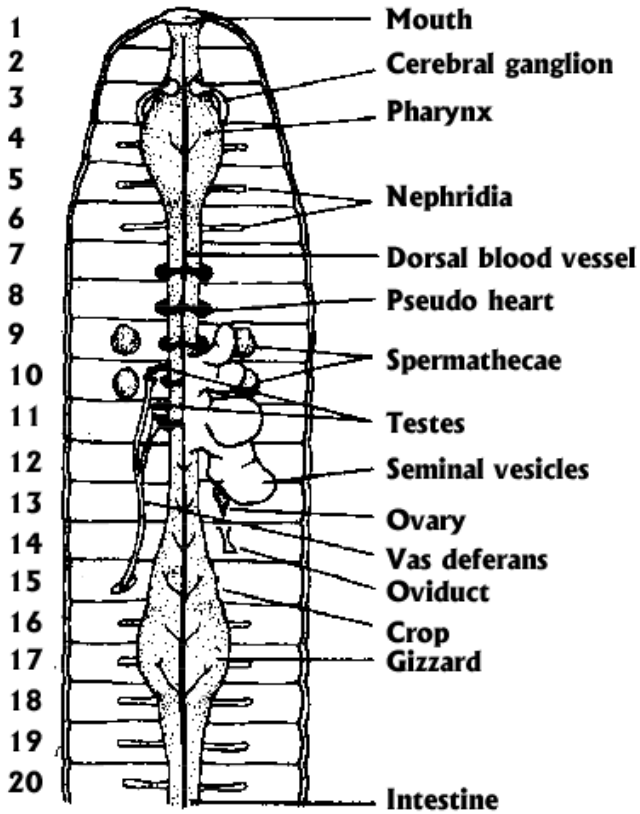

Planctomycetes
OP3
Chlamydia
Verrucomicrobia
OS-K
Nitrospira
Acidobacterium
Termite group I
OP8
Synergistes
Flexistipes
Cyanobacteria
Low G+C gram positive
Fibrobacter
Marine group A
Green sulfur
Cytophagales
Thermus/Deinococcus
Spirochetes
TM6
WS6
TM7
Proteobacteria
Fusobacteria
OP11
Archaea
0.10
Aquificales
Thermodesulfobacterium
Thermotogales
Coprothermobacter
Dictyoglomus
OP9
OP5
Green non-sulfur
Actinobacteria
WS1
OP10

Figure 1.2. Schematic of the reproductive, circulatory, and digestive system of the earthworm *Lumbricus terrestris* (from Edwards and Lofty, 1977).



(Reproductive structures not bilaterally complete. All nephridia not shown.)

CHAPTER II

QUANTITATIVE COMPARISONS OF 16S rDNA SEQUENCE LIBRARIES FROM ENVIRONMENTAL SAMPLES[1]

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[1] Singleton, D. R., M. A. Furlong, S. L. Rathbun, and W. B. Whitman. 2001. *Applied and Envionmental Microbiology*. 67:4374-4376. Reprinted here with permission of publisher.

ABSTRACT

To determine the significance of differences between clonal libraries of environmental rDNA, differences between homologous coverage curves $C_X(D)$ and heterologous coverage curves $C_{XY}(D)$ were calculated by a Cramér von-Mises type statistic and compared by a Monte Carlo test procedure. This method successfully distinguished rDNA libraries from soil and bioreactors and correctly failed to find differences between libraries of the same composition.

The sequencing of 16S rRNA genes from clone libraries of DNA from environmental samples has led to a wealth of information concerning prokaryotic diversity. However, in addition to methodological problems in producing libraries representative of the environmental sample (for a review see ref 6), this approach is also limited by the difficulty in comparing libraries and determining if they are significantly different.

This problem can be addressed quantitatively by an application of the formula for coverage as described by Good (3). Let "X" be a collection of sequences, such as a library of 16S rRNA genes. Define the “homologous” coverage of X (or C_X) by a sample from X to be equal to

$$C_X = 1 - (N_X/n)$$

where N_X is the number of unique sequences in the sample (i.e. sequences without a replicate) and n is the total number of sequences. In practice, the definition of N_X depends upon the criteria used to define uniqueness. For instance, McCaig et al. (5) considered sequences without a homolog of $\geq$ 97% similarity to be unique. Other

authors have used $\geq$ 99 % sequence similarity as the criterion. In principle, uniqueness can be defined at any level of sequence similarity or evolutionary distance (D), and a "homologous coverage curve" or $C_X(D)$ can be generated by plotting C_X vs. D (Figure 2.2). The coverage curve then describes how well the sample represents the entire library X at varying levels of relatedness. Typically, coverage might be low at high levels of relatedness (low values of D), indicating that only a small fraction of the sequences representing unique species are in fact sampled. In contrast, coverage might be much higher at low levels of relatedness, indicating that representatives of most of the deep phylogenetic groups present in X are found in the sample.

While C_X is the "homologous coverage" of X by a sample of X, it is also possible to calculate a "heterologous coverage" of X (or C_{XY}) by a sample Y from another collection of sequences:

$$C_{XY} = 1- (N_{XY}/n)$$

where N_{XY} is the number of sequences in a sample of X that are not found in a sample of Y, and n is the number of sequences in the sample of X. Similarly to N_X, N_{XY} can also be defined at different levels of D to generate a coverage curve $C_{XY}(D)$. Moreover, if X = Y, one might expect the coverage curves $C_X(D)$ and $C_{XY}(D)$ [as well as $C_Y(D)$ and $C_{YX}(D)$] to be similar. Thus, a test for differences between these coverage curves is also a test for differences between X and Y. To determine if the coverage curves of $C_X(D)$ and $C_{XY}(D)$ are significantly different, the distance between the two curves are first calculated using the Cramér von-Mises test statistic:

$$\Delta C_{XY} = \sum_{D=0.0}^{0.5} (C_X - C_{XY})^2$$

where D increases in increments of 0.01. If X = Y, then ΔC_{XY} should not be significantly different than a ΔC calculated after randomly shuffling sequences between the two samples X and Y. Typically, the sequences are randomly shuffled a large number of N times (say N = 999), and ΔC_{XY} is calculated after each shuffling. The randomized values plus the empirical value of ΔC_{XY} are ranked from largest to smallest, then the p-value is estimated to be r/(N+1), where r denotes the rank of the empirical value of ΔC_{XY} (4). We have created a computer program (LIBSHUFF) which uses a sorted distance matrix containing both X and Y as input and returns the coverage curves $C_X(D)$, $C_Y(D)$, $C_{XY}(D)$ and $C_{YX}(D)$ as well as the p-values for both ΔC_{XY} and ΔC_{YX} from the distribution of ΔC. In addition, the distribution of $(C_X - C_{XY})^2$ with D appears to be informative and is given as well (see below). The computer program LIBSHUFF was written in Perl, and can be downloaded along with more detailed instructions on its use at:

http://www.arches.uga.edu/~whitman/libshuff.html

A first test of this method was to insure that samples from the same library were not shown to be different. Thus, a collection of clonal sequences (n = 275) from a soil community study (5) was divided into two samples based upon accession numbers (odds and evens, n = 138 and 137, respectively). Although the study contained sequences from two sample sites (SL and SAF clones), sequences from both sites were placed in each data set to form nearly equivalent samples. A comparison of $\Delta C_{odds/evens}$ to ΔC values resulted in a p = 0.871, which indicated that the two samples were not significantly different (Figure 2.1A). Similar results were obtained for $\Delta C_{evens/odds}$ and other arbitrarily divided sequence libraries (Table 2.1). Thus, as expected, samples taken from the same library were not found to be different.

To demonstrate that this procedure could correctly differentiate samples from different libraries, sequences of clones obtained from an activated sludge (SBR1; n = 97; ref 1) were compared to grassland soil SL clones. The SBR1 clones were found to be significantly different from the SL clones (p = 0.001; Figure 2.1B). More information on the nature of this difference was obtained by examination of the distribution of $(C_X - C_{XY})^2$ with D (Figure 2.1B). At low D, the actual $(C_X - C_{XY})^2$ exceeded the comparable values at p = 0.05 obtained during the calculation of ΔC. This result suggested that the libraries differed greatly at D < 0.10 but shared many deep taxa. However, smaller differences at D > 0.3 suggested that not all deep phylogenetic groups were found in both libraries. Similar results were also obtained for comparisons of other soil and bioreactor libraries (Table 2.1 and data not shown).

Three sequence collections consisting of multiple samples were analyzed to determine if differences could be detected between the samples (Table 2.1). Clonal libraries derived from the microbial populations of phosphate-removing (SBR1) and non-phosphate-removing (SBR2) bioreactors differed in the abundance of certain taxa (1). However, these differences were not significant by our method (Table 2.1). The composition of libraries from the microbial communities of improved (SL) and unimproved (SAF) upland grass pasture soils were not found to be significantly different (5). We also obtained the same conclusion by our method (Table 2.1). Finally, comparisons of RFLP types from C0 and S0, two clonal libraries derived from arid soils, suggested that C0 was more diverse than S0 (2). Our analysis of the sequences obtained from this study was consistent with this conclusion and further suggested that S0 was a subset of C0. $\Delta C_{S0/C0}$ was not significant, which suggested that all taxa present in S0

were also present in C0 (Table 2.1). However, the reciprocal value $\Delta C_{C0/S0}$ was significant, therefore C0 also contained sequences of one or more taxa not found in S0. The distribution of $(C_X - C_{XY})^2$ with D further indicated that the additional taxa in C0 represented moderately deep phylogenetic groups, $0.15 < D < 0.25$ (Figure 2.1C).

Sample size was expected to have a major affect on comparisons of libraries. To examine this point in further detail, variable numbers of clonal sequences were randomly selected from either SBR1 or SL libraries (Y), compared to the opposite library (X), and p-values determined for 10 replicates. Approximately 20 and 25 sequences from SBR1 and SL, respectively, were required to differentiate the two libraries ($p < 0.05$) when X was represented by 97 and 137 sequences, respectively (Figure 2.2). Tests were also performed to investigate the required sample size of X (SBR1) when the size of Y (SL) was low. It was found that nearly all (≥ 90) sequences from the SBR1 library were required to distinguish these libraries when the SL library (Y) was represented by 20 sequences (data not shown). When the sizes of both libraries were varied, they were consistently detected as different when the SBR1 (X) and SL (Y) libraries were represented by ≥ 40 and ≥ 30 sequences, respectively (data not shown). While the number of sequences necessary to distinguish libraries depends to some extent upon the complexity of the libraries, these results suggest that modest sized libraries from many environmental samples will be distinguished by this method.

We would like to thank Kamyar Farahi and Rob Waldo for their help with programming in Perl. We would also like to thank Lihua Wang of the Statistical Consulting office at the University of Georgia for her help. This work was supported in

part by an award from the Division of Molecular and Cellular Biosciences at NSF (MCB-0084164).

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Table 2.1. Comparisons of environmental clone libraries.

Site (reference)	Homologous (X)		Heterologous (Y)	
	Clones	n	Clones	p[b]
Grassland soils (McCaig et al., 1999)	odds[a]	138	evens[a]	0.871
	evens[a]	137	odds[a]	0.933
	SAF	138	SL	0.120
	SL	137	SAF	0.135
Bioreactors (Bond et al., 1995)	odds[a]	95	evens[a]	0.853
	evens[a]	94	odds[a]	0.623
	SBR1	97	SBR2	0.308
	SBR2	92	SBR1	0.824
Arid soils (Dunbar et al., 1999)	odds[ac]	56	evens[a]	0.251
	evens[a]	56	odds[ac]	0.516
	C0-clones	59	S0-clones	0.042
	S0-clones	53	C0-clones	0.398
Grassland soil/ bioreactor	SAF	138	SBR1	0.001
	SBR1	97	SAF	0.002
	SL	137	SBR1	0.001
	SBR1	97	SL	0.001

[a] Sequences with an odd or even accession number. Contains mixtures of both libraries described in the reference and are not expected to be different.

[b] Value of r/(N+1) as described in the text.

[c] Accession number AF128647 could not be found and was not included.

Figure 2.1. Results of selected LIBSHUFF comparisons. Homologous coverage curves (❍) and heterologous coverage curves (●) for 16S rDNA libraries from environmental samples. Solid lines indicate the value of $(C_X - C_{XY})^2$ for the original samples at each value of D. Broken lines indicate the 950^{th} value (or $p = 0.05$) of $(C_X - C_{XY})^2$ for the randomized samples. (A) Comparison of clones from grassland soils with odd (X) and even (Y) accession numbers. (B) Comparison of bioreactor clones SBR1 (X) and grassland soil SL (Y) clones. (C) Comparison of C0 (X) and S0 (Y) clones from arid soils.

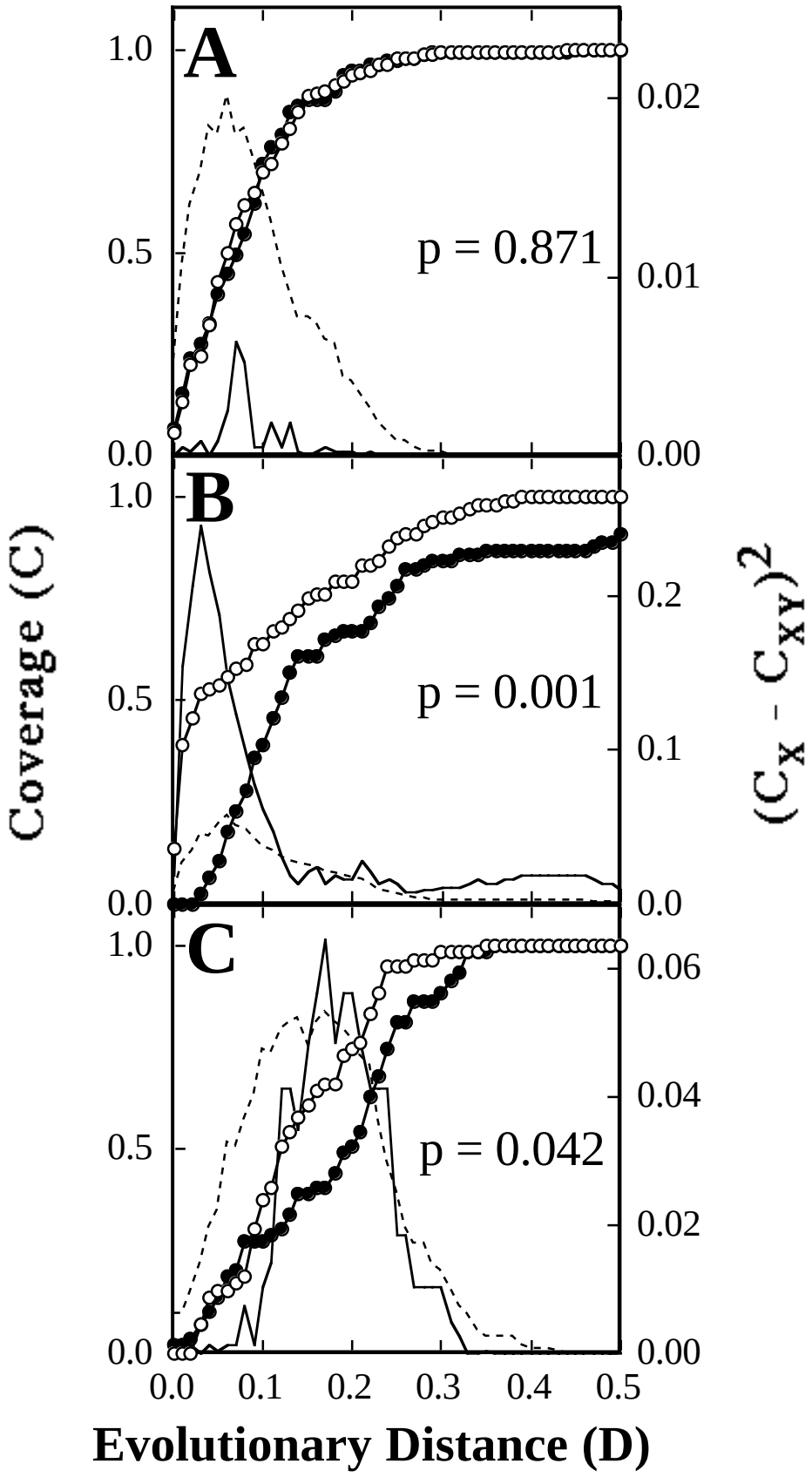
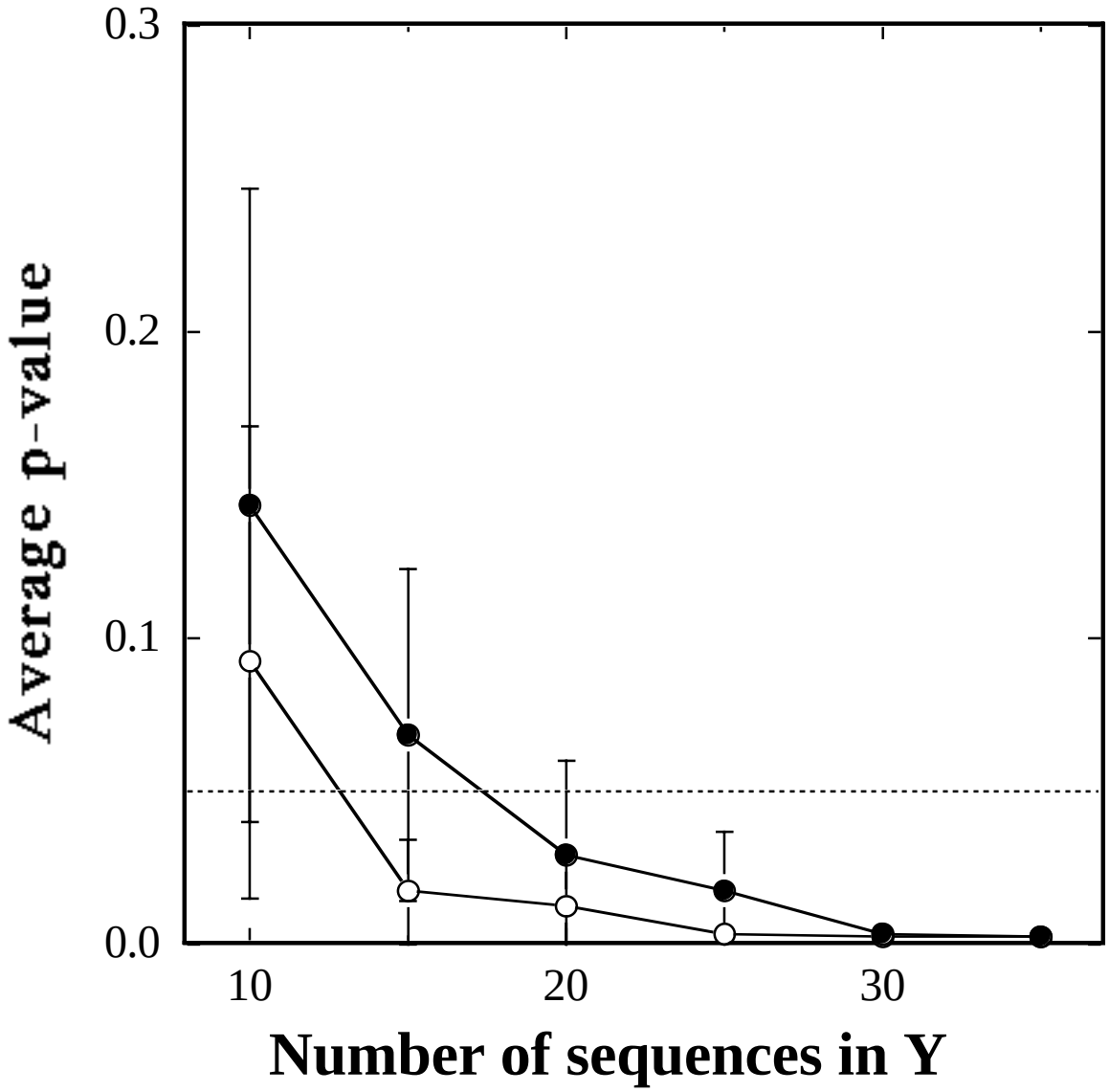

A
B
C
p = 0.871
p = 0.001
p = 0.042
1.0
0.5
0.0
0.02
0.01
0.00
0.2
0.1
0.06
0.04
0.0 0.1 0.2 0.3 0.4 0.5
Coverage (C)
$(C_X - C_{XY})^2$
Evolutionary Distance (D)

Figure 2.2. Effect of sample size on the discrimination of libraries. Comparison (●) of the SL library from grassland soil (Y; n = variable) to the bioreactor library SBR1 (X; n = 97) and the comparison (❍) of the SBR1 (Y; n = variable) to the SL (X; n = 137) library. Each point represents an average of 10 replicates and the error bars are one standard deviation. The broken line indicates p = 0.05.


0.3
0.2
0.1
0.0
Average p-value
10
20
30
Number of sequences in Y

CHAPTER III

MOLECULAR BASED ANALYSES OF PROKARYOTIC COMMUNITIES FROM AN AGRICULTURAL SOIL AND CASTS OF THE EARTHWORM *Lumbricus rubellus*[1]

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[1] Portions published in:

Furlong, M. A., D. R. Singleton, D. C. Coleman, and W. B. Whitman. 2001. *Applied and Environmental Microbiology*. 68:1265-1279. Reprinted here with permission of publisher.

ABSTRACT

The prokaryotic communities of a no-till agricultural soil and the casts of the earthworm *Lumbricus rubellus* from the Horseshoe Bend Agroecosystem Facility in Georgia were compared using 16S rDNA clone libraries. The soil bacterial clone library was similar to previously determined soil libraries and included 11 phyla and candidate divisions in addition to a number of sequences (18%) of for which taxa have not been described. The most abundant groups of organisms in the soil bacterial library grouped phylogenetically with the Acidobacteria (14%), Cytophagales (11%), Chloroflexi (8%), β-Proteobacteria (8%), an undescribed group (6%), Actinobacteria (5%), Nitrospira (5%), and the γ-Proteobacteria (5%). Although the cast bacterial library contained most of the same phyla as the soil library, larger numbers of sequences were recovered belonging to the γ-Proteobacteria (22%), Actinobacteria (17%), and Firmicutes (12%). Quantitative comparisons of the two bacterial libraries indicated that the cast library was derived from a population similar to that found in the soil except that it contained more representatives of the genus *Pseudomonas* as well as the phyla Firmicutes and Actinobacteria. In addition, the number of sequences from one deep undescribed phylogenetic group was reduced. The archaea detected were representative of a single lineage that has previously been detected in soil. The archaeal communities of the soil and cast were similar, except that one RFLP type was much more abundant in the cast. Bacterial isolates taken from the same site in a previous study were not well represented in the clonal libraries, and the two methods appeared to sample different portions of the soil and cast communities. Quantitative comparisons of the soil clone library to a clone library constructed from a

Scottish grassland soil indicated that the two samples were different, even though many of the same phyla were represented in both libraries.

INTRODUCTION

Earthworms can affect the soil environment in a variety of ways. Physically, earthworms can increase soil porosity (16, 38), aggregation (16, 17, 22, 64), pedogenesis, and litter breakdown (17), which are generally thought to promote soil health. The chemical characteristics of soil may also be influenced by earthworms, such as the varying concentrations of NO_3, NH_4, P, K, and organic C found in casts and drilosphere (burrow) soil (48, 63). In addition to the physical and chemical characteristics of soil, earthworms may also influence microbial populations by the comminution and transportation of litter in the soil profile (which may be a food source for microorganisms), selectively feeding on or dispersing microorganisms, or providing nutrients for the growth of organisms through secretions or passage through the intestinal cavity (reviewed in ref 8).

Studies of the earthworm gut and casts provide an opportunity to examine in detail the effects of soil and litter passage on microbial populations. Studies of various earthworm species have generally shown an increase in microbial numbers or activity either during or after passage through the gut (1, 12, 13, 18, 36, 49, 51, 58, 68, 72), although corresponding increases in microbial biomass were not always observed (12, 57). Specific groups of organisms have also been found in higher numbers in earthworm guts, casts, or burrows such as *Aeromonas hydrophila* in *Eisenia foetida* (66), fluorescent Pseudomonads in *Lumbricus terrestris* (13), and Actinobacteria in *Lumbricus rubellus* (36). Additionally, many studies have attempted to discern whether earthworms contain an indigenous microflora distinct from soil using culture-based techniques (11, 35, 42) and electron microscopy (31).

While agricultural soil has been studied previously using 16S rDNA cloning methods (7, 70), few studies have examined the prokaryotic communities of earthworms and earthworm casts in a culture-independent manner. In this study, we examine the casts of *L. rubellus* and the no-till soil that it inhabits using 16S rDNA clone libraries in order to detect what differences may exist between the two environments.

METHODS AND MATERIALS

Sample Site. Soil and earthworm samples were taken from the Horseshoe Bend Agroecosystem Facility (HSB). HSB is a NSF funded Long-Term Research in Environmental Biology (LTREB) site located in Athens, Georgia, USA. HSB is maintained by the Institute of Ecology at the University of Georgia and consists of agricultural plots subjected to long term (>20 years) no-till and conventional tillage regimes. Crops vary between season and year. Soil properties can be found in Furlong et al. (23) and Beare et al. (2).

Sample collection. Soil and earthworm samples were collected in April 1999 from a no-till plot (main plot, subplot 7) planted with clover. Soil samples were collected from 5 randomly chosen sites from a depth of 5-10 cm below the litter layer and put immediately on ice. The samples were stored at -20°C until processed. Two *Lumbricus rubellus* earthworms were collected from each of the five soil sample sites, placed into separate sterile Petri dishes, and taken to the laboratory. Earthworms were washed with sterile water and placed into new sterile Petri dishes until casts were collected. The first five casts were collected within four hours of sampling, combined into a single sample, and frozen at -20°C.

DNA extraction from soil and cast samples. Total DNA was extracted from the soil and cast samples by a modification of the protocols of Tsai and Olson (69) and Moré et al. (46). Soil, 1 gram wet weight, from each of the 5 sites were placed into 10 mL centrifuge tubes. The pooled casts from five earthworms (0.2 g wet weight) were placed into a 1.5 mL tube. To each tube was added 2 mL (g of sample)$^{-1}$ of a lysis buffer (0.15M NaCl, 0.1M disodium EDTA [pH 8.0], and lysozyme [1.5 mg/mL]). The samples were vortexed and placed in a shaking 37°C water bath for 1 hour, with additional vortexing every 15 minutes. The soil slurries were aliquoted into 500 µl portions in microfuge tubes. To each of the soil aliquots and the tube containing the cast material was added 400 µl of a bead beating solution (0.1M NaCl, 0.5M Tris·Cl [pH 8.0], and 10% SDS), and approximately 0.1 g of 0.1 mm diameter glass beads. These tubes were then placed into a TurboMix adaptor (Scientific Products) for a Vortex Genie 2 (Fisher Scientific) and treated for 3 minutes at maximum speed. The tubes were centrifuged for 3 minutes, and the supernatants were decanted into new tubes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Amresco) was added to each sample, vortexed briefly, and centrifuged for 10 minutes. The aqueous layer was transferred to a new tube, and extracted again with chloroform:isoamyl alcohol (24:1). The aqueous layer was transferred to a new tube, and the nucleic acids were precipitated with an equal volume of isopropanol at -20°C. After the precipitation, the tubes were centrifuged for 10 minutes. A dark brown pellet was visible in each tube. The pellets were vacuum dried and resuspended in 500 µl of dH_2O. These preparations were treated with RNAse (final concentration 10 µg/500 mL) at 37°C for 1 hour followed by passage through a

Wizard DNA Clean-Up Kit (Promega). The quality and concentration of DNA was confirmed via gel electrophoresis on a 1% agarose gel.

PCR amplification of 16S rRNA genes. 16S rRNA genes were amplified for construction of bacterial and archaeal clone libraries. Each PCR reaction consisted of 1 Ready-to-Go PCR bead (Amersham Pharmacia), 2 µl of forward primer (either 27f for bacterial libraries [5'-AGA GTT TGA TCM TGG CTC AG-3'] or 21f for archaeal libraries [5'-TTC CGG TTG ATC CYG CCG GA-3']), 2 µl of reverse primer (1392r universal [5'-ACG GGC GGT GTG TRC-3']), 1-2 µl sample DNA (4-20 ng), dH_2O to bring the reaction to 25 µl, and 30 µl mineral oil. Each PCR was 25 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1.75 minutes for denaturation, annealing, and extension steps, respectively, on a Thermolyne Temp·tronic thermocycler (Dubuque, IA). The mineral oil was removed, and the products were visualized on a 1% agarose gel prior to cloning. PCR products were either gel purified or cloned directly into the vector.

Construction of clone libraries. Clone libraries of archaeal and bacterial PCR products were constructed using a TA Cloning Kit (Invitrogen) with plasmid pCR®2.1. Successful transformants were inoculated into LB broth containing kanomycin and allowed to grow overnight. One library was prepared for each of the five soil samples. Similarly, the cast sample was amplified twice, and a library was prepared from each PCR product. Plasmids from the libraries were extracted using a QIAprep® Spin Miniprep Kit (Qiagen). Clones containing putative 16S rRNA genes were screened by *Eco*RI restriction endonuclease digestions. Each digestion reaction consisted of 2 µl of purified plasmid, 2 µl of Buffer H (Promega), 1 µl of *Eco*RI (Promega), and 18 µl dH_2O and was incubated at 37°C for 1 hour. Digestion products were visualized on a 1% agarose gel to

ensure the presence of inserts of the expected size. Clones which exhibited no insert or inserts much larger or smaller than expected were excluded from sequencing.

Screening of archaeal clones. Archaeal clones appeared less diverse than bacterial clones and were screened by restriction analysis prior to sequencing. Inserts in archaeal clones were reamplified using the PCR protocol described above. The restriction digestion was composed of 3 µl PCR product, 1 µl of 10X Multicore buffer (Promega), 1 µl of *Pvu*II (Promega), 1 µl of *Bgl*I (Promega), and 4 µl of dH_2O. The reaction was incubated at 37°C for 1 hour, and the entire reaction mixture run on a 2% agarose gel.

Sequencing of 16S rRNA genes. Partial sequencing of 16S rRNA genes was accomplished using an ABI 377 automated sequencer (Perkin-Elmer). Bacterial clones were sequenced using the primer 27f, while archaeal sequences were sequenced with the primer 21f. The average sequence length obtained was 550 bases. Selected clones were also sequenced using primer 1392r. Sequences were manually examined for quality, and those containing multiple ambiguous bases or were too short for analysis were resequenced.

Phylogenetic tree construction. Alignments of clone sequences and reference organisms were created using the PILEUP program of the GCG software package (24). Only sequence data corresponding to *E. coli* bases 81-459 were used for the phylogenetic trees. Trees were constructed using the PHYLIP software package (19). Distances were calculated using the Jukes-Cantor algorithm of DNADIST, and the branching order was determined via a Neighbor-joining algorithm using NEIGHBOR. Each tree was a consensus of 100 replicate trees (constructed by SEQBOOT) to obtain bootstrap values.

Analysis of 16S rDNA data. The closest database relatives of all clone sequences were obtained using all available sequence information by FastA searches of GenBank (50). The low sequence similarity of many of the clones to known organisms made classification into phyla based solely on these searches difficult, therefore, all clone sequences were plotted on a phylogenetic tree with representative sequences from known phyla. Phylogenetic trees specific for phyla and candidate divisions were then constructed to insure support for the positioning of clone sequences. Sequences which did not associate with any of the formally described lineages were classified as "undescribed" taxa for this analysis.

Chimera checking. All clones exhibiting less than 95% sequence similarity to an existing GenBank sequence were examined as potential chimeras. Sequences were first analysed with the CHIMERA_CHECK program at the Ribosome Database Project II (RDPII; ref 41) using the default settings. Potential chimeric sequences were then reanalyzed excluding RDP sequences shorter than 1000 nucleotides so that the analysis would not be biased by shorter entries that contained sequence information homologous to only a portion of the clone sequence. Clones that were not excluded as possible chimeras at this step were then examined through the use of FastA searches of the entire available sequence and of the 5' and 3' sequence fragments as previously suggested by CHIMERA_CHECK. Sequences where the 5' and 3' ends were closely related to different phylogenetic groups and did not have a close relative in this study from an independent PCR reaction were considered chimeric and were excluded from the study.

Coverage and diversity comparisons. For the purposes of this study, an OTU was defined as a group of sequences with ≥ 99% sequence similarity. Coverage for the

libraries was determined by the formula of Good (26). The Shannon-Weaver diversity index and eveness were calculated using standard equations (53, 59). In order to directly compare soil and cast libraries, the cast library was artificially reduced in number by randomly removing 7 sequences before calculating diversity indices. This process was repeated 10 times and the average value was reported.

LIBSHUFF comparisons. All sequences to be compared were first aligned using the PILEUP program of the GCG software package (24). The resulting file was formatted such that only sequence data available for the majority of clones (> 95%) was used. Evolutionary distances were acquired using the Jukes-Cantor algorithm of DNADIST in the PHYLIP software package (19). The output was converted into a distance matrix using a spreadsheet program, reformatted such that sequences belonging to each library were sorted together in both the x and y coordinates, and the names of the sequences were removed. The resulting file was then run through the LIBSHUFF analysis (60).

Nucleotide sequence accession numbers. Clones from the soil and *L. rubellus* cast were deposited in GenBank with the accession numbers AY037556-AY037762.

RESULTS

Construction of the bacterial libraries. A total of 100 and 104 bacterial 16S rDNA clones were sequenced from DNA extracted from HSB agricultural soil and *L. rubellus* casts, respectively. Five soil and two cast clones were identified as chimeras and removed from further analyses, resulting in 95 and 102 clones for the libraries, respectively.

Comparison of the replica libraries suggested that the methods for their construction were reproducible because most phyla were evenly represented in the replicates (Table 3.1). A notable exception was the presence of relatively high numbers of Firmicutes sequences in only one of the five soil libraries and one of the two cast libraries. As all reactions were treated in a similar fashion we cannot explain this disproportionate amplification of Firmicutes sequences. Perhaps, the distribution of this taxon was uneven in the soil samples as well, although this would not explain the increased occurrence of Firmicutes in one of the cast libraries, which were both were constructed from the same sample. Additionally, both the cast and soil libraries contained representatives of phyla difficult to lyse in pure culture (e.g. Actinobacteria), so biases arising from incomplete cell lysis were probably minimal (46).

Comparison of soil and earthworm cast libraries: Sequences with ≥ 99% sequence similarity over the region of the 16S rRNA gene used for phylogenetic analyses were combined into operational taxonomic units (OTUs). The soil library contained 86 OTUs (out of 95 clones), which were classified into 11 phyla (or candidate divisions) and 9 taxa which have not been formally described or "undescribed" groups. No single phylum dominated the soil library, but the largest number of clones were from the Acidobacteria (14%), Cytophagales (13%), Chloroflexi (8%), and β-Proteobacteria (8%). The cast library contained 81 OTUs (out of 102 clones), which were grouped into 10 phyla (or candidate divisions) and 2 undescribed taxa. The library included comparable numbers of Acidobacteria (10%) and Cytophagales (13%) as the soil library, but it also contained a large number of clones from the γ-Proteobacteria (22%), Actinobacteria (17%), Firmicutes (12%), and Alpha Proteobacteria (10%), groups that were not well represented

in the soil. All phyla and undescribed taxa found in the cast library were also present in the soil library, although the soil library contained a number of groups that were not found in the cast library.

A number of clones could not be placed into a described phylum or candidate division (Table 3.1) and were classified into undescribed groups A - I. Group A was the largest and was found only in soil. It possessed and average 87% sequence similarity to a clone obtained from an environment contaminated with pentachlorophenol (PCP; ref 3). Because these clones, which possessed 85 - 93% sequence similarity to each other, were obtained from more than one sample, they were unlikely to be PCR artifacts. Group B included two cast clones and a soil clone (84 - 89% similarity) with an average sequence similarity of 91% to clones from rhizosphere (43) and arid soil (37). Group C contained three soil clones (88 - 89% similarity) which possessed an average sequence similarity of 86% to hot spring clones (30). Group D contained two clones with an average similarity of 86% to each other and clones from an arid (15) and a metal-contaminated soil. Group E consisted of a pair of soil clones (85% similarity) with an average similarity of 83% to Chloroflexi clones from an anaerobic bioreactor (71) and Antarctic ice (27). However, in our analyses these sequences did not possess a strong affiliation with the Chloroflexi phylum. Group F included two identical soil clones with 77% similarity to a freshwater bacterium clone (25). Groups G, H, and I each consisted of a single soil clone with sequence similarity to *Acidosphaera rubrifaciens* (74%), a subsurface clone (80%; ref 10), and a deep sea bacterium (89%; ref 39), respectively.

While the abundance of many of the taxa in the soil and cast libraries were similar, the abundance of other taxa changed dramatically. For instance, the γ-

Proteobacteria increased from 4% of the soil library to 22% of the cast library (Table 3.1). The majority of cast clones in this group were closely related to a number of *Pseudomonas* spp. (Figure 3.1). Although numerically less abundant, closely related soil clones were also observed. The Firmicutes clones increased from 4% in the soil to 12% in the casts (Table 3.1), and most of the clones were phylogenetically related to *Bacillus* spp. and similar genera (Figure 3.2). Although none of the cast clones were closely related to the soil clones, this observation may be due to the low representation of soil clones in this taxon. The Actinobacteria also increased from 5% in the soil to 17% in the cast (Table 3.1). While individual clones were associated with a number of different genera within the phylum (Figure 3.3), the largest group of nine clones (2 soil and 7 cast) were related to the Rubrobacteria, which have been found in a number of clone libraries previously (Figure 3.4; refs 6, 28, 40, 44, 55).

The hypothesis that the soil and cast libraries were not significantly different was tested by the LIBSHUFF method (60). The heterologous coverage of the cast library by the soil library was not significantly different ($p = 0.431$) from the homologous coverage of the cast library by itself, indicating that most sequences from the cast library had high similarity to sequences in the soil library (Figure 3.5A). Additionally, a comparison of the calculated value of $(C_X - C_{XY})^2$ to the 95% value of $(C_X - C_{XY})^2$ from the random shufflings showed differences between the libraries only when $D \leq 0.02$ (Figure 3.5A). This result suggested that differences that did occur were mostly among closely related sequences. This result was also expected from the low coverage of the libraries at high levels of relatedness (see also Table 3.2). These conclusions were supported by phylogenetic trees in which cast sequences often grouped near soil sequences (Figures 1-

4 and data not shown) but were rarely identical. In fact, the average evolutionary distance from a cast clone to its closest relative in the soil libraries was only 0.09 ± 0.07, compared to a distance of 0.18 ± 0.14 for a soil clone to its nearest relative in the cast libraries. In contrast to the high similarity of the cast library to the soil library, the heterologous coverage of the soil library by the cast library was significantly different ($p = 0.001$) from the homologous coverage of the soil library. This result indicated that some sequences in the soil library had no close relatives in the cast library (Figure 3.5B). Differences between the libraries were especially apparent when D was between 0.03 and 0.05 and well as when $D \geq 0.14$ (Figure 3.5B), which suggested that in addition to differences within phyla, at least one deep phylogenetic group was present in the soil library that was absent in the cast library.

Examination of Table 3.1 suggested that the increased abundance of the Actinomyces, Firmicutes, and Gamma Proteobacteria in the cast library as well as the presence of six sequences in undescribed group A in the soil library that were absent from the cast library may have been responsible for the differences in the two libraries. When these four groups were removed from the LIBSHUFF comparison, the cast and soil libraries were no longer significantly different (X = cast library, Y = soil library; $p_{XY} = 0.421$ and $p_{YX} = 0.144$). However, removal of any three of these four groups from the analysis still yielded p values < 0.05. Thus, all four taxa combined were responsible for the differences between the soil and cast bacterial libraries.

Various diversity indices were calculated for the soil and cast bacterial libraries (Table 3.2). The diversity and eveness indices approached the maximum for both the soil and cast libraries, although the values for the soil library were slightly higher. The higher

diversity of the soil library was reflected in the phylogenetic distribution. The soil library contained representatives of more phyla and other deep phylogenetic groups than the cast clones (Table 3.1). Coverage values were low for both samples, which would be expected for libraries constructed from populations of high diversity. However, the coverage of the cast library was slightly higher than the soil library, which was consistent with the somewhat lower diversity in the cast library. Additionally, the large number of nearly identical sequences representing the genus *Pseudomonas* (Figure 3.1) was also consistent with the higher overall coverage of the cast library.

Archaeal clone libraries. Archaeal PCR products were obtained from DNA extracted from four of the five soil samples as well as the pooled cast sample. Preliminary sequencing of some of these archaeal clones indicated that the archaeal libraries contained limited diversity. Therefore, a number of clones from the four soil samples (n = 27) and the cast sample (n = 18) were screened by RFLP analysis. Five patterns (designated types A - E) were observed, and partial sequencing of representative clones indicated that they constituted a single archaeal lineage which had been detected previously in soil (Figure 3.6A; ref 5). The RFLP types were not distributed evenly between the soil and cast clones (Figure 3.6B). Pattern B dominated in the soil clones (70%), while pattern D accounted for the majority of the cast clones (72%). Additionally, it is possible that pattern E may have been more highly represented in the cast library than the soil library (Figure 3.6B). Although only three of the five RFLP types were found in the cast library fewer cast clones were screened, and it was not surprising that less abundant RFLP types (types A and C) were not detected in the cast sample.

Comparisons of the clone libraries to isolates from the same site. Bacterial isolates were previously obtained from soil and *L. rubellus* burrows and casts taken from the same site, although most of the isolates were obtained in a different year (23). Although the nature of the cast isolates may have changed during the three year study period, the phenotypic properties of the soil isolates appeared to remain the same (23). Therefore, it was of particular interest to compare the soil isolates to the soil clone library by the LIBSHUFF method. The isolate and clone libraries were significantly different ($p = 0.001$ for both comparisons; data not shown) even though the same phyla represented by the isolates were also found in the clone libraries. Further analysis of the LIBSHUFF results and phylogenetic trees suggested that, for the most part, different genera and species were isolated than were sequenced from the clone libraries for both the soil and cast (Figures 1-4 and data not shown). Specific exceptions to this included one isolate in the Firmicutes, and two isolates in the γ-Proteobacteria that possessed nearly identical sequences to those found in the clone libraries.

The clone libraries contained representatives of many more deep phylogenetic groups than the collection of isolates. For instance, the Acidobacteria, Cytophagales, and Chloroflexi were all well represented in the soil clones, but nearly or entirely absent from the isolates. Similar observations are common, suggesting that soil contains a large number of taxa that are not readily culturable by common laboratory techniques. Moreover, even among the phyla well represented in both the clone and isolate collections, the overlap was less than expected. For instance, from the coverage curves, the collection of isolates was expected to be 75% complete when $D = 0.03$ (23). Nevertheless, only 2 sequences of the 25 soil clones in the Actinobacteria, Firmicutes,

and Proteobacteria possessed an evolutionary distance of ≤ 0.03 to an isolate sequence (Figures 1-4). Similary, the LIBSHUFF analysis suggested that both collections were different ($p = 0.001$). Thus, the overlap even within the common phyla was low. However, while previous studies utilizing both molecular and cultivation methods have produced similar findings (4, 10, 15, 45, 52, 61, 62), the interpretation of the results presented in this study is ambiguous because the clones and isolates were not from samples collected at the same time. Therefore, it is possible that changes in the ribotypes of the microbial population of soil that were not reflected in changes in the phenotype contributed to the observed differences.

Comparison of Horseshoe Bend clones to Scottish soil clones. Based on phyla level classification, many soil clone libraries appear similar (29). In order to examine this apparent similarity in greater detail, a clone library constructed from Scottish grassland soils (44) was compared to the soil clone library from HSB. The clone libraries constructed from Scottish and HSB soils contained most of the same phyla, although the α-Proteobacteria were much more abundant in the library from the Scottish soils (44). Additionally, each library contained a number of clones from undescribed taxa.

LIBSHUFF analyses were performed comparing the HSB soil clone library ($n = 95$) to the Scottish soil SAF clone library ($n = 138$; ref 44). As previous comparisons have shown that the SAF and SL libraries of McCaig et al. (44) were not significantly different (60), only comparisons to the one Scottish library were performed. The HSB and Scottish libraries were significantly different ($p = 0.001$; Figure 3.7). When comparing the SAF library (X) to the HSB library (Y), the main differences occurred only when $D \leq 0.15$, indicating that all the phyla present in the SAF library were also in

the HSB library (Figure 3.7A). However, within these phyla, the SAF and HSB libraries contained different OTUs. In contrast, the differences between the HSB library (X) and the SAF library (Y) occurred over a larger range ($D \leq 0.42$), indicating that at least one deep taxon was present in the HSB library which was not present in the SAF library (Figure 3.7B). Because the Scottish library contained a much higher number of α-Proteobacteria than the HSB library, a second LIBSHUFF analysis was performed with all sequences belonging to this taxon removed. Significant differences were still detected between the libraries (X = SAF, Y = HSB, $p_{XY} = 0.003$, $p_{YX} = 0.001$), and the nature of the differences were similar to those observed when considering the entire libraries.

Estimation of OTUs present in HSB soils. The number of OTUs expected in the soil population was estimated by calculating the number of OTUs present in the soil library at each value of D and multiplying by the coverage. At low values of D, nearly 1000 OTUs are expected in the soil population (Figure 3.8). This is in agreement with other methodologies that predicted that thousands of genomes may exist in soil (65).

DISCUSSION

L. rubellus is an epigeic earthworm that typically inhabits and feeds within the litter layer of the soil. However, nutritional studies have shown that *L. rubellus* ingests soil when feeding and actually prefers a combination of soil and litter to litter alone (14). Indeed, the cast samples taken in this study were from earthworms found in the soil profile, well below the litter layer. Thus, it would not be unreasonable to hypothesize that many, if not all, of the microoganisms deposited in the cast may have originated in

the soil. In fact, close relatives of nearly all clone sequences from the *L. rubellus* cast library were found in the soil library, including those which could not be placed into a known phylum or candidate division. This result was confirmed by the LIBSHUFF comparison of the cast clones to the soil clones which indicated no significant difference existed. Moreover, some prokaryotic groups were more abundant in the cast than soil libraries, suggesting that these groups may have been specifically amplified by passage through the earthworm gut. The alternative, that all other taxa were selectively digested seems unlikely. If degradation was the major process, taxa with the same cell wall type might be expected to fare similarly. This was not observed. For example, the γ-Proteobacteria dramatically increased in abundance while the other proteobacteria increased modestly or not at all. If the numbers of some taxa increased, the abundance of others would be expected to decrease just through dilution. This model alone would appear sufficient to explain the absence of many of the less common soil taxa in the cast library and the lower diversity of the cast library. However, it is also possible that some groups, such as undescribed group A, whose abundance was dramatically lower in casts, were either selectively degraded or avoided during feeding.

The increased numbers of γ-Proteobacteria was the most startling difference between the cast and soil libraries. This phenomenon has been observed previously in *Lumbricus terrestris*, in which members of the γ-Proteobacteria increased nearly 20-fold between the foregut and the cast (20). Of the γ-Proteobacterial sequences taken from the cast library, 91% were highly related to *Pseudomonas* species. Examinations of the numbers of *Pseudomonas* spp. associated with earthworms sometimes contradict. In one study, one day old casts of *L. terrestris* contained up to 141 times more fluorescent

pseudomonads than a corresponding untreated soil (13). In contrast, a study by Pedersen and Hendriksen (51) found that numbers of *Pseudomonas putida* decreased after passage through the gut of various *Lumbricus* earthworm species. *Pseudomonas* spp. utilize a wide variety of growth substrates and can grow well in the presence of other organisms (47), which could be important given that the alimentary canal of the earthworm contains higher levels of moisture, organic carbon, and total nitrogen than soil (12, 33). Additionally, many pseudomonads are also resistant to antibiotics produced by Actinobacteria (47), a group which also constituted a significant portion of the cast clone and isolate libraries. Given these characteristics, members of the *Pseudomonas* genus are likely candidates for amplification by passage through the earthworm.

Isolates from the cast also contained significant numbers of γ-Proteobacteria (23). However, while a number of *Pseudomonas* spp. were isolated from casts, the dominant cast isolates showed high similarity to *Aeromonas* spp., which was not found in the clone libraries. *Aeromonas* spp. have been previously identified as a dominant isolate in the casts of the earthworm *Eisenia foetida* (66) and have been associated with the gut of *Pheretima* sp. (67). Given the isolation of large numbers of *Aeromonas* spp. from earthworms previously and from *L. rubellus* casts at HSB, it was somewhat surprising that no clones associated with this lineage were found in either of the clone libraries. Because the isolate and clone library samples were taken in different years, it is possible that there may have been a shift in the microbial community structure (23). Alternatively, *Aeromonas* spp., while readily culturable, may not have composed a large enough percentage of the total community to have been readily detected in the clone libraries.

Members of the Actinobacteria and Firmicutes phyla also appeared in greater numbers in the cast library as compared to the soil library (Table 3.1). Actinobacteria have been commonly isolated from earthworms and the genera *Streptomyces* and *Micromonospora* have been associated with *L. rubellus* (11, 35, 49). In contrast, only two cast clones were closely related to these genera. Moreover, no single organism dominated the Actinobacteria cast clones, although the largest number of sequences were associated with the Rubrobacteria. Brown (8) hypothesized that antibiotic production by Actinobacteria in the earthworm gut may inhibit the growth of other organisms, particularly other Gram positive and sensitive Gram negative organisms. Our observations were consistent with this hypothesis, where two of the three taxa with increased abundance in the cast libraries are related to groups that are commonly antibiotic resistant. If this hypothesis is correct, the apparent increase in Firmicutes clones in the cast library may have resulted from endospore germination but not growth in the gut. When Fisher et al. (21) examined the passage of *Bacillus megaterium* endospores through *L. terrestris*, they found that spores germinated in the gut but found little evidence for the division of vegetative cells. Another study followed the passage of *Streptomyces* spores through the earthworm *Eisenia foetida* and found that, while cell numbers decreased in the gut of the worm, numbers of cells and mycelium length significantly increased in the cast (54). The gut and cast conditions of *L. rubellus* could similarly induce germination of endospores. The loss of the spore coat could then have rendered the cells more susceptible to cellular lysis and increased their representation in the cast libraries.

Although Archaea in soils appear to have limited diversity, they include a number of deep phylogenetic groups that have never been cultivated (5, 9, 32, 34, 56, 70). The archaeal sequences recovered in this study were similar to sequences found in other agricultural soils (5, 70). The sequenced representative of the dominant soil library RFLP type (B) was a close relative of archaeal clones SCA1154 and FIE16 from other soils (5, 70) . Although RFLP type B accounted for 47% of all archaeal clones screened in this study (soil and cast), soil clone SCA1154 was found only once in a screening of 34 archaeal sequences from a Wisconsin soil. Thus, it seems likely that this organism is a larger fraction of the archaeal community in HSB than the Wisconsin soil. In contrast, RFLP type (D) was abundant at HSB and was similar to clone SCA1145, which accounted for 47% of the archaeal sequences obtained in the Wisconsin soil (5) and was similar to a ribotype common in soils from Michigan (9). Therefore, this phylogenetic group appears to be widely distributed. Interestingly, RFLP type D also dominated the libraries constructed from cast material, and it would be interesting to know if this group was associated with earthworms at other sites as well.

The LIBSHUFF analysis allows direct comparison of clone libraries without prior assignment of the clones into phylogenetic groups, which is a great advantage when comparing libraries from studies that often use different classifications. Unfortunately, other prominent agricultural soil clone libraries (e.g. refs 7, 70) could not be compared to the HSB soil clone library because different portions of the gene were sequenced. However, the grassland soil clone libraries of McCaig et al. (44) contained a large number of sequences which shared the same region of the 16S rRNA gene as our own libraries. The HSB and Scottish soil clone libraries were found to be significantly

different, which, given the differences in geography, climate, and vegetation may not be surprising. Although methodological differences between the laboratories may also have contributed to the observed differences, the results suggest that the prokaryotic populations of soils may be highly variable. Thus, not only is the diversity within one location high (65), but the prokaryotic community is also likely to be different and just as diverse in other types of soil.

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Table 3.1. Distribution of clone sequences into phylogenetic groups.

Phylogenetic group	Number of clones							% of Total Library	
	Agricultural soil					Earthworm cast			
	1	2	3	4	5	1	2	Soil	Cast
Acidobacteria	3	2	3	4	1	3	7	14	10
Actinobacteria	1	1		2	1	10	7	5	17
Candidate Division TM7	1		1			3	3	2	6
Cytophagales	3	2	3	1	1	8	5	11	13
Fibrobacter			1	2	1	1		4	1
Firmicutes		4				11	1	4	12
Chloroflexi	2	1	3	2			2	8	2
Nitrospira			2	1	2		1	5	1
Planctomycetes			1	2				3	
Proteobacteria									
α–	1	1			2	8	2	4	10
β–	1	3	3	1		1	2	8	3
γ–	1	1	1		1	15	7	4	22
δ–		2	2	1		1		5	1
Verrucomicrobia		2				1		2	1
Undescribed									
Group A	3	1		2				6	
Group B				1		1	1	1	2
Group C	1	1		1				3	
Group D		1				1		1	1
Group E		1			1			2	
Group F		2						2	
Group G		1						1	
Group H		1						1	
Group I				1				1	
Sample size (n)	17	27	20	21	10	64	38	95	102

Table 3.2. Diversity indices for soil and cast clone libraries[a].

Index	Soil Library[b]	Cast Library[c]	Maximum[d]
Shannon-Weaver	4.40	4.11	4.61
Evenness	2.27	2.19	2.33
Coverage	0.14	0.20	1.00

[a] All calculations based on OTU of ≥99% sequence similarity over positions 81-459.
[b] All 95 clones were used in the analysis.
[c] Value shown is average of 10 replicates of the cast library randomly reduced from 102 to 95 sequences.
[d] Maximum value for each index. The minimum values for each index were 0.

Figure 3.1. Phylogenetic tree of the γ-Proteobacteria clones and isolates from HSB. Clone sequences from this study are printed in bold. HSB isolate names follow the code of one or two letters indicating the habitat, number, letter indicating the isolation media ("M" for a minimal media with acetate and "D" for 50% Difco nutrient broth), and number (e.g. S84D1). Clone and isolate sequences from the soil begin with "S", from the cast with "C", from the earthworm burrow with "B", except for isolates taken in the same year as the clones, which are denoted with "NS" or "NC" for the "new" soil and "new" cast, respectively. Brackets after HSB sequences denote the total number of sequences in the OTU. Accession numbers appear in parenthesis for all reference sequences. Open circles (❍) denote bootstrap support of ≥ 95% and closed circles (●) denote bootstrap support of ≥ 50%. The scale bar represents Jukes-Cantor evolutionary distance.

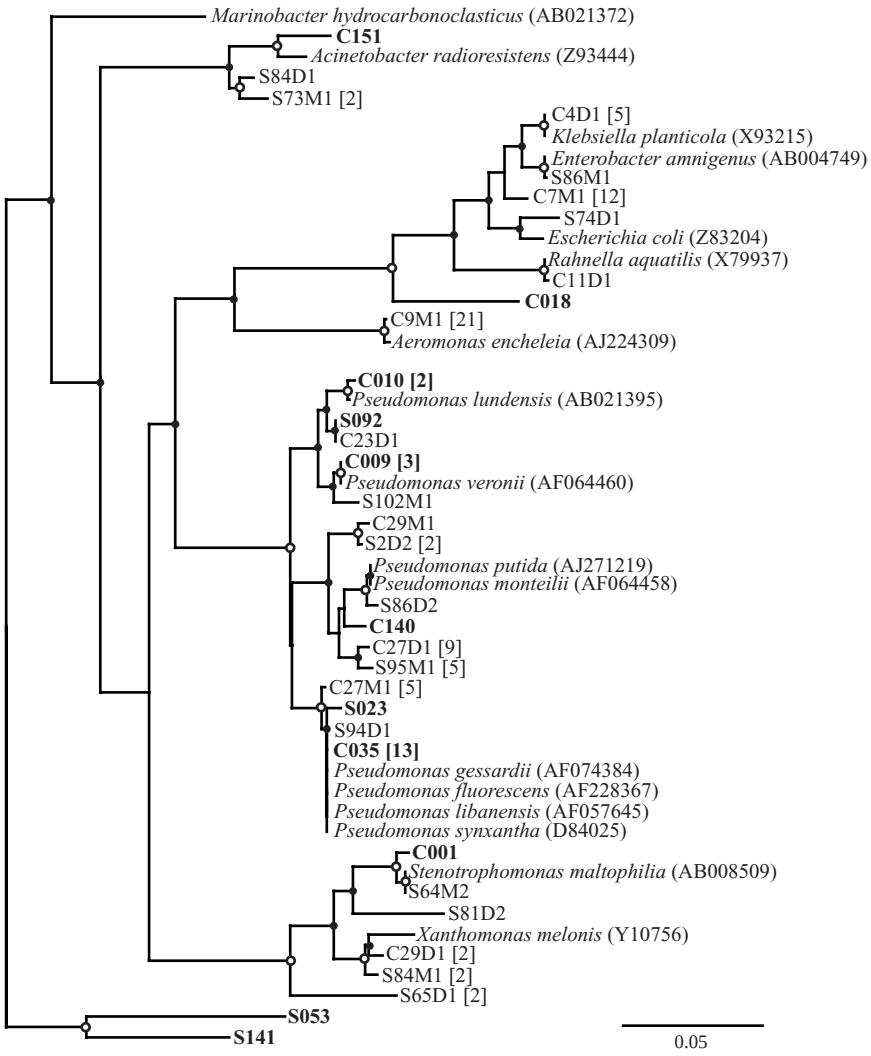

Marinobacter hydrocarbonoclasticus (AB021372)
C151
Acinetobacter radioresistens (Z93444)
S84D1
S73M1 [2]
C4D1 [5]
Klebsiella planticola (X93215)
Enterobacter amnigenus (AB004749)
S86M1
C7M1 [12]
S74D1
Escherichia coli (Z83204)
Rahnella aquatilis (X79937)
C11D1
C018
C9M1 [21]
Aeromonas encheleia (AJ224309)
C010 [2]
Pseudomonas lundensis (AB021395)
S092
C23D1
C009 [3]
Pseudomonas veronii (AF064460)
S102M1
C29M1
S2D2 [2]
Pseudomonas putida (AJ271219)
Pseudomonas monteilii (AF064458)
S86D2
C140
C27D1 [9]
S95M1 [5]
C27M1 [5]
S023
S94D1
C035 [13]
Pseudomonas gessardii (AF074384)
Pseudomonas fluorescens (AF228367)
Pseudomonas libanensis (AF057645)
Pseudomonas synxantha (D84025)
C001
Stenotrophomonas maltophilia (AB008509)
S64M2
S81D2
Xanthomonas melonis (Y10756)
C29D1 [2]
S84M1 [2]
S65D1 [2]
S053
S141
0.05

Figure 3.2. Phylogenetic tree of the HSB clones and isolates associated with the Firmicutes phylum. Notation is as described in Figure 3.1.

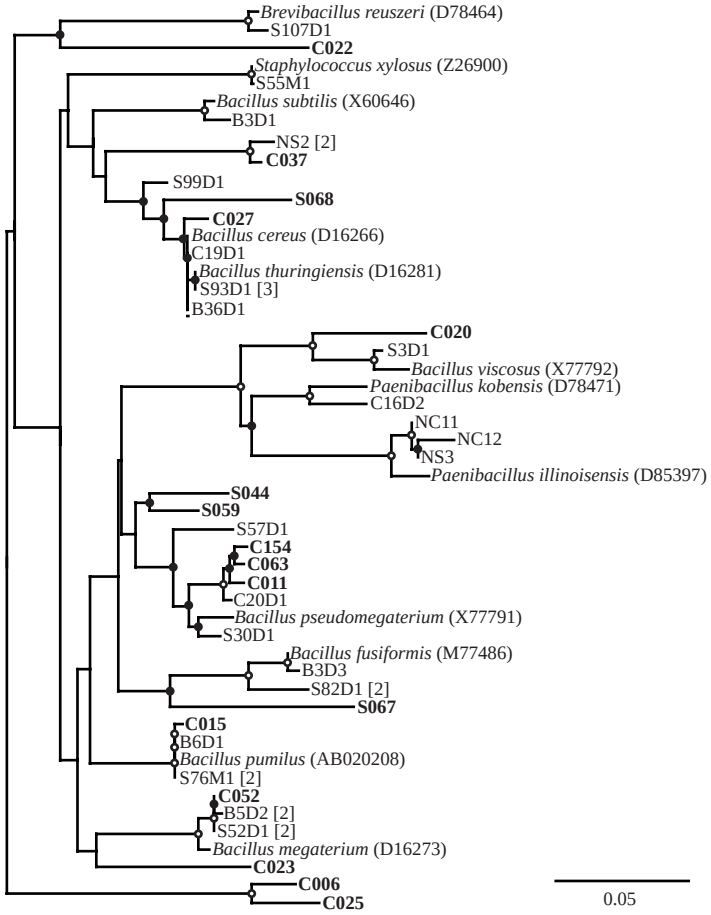

Brevibacillus reuszeri (D78464)
S107D1
C022
Staphylococcus xylosus (Z26900)
S55M1
Bacillus subtilis (X60646)
B3D1
NS2 [2]
C037
S99D1
S068
C027
Bacillus cereus (D16266)
C19D1
Bacillus thuringiensis (D16281)
S93D1 [3]
B36D1
C020
S3D1
Bacillus viscosus (X77792)
Paenibacillus kobensis (D78471)
C16D2
NC11
NC12
NS3
Paenibacillus illinoisensis (D85397)
S044
S059
S57D1
C154
C063
C011
C20D1
Bacillus pseudomegaterium (X77791)
S30D1
Bacillus fusiformis (M77486)
B3D3
S82D1 [2]
S067
C015
B6D1
Bacillus pumilus (AB020208)
S76M1 [2]
C052
B5D2 [2]
S52D1 [2]
Bacillus megaterium (D16273)
C023
C006
C025
0.05

Figure 3.3. Phylogenetic tree of the HSB clones and isolates associated with the Actinobacteria phylum except Rubrobacteria. Sequences associated with the Rubrobacteria are shown in Figure 3.4. Notation is as described in Figure 3.1.

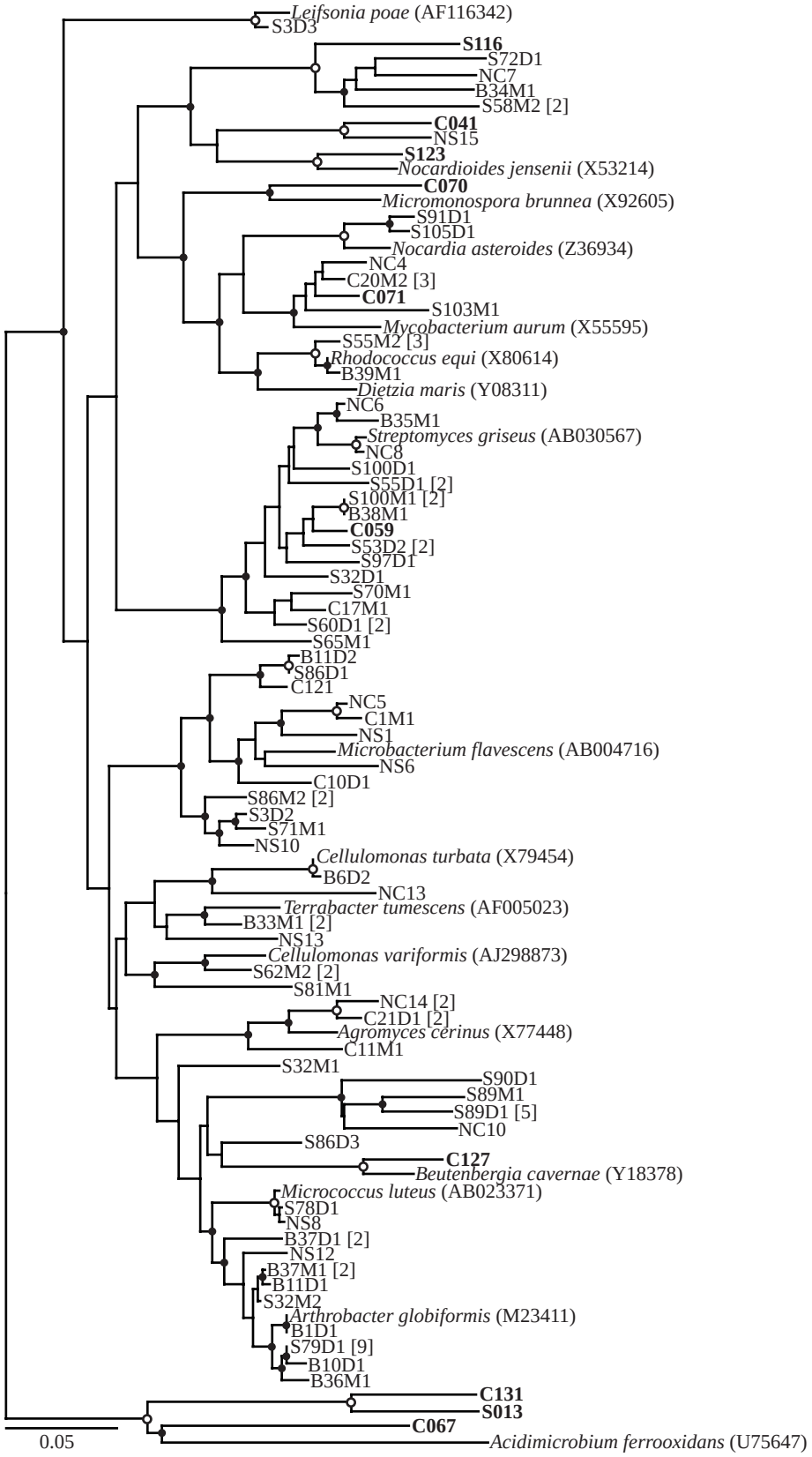

Leifsonia poae (AF116342)
S3D3
S116
S72D1
NC7
B34M1
S58M2 [2]
C041
NS15
S123
Nocardioides jensenii (X53214)
C070
Micromonospora brunnea (X92605)
S91D1
S105D1
Nocardia asteroides (Z36934)
NC4
C20M2 [3]
C071
S103M1
Mycobacterium aurum (X55595)
S55M2 [3]
Rhodococcus equi (X80614)
B39M1
Dietzia maris (Y08311)
NC6
B35M1
Streptomyces griseus (AB030567)
NC8
S100D1
S55D1 [2]
S100M1 [2]
B38M1
C059
S53D2 [2]
S97D1
S32D1
S70M1
C17M1
S60D1 [2]
S65M1
B11D2
S86D1
C121
NC5
C1M1
NS1
Microbacterium flavescens (AB004716)
NS6
C10D1
S86M2 [2]
S3D2
S71M1
NS10
Cellulomonas turbata (X79454)
B6D2
NC13
Terrabacter tumescens (AF005023)
B33M1 [2]
NS13
Cellulomonas variformis (AJ298873)
S62M2 [2]
S81M1
NC14 [2]
C21D1 [2]
Agromyces cerinus (X77448)
C11M1
S32M1
S90D1
S89M1
S89D1 [5]
NC10
S86D3
C127
Beutenbergia cavernae (Y18378)
Micrococcus luteus (AB023371)
S78D1
NS8
B37D1 [2]
NS12
B37M1 [2]
B11D1
S32M2
Arthrobacter globiformis (M23411)
B1D1
S79D1 [9]
B10D1
B36M1
C131
S013
C067
Acidimicrobium ferrooxidans (U75647)
0.05

Figure 3.4. Phylogenetic tree of the HSB clones and isolates associated with the Rubrobacteria. Groups denoted on the right side of the tree follow the nomenclature of Holmes et al. (28). Other notation is as described in Figure 3.1.

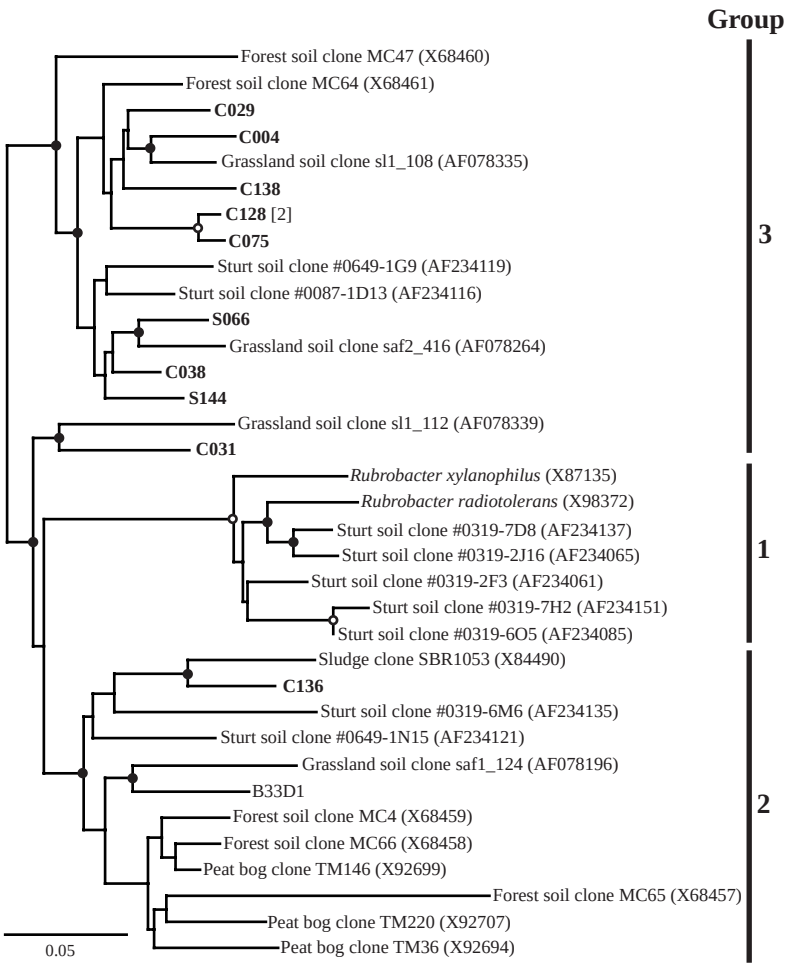

Group
Forest soil clone MC47 (X68460)
Forest soil clone MC64 (X68461)
C029
C004
Grassland soil clone sl1_108 (AF078335)
C138
C128 [2]
C075
Sturt soil clone #0649-1G9 (AF234119)
Sturt soil clone #0087-1D13 (AF234116)
S066
Grassland soil clone saf2_416 (AF078264)
C038
S144
Grassland soil clone sl1_112 (AF078339)
C031
Rubrobacter xylanophilus (X87135)
Rubrobacter radiotolerans (X98372)
Sturt soil clone #0319-7D8 (AF234137)
Sturt soil clone #0319-2J16 (AF234065)
Sturt soil clone #0319-2F3 (AF234061)
Sturt soil clone #0319-7H2 (AF234151)
Sturt soil clone #0319-6O5 (AF234085)
Sludge clone SBR1053 (X84490)
C136
Sturt soil clone #0319-6M6 (AF234135)
Sturt soil clone #0649-1N15 (AF234121)
Grassland soil clone saf1_124 (AF078196)
B33D1
Forest soil clone MC4 (X68459)
Forest soil clone MC66 (X68458)
Peat bog clone TM146 (X92699)
Forest soil clone MC65 (X68457)
Peat bog clone TM220 (X92707)
Peat bog clone TM36 (X92694)
0.05
3
1
2

Figure 3.5. LIBSHUFF comparison of HSB soil clones and cast clones. Homologous coverage curves are shown with open circles (❍) and heterologous coverage curves with closed circles (●). Solid lines indicate the difference between the homologous and heterologous coverage curves at each value of D as determined by the Cramér von-Mises test statistic, and broken lines denote the 95% value of the random shufflings. (A) Comparison of the HSB cast clones [X] to the HSB soil clones [Y], and (B) comparison of the HSB soil clones [X] to the HSB cast clones [Y].

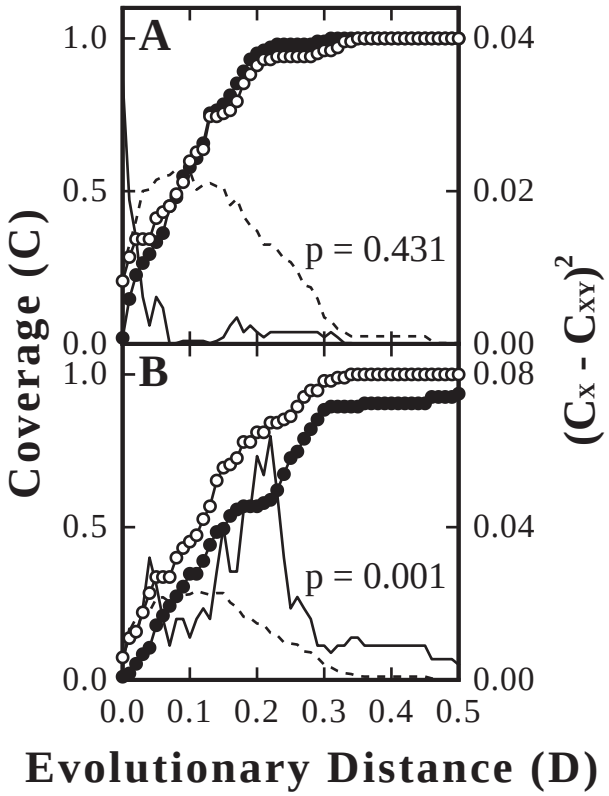

A
B
p = 0.431
p = 0.001
1.0
0.5
0.0
0.04
0.02
0.00
0.08
0.04
0.00
0.0 0.1 0.2 0.3 0.4 0.5
Coverage (C)
(Cx - Cxy)2
Evolutionary Distance (D)

Figure 3.6. (A) Phylogenetic tree of the soil archaea clones from HSB. RFLP patterns are in parenthesis after clone names. Other notation is as described in Figure 3.1. (B) Distribution of RFLP patterns of archaeal clones in soil and cast clone libraries.

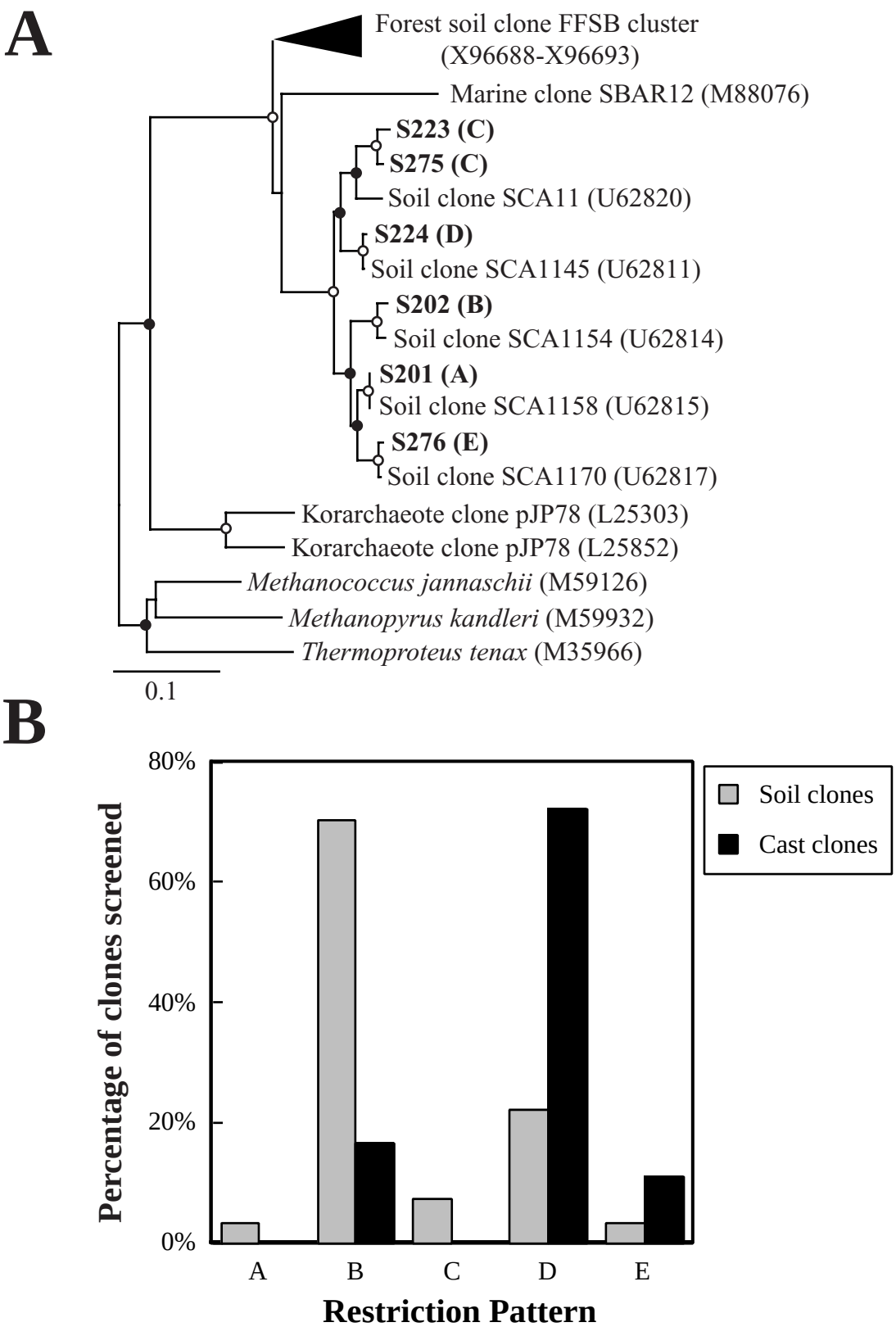

A
Forest soil clone FFSB cluster
(X96688-X96693)
Marine clone SBAR12 (M88076)
S223 (C)
S275 (C)
Soil clone SCA11 (U62820)
S224 (D)
Soil clone SCA1145 (U62811)
S202 (B)
Soil clone SCA1154 (U62814)
S201 (A)
Soil clone SCA1158 (U62815)
S276 (E)
Soil clone SCA1170 (U62817)
Korarchaeote clone pJP78 (L25303)
Korarchaeote clone pJP78 (L25852)
Methanococcus jannaschii (M59126)
Methanopyrus kandleri (M59932)
Thermoproteus tenax (M35966)
0.1
B
80%
60%
40%
20%
0%
Percentage of clones screened
Soil clones
Cast clones
A
B
C
D
E
Restriction Pattern

Figure 3.7. LIBSHUFF comparison of HSB soil clones and Scottish soil clones (SAF). Notation is as described in Figure 3.5. (A) Comparison of SAF [X] to HSB clones [Y], (B) comparison of HSB [X] to SAF clones [Y].

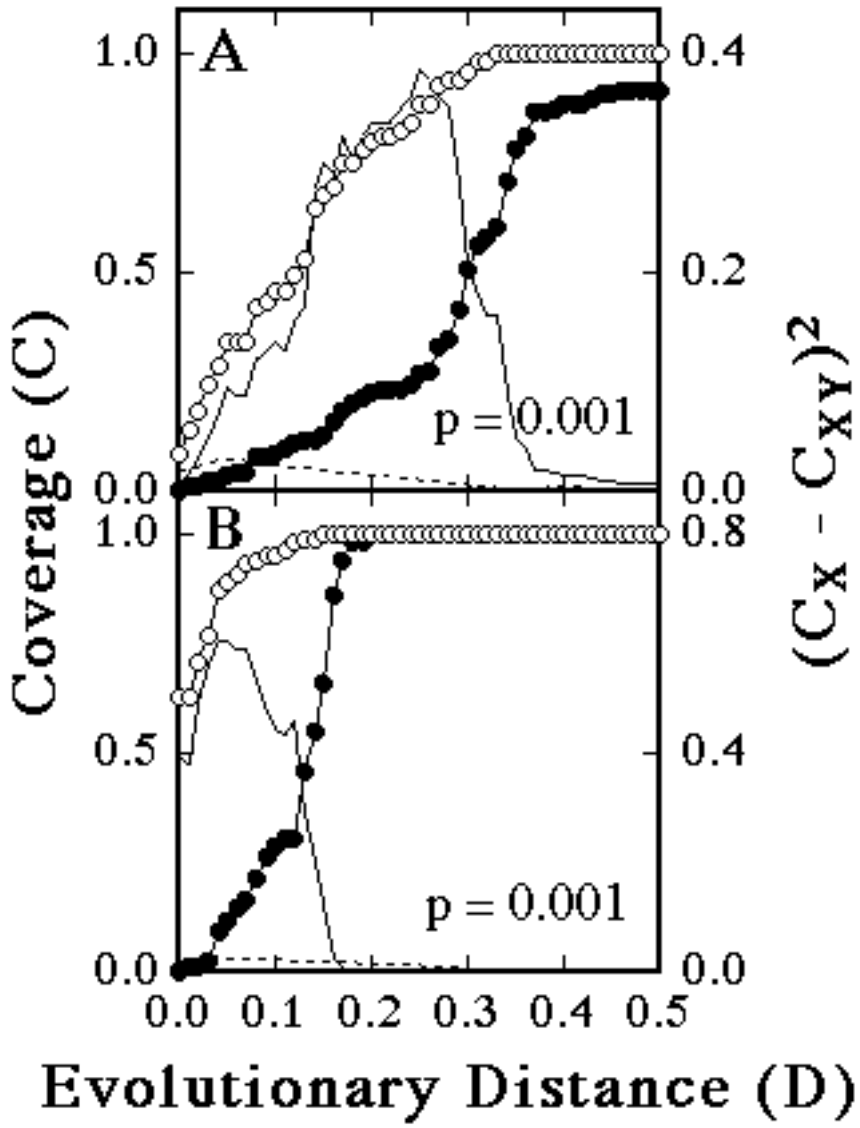
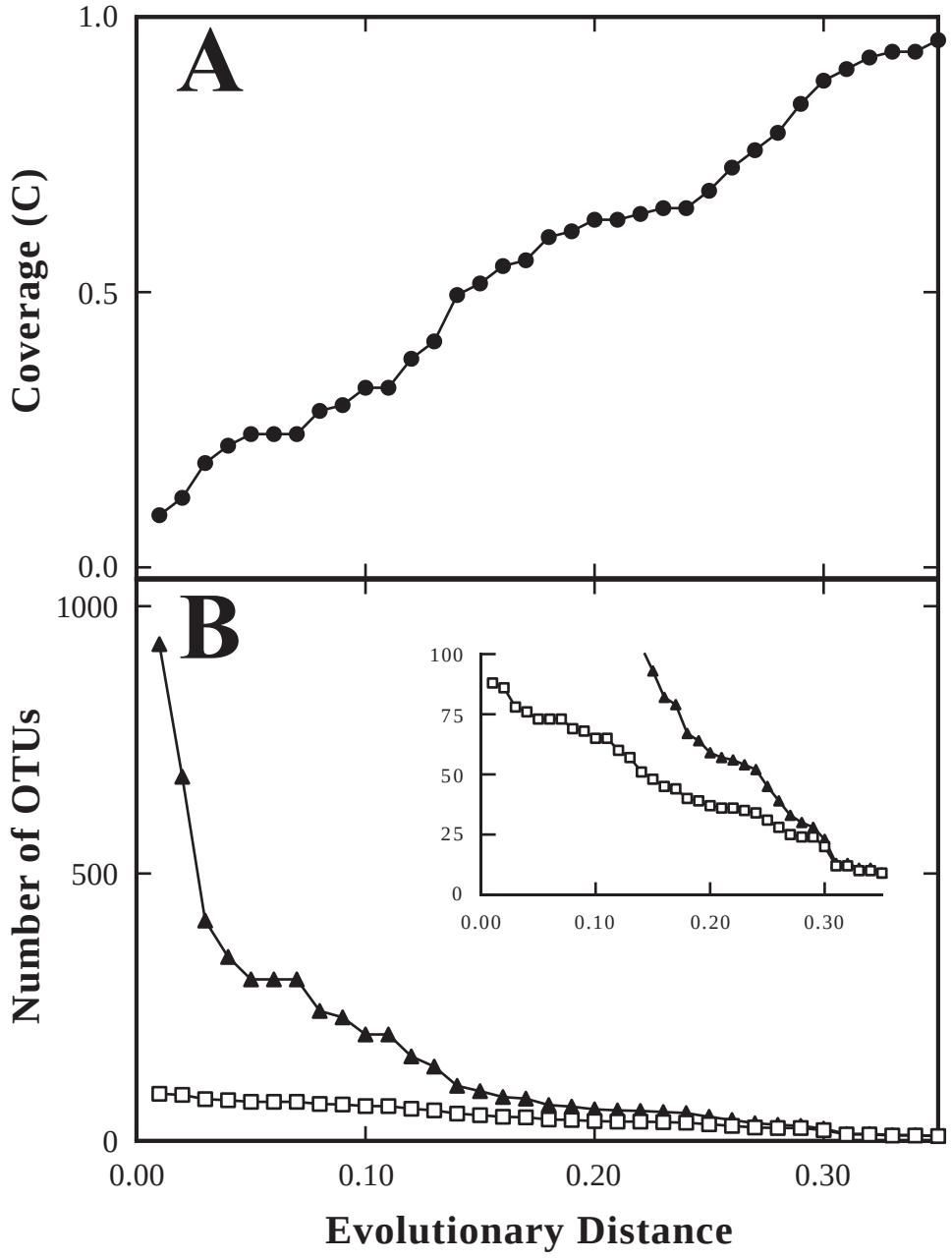

A
B
p = 0.001
p = 0.001
1.0
0.5
0.0
0.4
0.2
0.0
1.0
0.5
0.0
0.8
0.4
0.0
0.0 0.1 0.2 0.3 0.4 0.5
Coverage (C)
$(C_X - C_{XY})^2$
Evolutionary Distance (D)

Figure 3.8. Calculated OTUs (❑) for the soil clone library and estimated OTUs (▲) present in the soil bacterial population. The inset shows the same data on a smaller scale.


A
B
Coverage (C)
Number of OTUs
Evolutionary Distance
1.0
0.5
0.0
1000
500
0
0.00
0.10
0.20
0.30
100
75
50
25
0
0.00
0.10
0.20
0.30

CHAPTER IV

BACTERIAL COMMUNITIES ASSOCIATED WITH THE INTESTINE OF THE EARTHWORM *Lumbricus rubellus*

Abstract

The bacterial communities associated with the intestine and casts of the earthworm *Lumbricus rubellus* were examined by direct counts, culturability studies, 16S rRNA gene clone libraries, and fluorescent in situ hydridization (FISH). While the number of the organisms in each earthworm tightly associated with the intestine wall was lower than that of the cast; the number was a significant fraction of the total numbers of prokaryotes remaining in the intestine after casting (24 - 47%). Clone libraries constructed from prokaryotes tightly-associated with intestines in April and November of 2001 suggested that this group was dominated by a few ribotypes that were either absent or in low abundance in the cast. Depending on the date of sampling, these ribotypes were associated with the Acidobacteria, *Firmicutes*, β-proteobacteria, and one phylogenetically deep, unclassified group. Juvenile worms collected in Februrary 2002 contained the *Firmicutes* ribotype that was abundant in the library from April 2001. Upon examination by FISH, this group of bacteria was found to be only a minor percentage of the total population. The February 2002 worms also contained a β-Proteobacterium that was observed in both of the previous libraries, and one worm tested positive for the unclassified sequence that dominated the November 2001 library. A cast library from April 2001 was significantly different from a cast library constructed two years earlier from a similar site. These results suggest that the microbial population of the intestine of the earthworm *L. rubellus* is dominated by a small number of ribotypes which rise to abundance under varying, as of yet uncertain, conditions.

Introduction

A number of invertebrates possess an intestinal prokaryotic community. For example, *Aeromonas* spp. are the dominant intestinal organisms in leeches (*Hirudo medicinalis*; Graf, 1999). The intestine of the termite *Reticulitermes speratus* contains a complex community composed of proteobacteria, *Bacteroides*, *Firmicutes*, spirochetes, and a unique termite ribotype (Okhuma and Kudo, 1996). Novel species of *Methanobrevibacter* have also been isolated from *R. speratus* (Leadbetter and Breznak, 1996). Similarly, a clone library from another termite, *Cryptotermes domesticus*, identified a number of unique termite ribotypes as well as ribotypes associated with *Bacteroides*, *Treponema*, *Leuconostoc*, and *Methanobrevibacter* (Ohkuma and Kudo, 1998). Culture-independent analyses of spirochetes in the hindgut of the termite *Nasutitermes lujae* have revealed novel *Treponema* ribotypes (Paster et al., 1996). The gut of the soil microarthropod *Folsomia candida* also contains a number of different types of bacteria. Upon ingestion, some bacterial species thrive in the midgut, but others are selectively digested or retained (Thimm et al., 1998).

The associations of prokaryotes with a variety of earthworm species have been investigated previously, although most studies relied on culture-based methods (e.g. Kristufek et al., 1993; Contreras, 1980; Márialigeti, 1979; Parle 1963). A few studies have examined the prokaryotic diversity using molecular techniques, including 16S rRNA gene clone libraries of earthworm casts (Furlong et al., 2002), fluorescent in situ hybridization (FISH) of major groups associated with litter, soil, casts, and the intestinal tract (Schönholzer et al., 2002; Fisher et al., 1995), and electron microscopy of gut walls (Jolly et al., 1993; Vinceslas-Akpa and Loquet, 1995). Fischer et al. (1995) found that

numbers of α-, β-, and γ-proteobacteria generally increased during passage through the intestine of *Lumbricus terrestris*. The largest difference was the γ-proteobacteria, which increased nearly 28-fold from the foregut to the hindgut. In contrast to the results of that study, similar procedures performed later did not show this proteobacterial increase in the intestine (Schönholzer et al., 2002). However, an increase in the numbers of bacteria associated with the δ-proteobacteria and *Cytophaga-Flavobacteria* in the cast was observed. Unfortunately, while these methods provided information on the abundance of taxonomic groups in various regions of the earthworm intestine and casts, genus and species level identification of the cells was not made. Likewise, scanning electron microscopy (SEM) of the intestinal walls of the earthworms *Eisenia fetida andrei, Lumbricus terrestris,* and *Octolasion cyaneum* did not identify specific organisms attached to the earthworm intestinal walls (Vinceslas-Akpa and Loquet, 1995; Jolly et al., 1993). They did, however, describe a number of bacterial morphologies, particularly a filamentous organism with an apparent socket-like structure that appeared to attach to the intestinal wall. To the best of our knowledge, no studies have used molecular techniques to identify specific bacteria associated with the earthworm intestine.

In this work, the bacterial populations associated with the intestine of the epigeic earthworm *Lumbricus rubellus* were examined. Clone libraries of the 16S rRNA genes were used to compare the populations in the casts with the bacteria tightly associated with the intestinal wall, fluorescent in situ hybridization was used to visualize one of the uncultured bacteria, and direct counts and culturability studies were used to determine the extent of bacterial association with intestines.

Methods and Materials

Sample collection. Worms were collected from the Horseshoe Bend Agroecosystem Facility in Athens, Georgia from soil under the deciduous leaf litter just outside of no-till subplot 3 of the main plot. The three worms collected in April, 4 in May, and 2 in November of 2001 were adults. Three worms recovered in February 2002 were juveniles. To collect casts from the April and May 2001 earthworms, the earthworms were washed with sterile water and placed into separate sterile Petri dishes on a piece of moist filter paper.

Intestine washes and clone library construction. The 3 worms collected in April 2001 were sacrificed by brief immersion in 50° C water. The intestine was removed from below the clitellum to the end of the worm. Each intestine was placed in a microcentrifuge tube containing 1 mL of saline solution (0.85% NaCl) and vortexed for 30 seconds to remove soil and cast debris. These washes were repeated a total of 8 times. The intestinal walls were sliced open at various points early in the washes to facilitate removal of soil particles during the washes. The first four washes for each of the worms were combined into a single sample and designated "early washes". The last four washes were combined and designated "late washes". The intestinal tissue was resuspended in saline buffer and physically disrupted by treatment in a mini bead-beater (Biospec Products) for 10 seconds at maximum speed. DNA was extracted from the material as described previously (Furlong et al., 2002). Two worms collected in November 2001 were dissected similarly except that phosphate buffered saline (PBS; pH 7.2) was used in place of the saline solution for the washes. In addition, a sterile cylinder was rolled along the length of the removed intestine to physically force out cast material prior to the

washes. Only the washed intestine was used for DNA extraction in the November samples. The juvenile worms from February 2002 were too small for dissection, and the posteriors of the worms were removed in their entirety, washed in PBS, and homogenized in a mini bead-beater for 30 seconds to disrupt the eucaryotic tissue. A portion of the disrupted tissue was fixed in 4% paraformaldehyde for later examination by FISH. DNA was extracted from the remainder of the juvenile tissue as previously described (Furlong et al., 2002).

Direct counts and culturability studies. To examine the numbers and culturability of bacteria in washed earthworm intestines, 3 worms collected in May 2001 were allowed to cast, were sacrified and dissected as described earlier, and the washed intestines were divided into six sections. Alternate sections were combined into two samples of three sections each. To test for consistency in the counting, one sample was not washed while the other half was washed as described previously. It was expected that the total number of cells in all washes would be equivalent to the unwashed sample. The washed intestinal tissue and the entire unwashed sample was disrupted by homogenization in a mini bead-beater for 10 seconds at maximum speed.

Portions of each washed sample (cast, early washes, late washes, intestine), as well as the unwashed sample, were used for direct counts and culturability studies. Samples for staining were fixed in 1% glutaraldehyde and an aliquot was stained with DAPI (final concentration 1 μg ml^{-1}) in a total volume of 5 mL for 15-20 minutes. Three mL of the stained mixture were filtered onto Isopore 0.2 μm GTBP membrane filters (Millipore). The filters were washed three times with particle-free water. DAPI-stained cells were examined using an Olympus BH-2 epifluorescence microscope. Counts of

cells on particles which were opaque were doubled to account for the unobserved side. The culturability of organisms was determined by plating on Nutrient Broth (Difco; NB) plates (pH 7.0) and incubating at room temperature for two weeks.

Clone library construction and analysis. PCR reactions and 16S rRNA gene clone library constructions were performed as described previously (Furlong et al., 2002). For the April 2001 samples, the three worms were treated as a single sample, and a library was made for each of the four treatments (cast, early washes, late washes, and intestine). The genomic DNA of the cast and early wash samples required gel purification prior to successful PCR. Analysis of the resulting sequences was also performed as described earlier (Furlong et al., 2002). Partial sequences of the cloned 16S rRNA genes were obtained using primer 27f (Table 4.1). Nearly complete sequences were obtained for selected clones using primer 1392r (Table 4.1). Phylogenetic trees were constructed as described previously, except that the Fitch-Margoliash algorithm of PHYLIP (Felsenstein, 1989) was used rather than Neighbor-Joining (Furlong et al., 2002). LIBSHUFF analyses to statistically compare clone libraries were performed as previously described (Singleton et al., 2001). A total of five sequences were detected as possible chimeras and excluded from the analyses.

Primer and probe design, PCR screening. Specific PCR primers and oligonucleotide probes were designed for several of the intestine-associated bacteria (Table 4.1). The nearly complete 16S rRNA gene sequences were first obtained for abundant clones by sequencing with primer 1392r (Table 4.1). The sequences were then aligned with closely related rRNA genes to determine regions of high variability. Once putative probe sequences had been identified, they were examined for possible hairpin or dimer

formations, and checked against GenBank and the Ribosome Database Project-II (RDP-II; Maidak et al., 2001) for any exact or nearly exact complementary sequences. Only primer wi14p6 had an exact match to an existing sequence, a soil clone nearly identical to the target sequence from this study. All primers and probes were obtained from Integrated DNA Technologies, Inc. (IDT; Coralville, IA), and probes used in fluorescent in situ hybridization (FISH) studies were labeled with either 5'-Cy3 (BAC338) or 5'-FITC (fluorescein isothiocyanate; wi32p3.1, wi14p6). All PCR primers were designed to be used in conjunction with primer 27f. Primers used in this study are listed in Table 4.1. PCR conditions for the screening of intestine samples for the presence of selected sequences were performed as described previously, except that 30 cycles were used to increase the sensitivity of the screen. A band of the expected size was considered a positive result.

Fluorescent in situ hybridization (FISH). Washed intestines of the juvenile earthworms from February 2002 were examined by FISH with probe wi32p3.1 (*Firmicutes*) following the protocol of Snadir et al. (1997). The salt concentration of the wash buffer was 0.9 M NaCl. The hybrization was carried out in a microcentrifuge tube, and the stained sample was spread onto a glass slide and allowed to air dry prior to observation. Earthworm tissue and associated bacteria were observed using a Nikon TE 300 inverted microscope. Digital pictures were taken through the IP Lab Spectrum software package (version 3.4.5) using an attached Princeton Instruments MicroMax high resolution, cooled CCD camera. Images were overlayed in Adobe Photoshop version 5.5.

Accession numbers. Clone sequences were deposited in GenBank with the accession numbers XXXXXXX-YYYYYYY.

Cast temporal community stability study. Casts were collected from all earthworms as previously described. All worms were adults collected from no-till plot 7 at Horseshoe Bend. For the spring 1999 samples, a single clone library was made from DNA extracted from casts that had sat overnight as described previously (Furlong et al., 2002).

Casts were collected from six worms in March of 2000 and pooled. A portion of the casts was resuspended in saline solution and serially diluted onto enriched *Cytophaga* agar plates (2.0 g tryptone, 0.5 g beef extract, 0.5 g yeast extract, 0.2 g sodium acetate per liter). Culture-based studies of worms collected in April 2001 were performed similarly, except that nutrient broth plates (Difco) and Gould's S1 agar (Gould et al., 1985) were also used.

To determine the composition of the microbial community in casts for the March 2000 samples, PCR products were hybridized quantitatively to taxon-specific probes. DNA was extracted from the remainder of the cast sample, and a PCR using general bacterial primers was performed as described previously (Furlong et al., 2002). PCR product (5 - 15 ng) and control plasmids containing inserts that would bind the Cytophagales- and *Pseudomonas*-specific probes (CF319a and PSMg, respectively), were diluted into 0.2M NaOH, heated to 60°C for one hour, then cooled on ice. An equivalent volume of 6X SSC was then added to the samples, which were then filtered onto triplicate membranes using a Bio-dot ST apparatus (Bio-rad). The DNA was fixed to the membranes by baking at 80°C for 30 minutes. Each membrane was prehybridized in a solution containing 6X SSC, 0.5% SDS, herring sperm DNA (10 mg ml^{-1}), and 5X Denhardt's solution (100 µl cm^{-2} membrane) for 3 hours at 42°C. The prehybridization buffer was removed and replaced with a hybridization solution containing 6X SSC, 0.5%

SDS, herring sperm DNA (10 mg ml^{-1}), 5X Denhardt's solution, and 0.01M EDTA (150µl cm^{-2} membrane). T4-polynucleotide kinase ^{32}P-labeled oligonucleotide probes (EUB338, a general bacterial probe; CF319a; PSMg) were added to each of the membranes at a concentration of 20 ng ml^{-1} hybridization buffer, and the hybridization was incubated overnight at 42°C. The hybridization solution was removed, and each membrane was washed three times at room temperature with a solution containing 6X SSC and 1% SDS. The membranes were then washed twice in a solution containing 1X SSC and 0.5% SDS at 42°C (EUB338, PSMg) or 46°C (CF319a). The amount of binding of each of the probes to the PCR samples and controls was determined using a PhosphorImager SI (Molecular Dynamics) with ImageQuant v1.0 software. The relative abundance for Cytophagales and *Pseudomonas* sequences in the amplification products was determined by the equation:

$$\frac{(\text{Sample}_{\text{specific probe}}/\text{Sample}_{\text{bacterial probe}})}{(\text{Control}_{\text{specific probe}}/\text{Control}_{\text{bacterial probe}})}$$

The relative abundance of these taxa in the PCR used to construct the cast libraries from a previous study (Furlong et al., 2002) was determined using the same techniques.

Results and Discussion

Number of intestine-associated prokaryotes. The number of prokaryotic cells in each of the four samples (cast, early washes, late washes, and intestine) was determined by direct counts (Table 4.2). The cast contained the highest numbers of organisms, followed by the early washes (Table 4.2). As significant amounts of soil were present in the early washes, the high numbers of organisms in those washes was expected. Numbers of

organisms in the late washes and intestine, which were expected to be more tightly associated with the intestinal tissue, were smaller than those of the cast but were still significant. For instance, excluding the cast material, 24 - 47% of the cells found in the intestine were not removed by the washing (Table 4.2). The total number of bacteria associated with the intestine was calculated for each worm by combining the totals for the washes and washed intestine. To examine the efficiency of bacterial recovery in the washing protocol, an unwashed portion of the intestine was also examined. In general, the number of organisms determined by direct counts of the washed and unwashed intestines were similar (Table 4.2).

To evaluate the significance of the numbers of prokaryotes that were tightly-associated with the intestine, the fraction of the intestinal wall that might be covered was calculated. Assuming that the dimensions of the *L. rubellus* intestine were a cylinder 40 mm long with a diameter of 0.5 mm, the total interior surface area would be approximately 63 mm^2. Further assuming that the average dimensions of the bacterial cells in the intestine were 1.5 μm x 0.5 μm (see FISH description below), then approximately 28% of the intestinal surface would be covered by the tightly associated prokaryotic cells that remained after washing. Because the lumbricid earthworm intestine contains multiple invaginations as well as a typhlosole (Edwards and Lofty, 1972), can stretch to much larger diameters when filled with soil or litter, and the orientation of the prokaryotic cells attached to an intestinal wall can vary; this value should be considered an upper limit. Thus, the numbers of tightly associated bacteria is far less than necessary to form a monolayer. This conclusion is consistent with direct observation by SEM of

the hindgut of *L. terrestris*, which contained fewer cells than other examined invertebrates (Jolly et al., 1993).

The percent culturability of bacterial cells was highest in the cast and early washes for all samples and was lower in the later washes and intestine (Table 4.2). These results indicated that the organisms tightly associated with the intestinal wall either were not readily cultured, or contained fewer types able to be cultured on the medium used. Since it was possible that these organisms represented groups not typically observed by culture-based methods, clone libraries of the 16S rRNA gene were used to identify dominant members of the communities tightly associated with the intestine. For comparison to the intestine library, clone libraries of the cast and washes were also constructed.

Identification of intestinal bacteria. Ribosomal RNA libraries of 99, 45, 50, and 44 clones were constructed for the cast, early washes, late washes, and intestine samples of three worms collected in April 2001, respectively. All the clones from these libraries grouped within 11 taxa. The cast library was dominated by Actinobacteria and α-proteobacteria sequences (Table 4.3). Similarly to the cast library, the library from the early washes also contained large numbers of Actinobacteria (Table 4.3 and Figure 4.1). Because the early washes also contained soil, the clones were expected to be similar to the cast clones; and, in general, similar ribotypes were found in both libraries. This observation was supported by LIBSHUFF analyses in which the cast and early washes were not signficantly different (Table 4.4; comparison 1). The library constructed from the late washes was dominated by γ-proteobacteria (42%), notably *Aeromonas*, *Buttiauxella*, and *Pseudomonas* spp. These groups may have represented bacteria which

were moderately associated with the intestinal walls of the earthworms. However, Actinobacteria and other taxa common in the cast and early washes libraries were also represented, suggesting that this wash contained cast material as well (Table 4.3). LIBSHUFF analyses indicated that the library from the late washes was not significantly different from that of the early washes, suggesting that ribotypes found in one were also present in the other (Table 4.4; comparison 2). The LIBSHUFF comparison also indicated that the cast library was well represented by the late wash library ($p = 0.081$, Table 4.4, comparison 3). In contrast, the late washes contained ribotypes absent from the cast library, and the cast was not representative of the late washes (LIBSHUFF $p = 0.005$). This result was consistent with the distribution of clones in the late washes. Although many ribotypes similar to those found in the cast were observed in the late washes, the late washes also contained ribotypes not present in the cast.

In contrast to libraries of the cast and washes, the library constructed from the washed intestine contained ribotypes from only four phyla, and it was dominated by a single ribotype (wi14) that grouped within the Acidobacteria (Table 4.3 and Figure 4.1). This clone, which had 99% sequence similarity to clone Y14-5 from a petroleum-contaminated soil (Ralebitso et al., 2000), represented 19 of 44 clones examined in this sample. Since most sequences phylogenetically associated with the Acidobacteria have not yet been cultured, this observation was consistent with the low culturability of cells from the intestine (Table 4.2). Another group of ribotypes represented by wi32 and related clones composed 23% of the intestine library. These ribotypes possessed ~90% sequence similarity to *Paenibacillus* spp. within the *Firmicutes* phylum (Figure 4.2). Because of their high abundance, these two taxa were likely candidates for bacteria

specifically associated with the intestinal community. These intestine ribotypes were also observed in smaller numbers in the late washes (Figures 1-2). This observation was consistent with the LIBSHUFF comparison which indicated that the ribotypes in the intestinal library were represented in the late washes ($p = 0.140$), however the late wash library was not well represented in the intestinal library (Table 4.4; comparison 4). This result was most likely due to the fact that the late washes contained both residual cast material and earthworm intestinal tissue, which sloughed off during the washing process. Because the abundant intestine ribotypes were not observed in the early washes, both the intestine and early wash libraries were significantly different (Table 4.4; comparison 5).

Stability of intestinal bacteria. To determine if these two intestine-associated ribotypes were present in worms from other seasons, two adult *L. rubellus* worms were collected in November. The washed intestines were tested for the presence of the organisms represented by the clones wi14 and wi32 using a PCR screening procedure. For both samples, products of the expected size were generated from a general bacterial primer pair but not from reactions involving the wi14 and wi32 specific primers. As an additional control, the DNA used to construct the late wash and intestine libraries constructed in April of 2001 was positive for both organisms in the PCR screen. On the basis of these experiments, the dominant organisms observed in the clone library from earthworm intestines in April were not abundant in the earthworms from November.

To determine what ribotypes were present in the November sample, an additional library of 46 clones was constructed from the DNA from the intestine of one of the worms. One clone was a *Lumbricus* DNA fragment unrelated to rRNA genes. The remainder of this library consisted of two bacterial ribotypes, and was dominated by a

ribotype (85% of the clones; type clone wi128) which bore < 80% sequence similarity to any other GenBank sequence (Figure 4.3). The closest cultured relatives of this ribotype were *Mycoplasma* species. Interestingly, other clones (excepting *Mycoplasma* and *Spiroplasma*) affiliated with this lineage have been found in DNA from pig intestines (Leser et al., 2002), the bovine rumen (Tajimsa et al., 1999), and human feces (Suau et al., 1999); which are all environments associated with the digestive system of animals (Figure 4.3). The other bacterial ribotype in the November intestine library was closely related to a β-Proteobacterium, *Pseudomonas* sp. P51 (97% sequence similarity), and was identical to a number of clones in the April 2001 libraries. This ribotype, wi89, was found once from the intestine, once from the cast, and 4 times from the early wash libraries (Figure 4.4). In addition, it was closely related to a clade of four other ribotypes from the intestine library from April 2001 (Figure 4.4). Because this ribotype was also found in the cast and early wash libraries from the April libraries, it did not appear to be exclusively associated with the intestine. Therefore, in contrast to the April intestine library, different ribotypes dominated the November library, and the Acidobacteria and *Firmicutes* ribotypes were not detected. The diversity of the intestine library from November was even lower than the diversity of the library from April. This difference may have been due to a modification in the protocol used in which cast material was forced out of the intestine prior to washing.

Three juvenile *L. rubellus* worms were recovered in February 2002 and tested for the presence of the dominant ribotypes recovered from the April and November intestine libraries from 2001. Because of the small size of the recovered worms, the intestine was not dissected. Instead, the entire posterior of the worm was removed, and DNA extracted

from the entire tissue, including small amounts of cast material. A PCR screening tested for the presence of the dominant bacterial ribotypes from the previous two intestine libraries; wi14 (Acidobacteria), wi32 (*Firmicutes*), wi89 (β-proteobacteria), and wi128 (unclassified). No products were found for the wi14 specific primer in any of the worms. However, products were obtained for wi32 and wi89 specific primers in all three. A faint PCR product was also observed for the wi128 primer in one worm.

The fixed tissue from each of the juvenile worms was examined by fluorescent in situ hybridization (FISH) for the presence of cells that could bind the wi32 probe. A large number of cells (mainly long rods, although smaller rods and some cocci) were visible using the general bacterial probe Cy3-BAC338. Less than one percent of the observed cells appeared to bind the FITC-labled specific probe wi32p3.1. The few observable cells that did bind the specific probe were short rods. From these data, the organisms represented by wi32 appeared to be only a small portion of the prokaryotic community of the juvenile worms. However, because the entire posterior of the worm was used, it was not possible to conclude that this ribotype was only found in the intestine.

To test for the presence of each of the intestine-associated sequences determined by clone libraries, DNA from soil collected from the sample site in March 2002 was screened. None of the specific primers produced a product (data not shown). It is possible, however, that these organisms are present in soils, but in such low numbers that our PCR amplification procedure does not detect them.

Summary of intestine-associated bacteria. In order to show an indigenous intestinal population, several criteria must be met. First, a large population associated with the

intestine must exist. While the number of bacteria in the intestine was not sufficient to densely cover the intestinal walls, a large number of cells remained associated with the intestine even after washing. Second, specific taxa must be present in the intestine that are not found in higher numbers elsewhere. This study identified at least 3 such taxa, including members of the Acidobacteria, *Firmicutes*, and one deep *Mycoplasma*-associated group. An additional taxon within the β-proteobacteria was also seen in the intestine samples, but it was found in cast and early washes as well. A final criterion for an indigenous population is that it should be stable and widespread in worms from a variety of seasons or locations. Our data did not find this to be the case, although some taxa did appear in samples from different sampling times.

Whether the organisms identifed in this study represent the population typically associated with intestinal tissue of *L. rubellus* is unclear. One might propose, that because only a minor portion of the intestinal wall could be covered by bacteria, our washing technique was simply not stringent enough to remove all bacterial cells, and those that remained would simply be part of the flora that passed through with the cast material. However, if this was the case, then we would have expected to recover a higher diversity of taxa in the intestine clone libraries, similarly to the cast, since the removal of cells would have been a random process. As this was not the case, we hypothesize that the limited taxa that were detected were specifically associated with the intestine. Additionally, a sparse covering of the intestinal wall, especially the hindgut, agrees with the SEM observations of *L. terrestris* by Jolly et al. (1993).

The SEM studies of Jolly et al. (1993) also provided indications that earthworm intestine-associated bacteria may not be stable within the population. They reported that

a number of bacterial morphotypes were associated with mucopolysaccharide, the intestinal wall, or both. However, the most obviously attached filamentous bacteria observed in the hindgut of their samples were only seen in 4 out of 10 of their worms. Although they presumed that attachment of the bacteria to the intestinal wall was indicative of an indigenous microflora, the absence of this morphotype in a large fraction of the worms indicated that bacterial attachment was not obligatory for the earthworm. Similarly, while the taxa detected in our study may represent organisms tightly-associated with the intestine, they were not shown to be present in all earthworms, even within the same species at the same sample site. Thus, while particular taxa may be able to colonize the earthworm intestine, not all earthworms contain the same taxa. In our study, a limited number of earthworms were examined for the presence of particular intestine-associated taxa. Therefore, it may not be surprising that different taxa were found to dominate the intestinal community of different earthworms. Examination of larger numbers of earthworms may help to identify specific organisms that may be more commonly associated with the intestine. Alternatively, the possibility that the detected taxa may represent an infection cannot be dismissed, as we had no means of distinguishing healthy from potentially diseased earthworms.

Comparison of cast clone libraries. The library constructed from the casts of the spring 2001 earthworms in this study were compared to a similarly constructed library from casts in 1999 (Furlong et al., 2002). LIBSHUFF comparisons of the libraries indicated significant differences (Table 4.4; comparison 6). Earlier studies suggested that the bacterial communities of the cast were largely derived from the soil (Furlong et al., 2002). Because the earthworms in this study were collected from a different region of the

study site, with presumably different soil conditions, the observed difference in populations may reflect differences in the soil populations.

Temporal stability of cast bacterial populations. To examine how the bacterial community within the cast may change over time, a clone library was constructed from casts collected in Spring, 1999 that were up to 17 hours old (post-deposition), compared to the freshly deposited casts reported earlier (Furlong et al., 2002). In contrast to clones obtained from fresh casts, the 36 sequenced clones from the older casts represented only two taxa; the *Firmicutes* phylum (9 of 36 clones) and the Cytophagales (27 of 36 clones). The abundant *Pseudomonas* spp. observed in the fresh cast samples (Furlong et al., 2002) were not detected at all in the library constructed from older cast samples.

To investigate this apparent increase in Cytophagales and decrease in *Pseudomonas* representation in older casts, six worms were collected in March 2000, their casts were collected and pooled, and samples were taken at four time points up to 21.5 hours after deposition. The bacteria in these samples were enumerated on an enriched *Cytophaga* medium (ECA; Table 4.5). The number of organisms capable of growing on ECA over the course of the experiment increased by nearly two orders of magnitude. Although this may be some indication of increased numbers of Cytophagales organisms in the cast samples, other taxa can utilize this particular medium as well and their numbers no doubt accounted for a percentage of the observed colonies.

To analyze this phenomenon in a more quantitative fashion, the percentages of *Pseudomonas* and Cytophagales sequences in PCR amplifications made from DNA extracted from the cast samples were determined by slot-blots with radiolabeled oligonucleotide probes (Table 4.5). The relative percentage of *Pseudomonas* organisms

decreased for the first three time points (up to 8.33 hours), but were sharply higher in the oldest cast samples (21.5 hours). The Cytophagales abundance was negatively correlated with the *Pseudomonas* abundance. As a control, the DNA used to construct the freshly deposited cast library previously was screened with the same procedure. The abundance of *Pseudomonas* sequences was determined to be 18% by hybridization intensity compared to 20% based upon the fraction of clones, and Cytophagales sequences were estimated to make up 6% by hybridization compared to 5% of the clones. Thus, the slot-blot method was consistent with fraction of clones by sequencing clone libraries. These data suggest that large changes in the abundances of specific taxa can occur within a short time after the cast has been deposited. Specifically, because many organisms within the Cytophagales can utilize cellulose and related substrates (Reichenbach, 1992) that are in higher concentration in the cast, the bacterial population may be responding to this substate availability. *L. terrestris* casts of varying ages were examined in a previous study using FISH (Schönholzer et al., 2002). Cast samples in that study were maintained at 90-100% relative humidity over 20 days. The percentage of DAPI stained cells in the casts that hybridized to the same Cytophagales probe used in our experiments was determined to be 28% (fresh casts), 15% (after 5 days), and 8% (after 20 days). Therefore, Cytophagales composed a significant portion of the community in their cast samples as well.

A further study was conducted in May of 2001 with three media types to examine the culturable bacterial population in casts of varying age. The casts of three different worms were allowed to sit for up to 45 hours, and cells were enumerated on a general heterotrophic medium (nutrient broth; NB), ECA, and Gould's S1 agar (selective for

pseudomonads; Gould et al., 1985). Only one of the three cast samples (worm 1) showed a general increase in bacterial numbers on all three media types (Figure 4.5). The most apparent increase for worm 1 was on Gould's S1 agar, although the numbers of CFUs on NB and ECA increased as well. Casts from the the other 2 worms showed a slight to no increase, or a slight decrease on all media types (Figure 4.5). As the cast from worm 1 had a higher moisture content than the other two casts (% not determined), it seems likely that the amount of time that a cast remains moist after deposition may have a significant affect on the bacterial community. In support of this hypothesis, the number of bacteria in *L. terrestris* casts from a another study that were maintained at 90-100% relative humidity doubled over two days (Schönholzer et al., 2002). If the cells that divide in the cast are culturable then this could help explain the increased number of CFUs observed in the moist cast sample.

The temporal variation of microbial populations in the casts of *Lumbricus terrestris* was previously studied by Tiunov and Scheu (2000). They found that initial microbial respiration rates were high in the cast, although they dropped after 10 days. They attributed the high repiration rates to the availability of easily utilized carbon sources. Our data suggest that the bacterial communities in the cast can change much faster than days, and, in the right conditions, hours may be sufficient for particular taxa to rise to abundance.

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Table 4.1. Primers used in this study.

Name	Sequence (5' - 3')	Base positions[a]	Specificity	Reference
27f	AGA GTT TGA TCM TGG CTC AG	8 - 27	Bacteria	Lane (1991)
1392r	ACG GGC GGT GTG TRC	1392-1406	Universal	Lane (1991)
wi14p6	CGG AGC ATT GAA ACT CC	826-841	Acidobacteria clone	This study
wi32p3.1	TAA GCG GCA GCC CGA ATT	204-214	Firmicutes clone	This study
wi89p1	TCA GCA ACC CCA GCT ATT CAC C	446-467	β-Proteobacteria clone	This study
wi128p1	ACT GCC TAC GCT ATC ATT	706-720	Unclassified clone	This study

[a] *Escherichia coli* numbering. Primers wi32p3.1 and wi128p1 contained positions not present in *E. coli.*

Table 4.2. Direct counts and culturability of prokaryotes in earthworm intestines, washes, and cast samples.[a]

Sample	Cast		Early washes		Late washes		Washed Intestine		Total cells in intestine (10^7)[b]	
	DAPI (10^7)	Percent culturability[c]	DAPI (10^7)[d]	Percent culturability[c]	DAPI (10^7)[d]	Percent culturability[c]	DAPI (10^7)[d]	Percent culturability[c]	Washes + intestine[e]	Unwashed control[f]
Worm 1	30.40	0.25	2.94	0.31	1.38	0.001	1.38	0.01	5.70	4.82
Worm 2	62.50	2.29	5.48	1.83	0.78	0.26	5.44	0.01	11.70	9.36
Worm 3	38.90	8.46	8.16	4.90	2.34	0.11	7.34	0.53	17.84	15.74

[a] The worms were collected in May 2001.
[b] Cells per worm intestine, not including casts
[c] Equal to CFUs on Difco Nutrient Agar plates incubated at room temperature divided by DAPI direct counts.
[d] Direct counts per worm calculated from the washed half of intestine.
[e] Sum of early washes, late washes, washed intestine, but not including casts.
[f] Direct counts of the unwashed half of the intestine and doubled to account for the entire sample.

Table 4.3. Phylogenetic distribution of clones from the casts, washes and intestine[a].

Sample type and season[b]	Casts	Early washes	Late washes	Intestine	Intestine
	Apr. 2001	Apr. 2001	Apr. 2001	Apr. 2001	Nov. 2001
Acidobacteria	3	7	6	43	
Actinobacteria[c]	32	33	14	14	
Rubrobacteria	15	11	8		
Candidate Division TM7	1				
Chloroflexi	5				
Cytophagales	6	2	6		
Fibrobacter	2				
Firmicutes	6	2	8	23	
Proteobacteria					
α -	15	7	8		
β -	6	18	6	16	13
γ -	7	18	42	5	
δ -	1	2	2		
Unclassified[d]					85
Sample size (n)	99	45	50	44	46[e]

[a] Percent of total clones for each sample.
[b] Apr., April; Nov., November
[c] Not including Rubrobacteria
[d] This ribotype was not affiliated with previously described taxa.
[e] Includes one eucayotic clone, not included in percentile calculations.

Table 4.4. LIBSHUFF comparisons of clone libraries[a].

	Homologous (X)		Heterologous (Y)	
Comparison	Library	n	Library	p-value
1	Cast - 2001	99	Early Washes	0.284
	Early Washes	45	Cast - 2001	0.704
2	Early Washes	45	Late Washes	0.262
	Late Washes	50	Early Washes	0.163
3	Cast - 2001	99	Late Washes	0.081
	Late Washes	50	Cast - 2001	0.005
4	Late Washes	50	Intestine	0.001
	Intestine	44	Late Washes	0.140
5	Early Washes	45	Intestine	0.007
	Intestine	44	Early Washes	0.001
6	Cast - 1999	102	Cast - 2001	0.001
	Cast - 2001	99	Cast - 1999	0.006

[a] Washes and intestine refer to April 2001 libraries.

Table 4.5. Culturability of bacteria and abundance of Cytophagales and *Pseudomonas* in casts of varying age, March 2000 samples.

Time (hours)[a]	0	4.25	8.33	21.5
ECA CFUs[b]	4.05×10^5	8.29×10^5	1.60×10^6	1.20×10^7
% *Pseudomonas* [c]	19.8	14.6	7.6	23.5
% Cytophagales[c]	4.2	11.8	16.8	6.9

[a] Time after deposition.
[b] CFUs on enriched Cytophaga agar plates per gram (wet weight) sample
[c] As determined by slot-blots of amplified PCR product, average of three PCR reactions.

Figure 4.1. Phylogenetic tree of clones affiliated with the Acidobacteria phylum. Sequences from this study are printed in bold. All clone sequences are from April 2001 samples. Intestinal clone wi14 was identical to 18 other intestinal clones and 3 late wash clones. All other ribotypes were found only once. This tree was based on 550 positions of aligned sequence and branching order was determined using a Fitch-Margoliash algorithm. Numbers in parentheses following reference sequences indicate GenBank accession numbers. Closed (●) and open (❍) circles represent bootstrap values of ≥ 50 and ≥ 95, respectively. The scale bar represents Jukes-Cantor evolutionary distance.

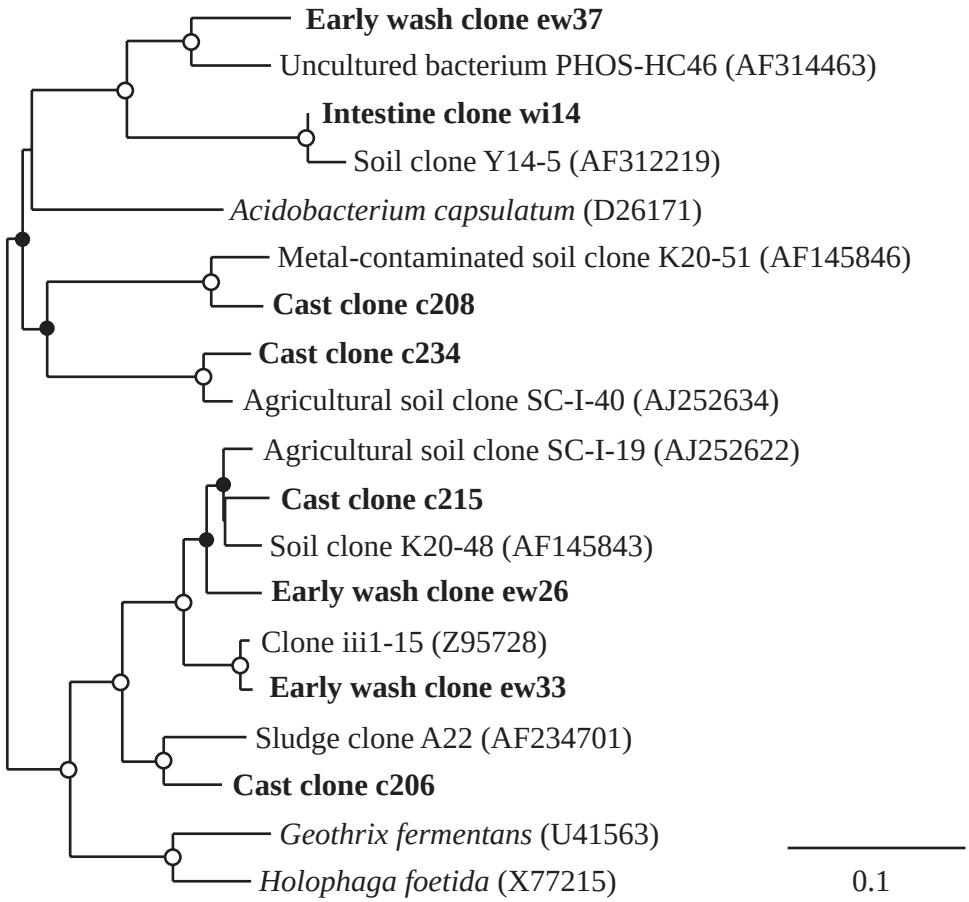

Early wash clone ew37
Uncultured bacterium PHOS-HC46 (AF314463)
Intestine clone wi14
Soil clone Y14-5 (AF312219)
Acidobacterium capsulatum (D26171)
Metal-contaminated soil clone K20-51 (AF145846)
Cast clone c208
Cast clone c234
Agricultural soil clone SC-I-40 (AJ252634)
Agricultural soil clone SC-I-19 (AJ252622)
Cast clone c215
Soil clone K20-48 (AF145843)
Early wash clone ew26
Clone iii1-15 (Z95728)
Early wash clone ew33
Sludge clone A22 (AF234701)
Cast clone c206
Geothrix fermentans (U41563)
Holophaga foetida (X77215)
0.1

Figure 4.2. Phylogenetic tree of clones affiliated with the *Firmicutes* phylum. All clone sequences are from April 2001 samples. Intestine clone wi32 was identical to one other intestine clone and 2 late wash clones. Intestine clones wi28 and wi21 were each identical to 2 other intestine clones. All other ribotypes were found only once. The tree was based on 572 positions of aligned sequence. Other notation is as described in Figure 4.1.

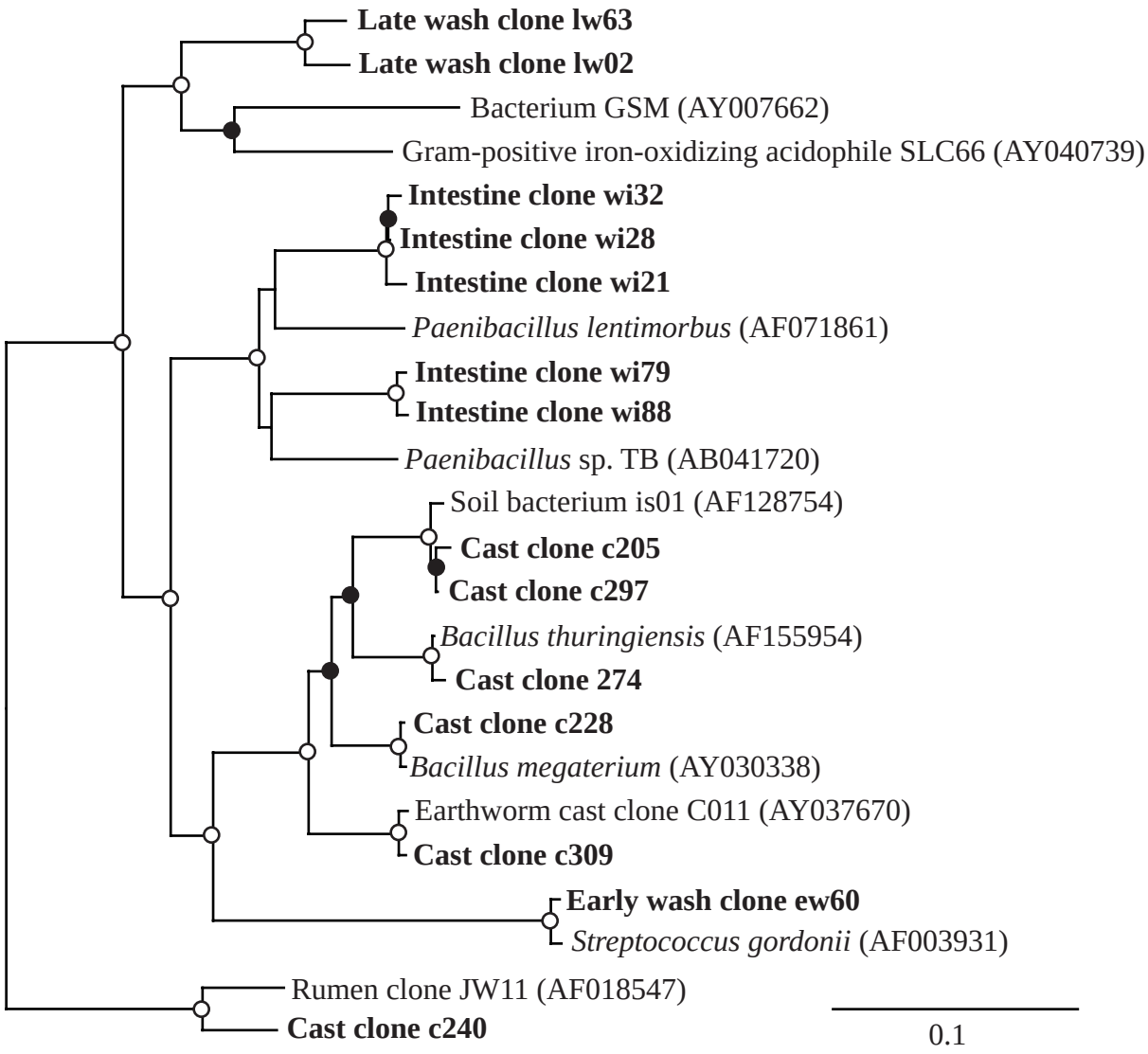

Late wash clone lw63
Late wash clone lw02
Bacterium GSM (AY007662)
Gram-positive iron-oxidizing acidophile SLC66 (AY040739)
Intestine clone wi32
Intestine clone wi28
Intestine clone wi21
Paenibacillus lentimorbus (AF071861)
Intestine clone wi79
Intestine clone wi88
Paenibacillus sp. TB (AB041720)
Soil bacterium is01 (AF128754)
Cast clone c205
Cast clone c297
Bacillus thuringiensis (AF155954)
Cast clone 274
Cast clone c228
Bacillus megaterium (AY030338)
Earthworm cast clone C011 (AY037670)
Cast clone c309
Early wash clone ew60
Streptococcus gordonii (AF003931)
Rumen clone JW11 (AF018547)
Cast clone c240
0.1

Figure 4.3. Phylogenetic tree of clones affiliated with a deep, *Mycoplasma*-associated lineage. Intestine clone wi128 was identical to 38 other intestine clones from the November sample. The tree was based on 1301 positions of aligned sequence. Other notation is as described in Figure 4.1.

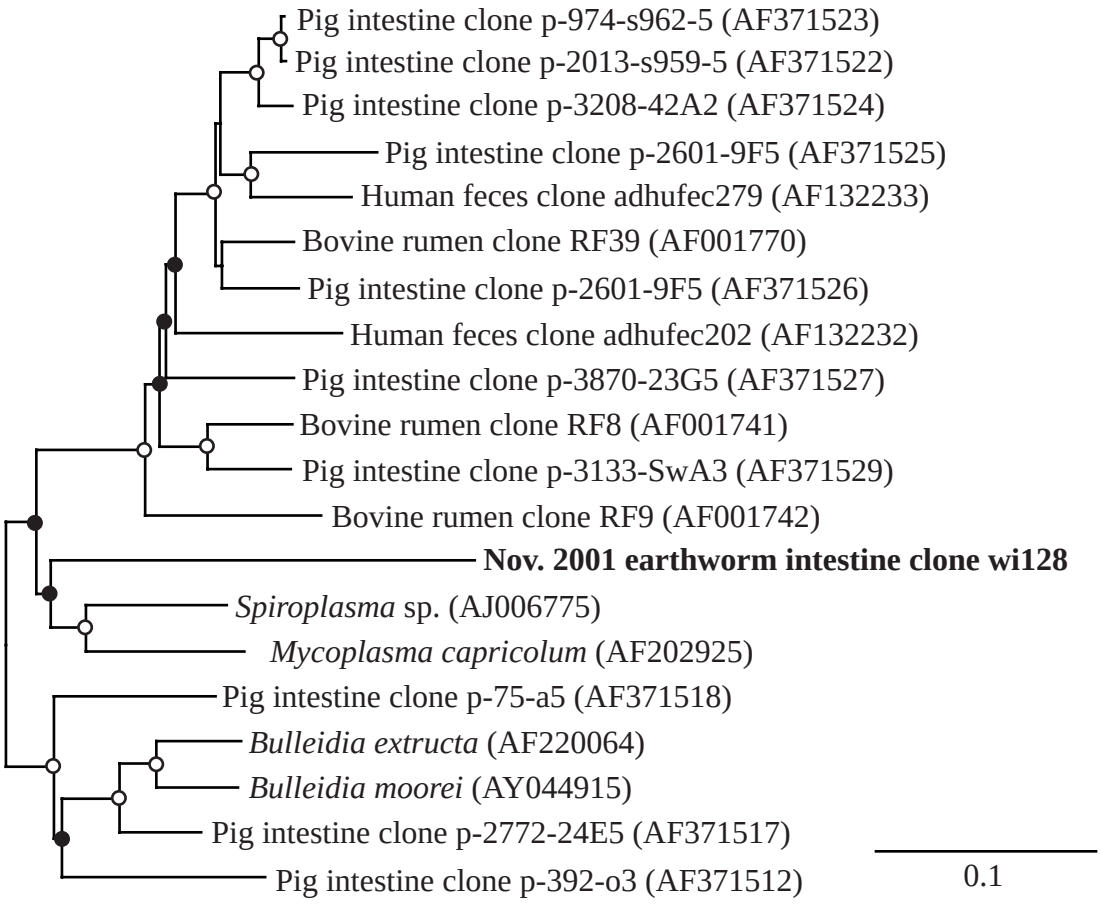

Pig intestine clone p-974-s962-5 (AF371523)
Pig intestine clone p-2013-s959-5 (AF371522)
Pig intestine clone p-3208-42A2 (AF371524)
Pig intestine clone p-2601-9F5 (AF371525)
Human feces clone adhufec279 (AF132233)
Bovine rumen clone RF39 (AF001770)
Pig intestine clone p-2601-9F5 (AF371526)
Human feces clone adhufec202 (AF132232)
Pig intestine clone p-3870-23G5 (AF371527)
Bovine rumen clone RF8 (AF001741)
Pig intestine clone p-3133-SwA3 (AF371529)
Bovine rumen clone RF9 (AF001742)
Nov. 2001 earthworm intestine clone wi128
Spiroplasma sp. (AJ006775)
Mycoplasma capricolum (AF202925)
Pig intestine clone p-75-a5 (AF371518)
Bulleidia extructa (AF220064)
Bulleidia moorei (AY044915)
Pig intestine clone p-2772-24E5 (AF371517)
Pig intestine clone p-392-o3 (AF371512)
0.1

Figure 4.4. Phylogenetic tree of clones affiliated with the β-proteobacteria. Intestine clone wi89 was from the April samples and was identical to 4 early wash clones and 1 cast clone from April, as well as 6 intestine clones from November. All other clones are from the April samples and were found only once. The tree was based on 587 positions of aligned sequence. Other notation is as described in Figure 4.1.

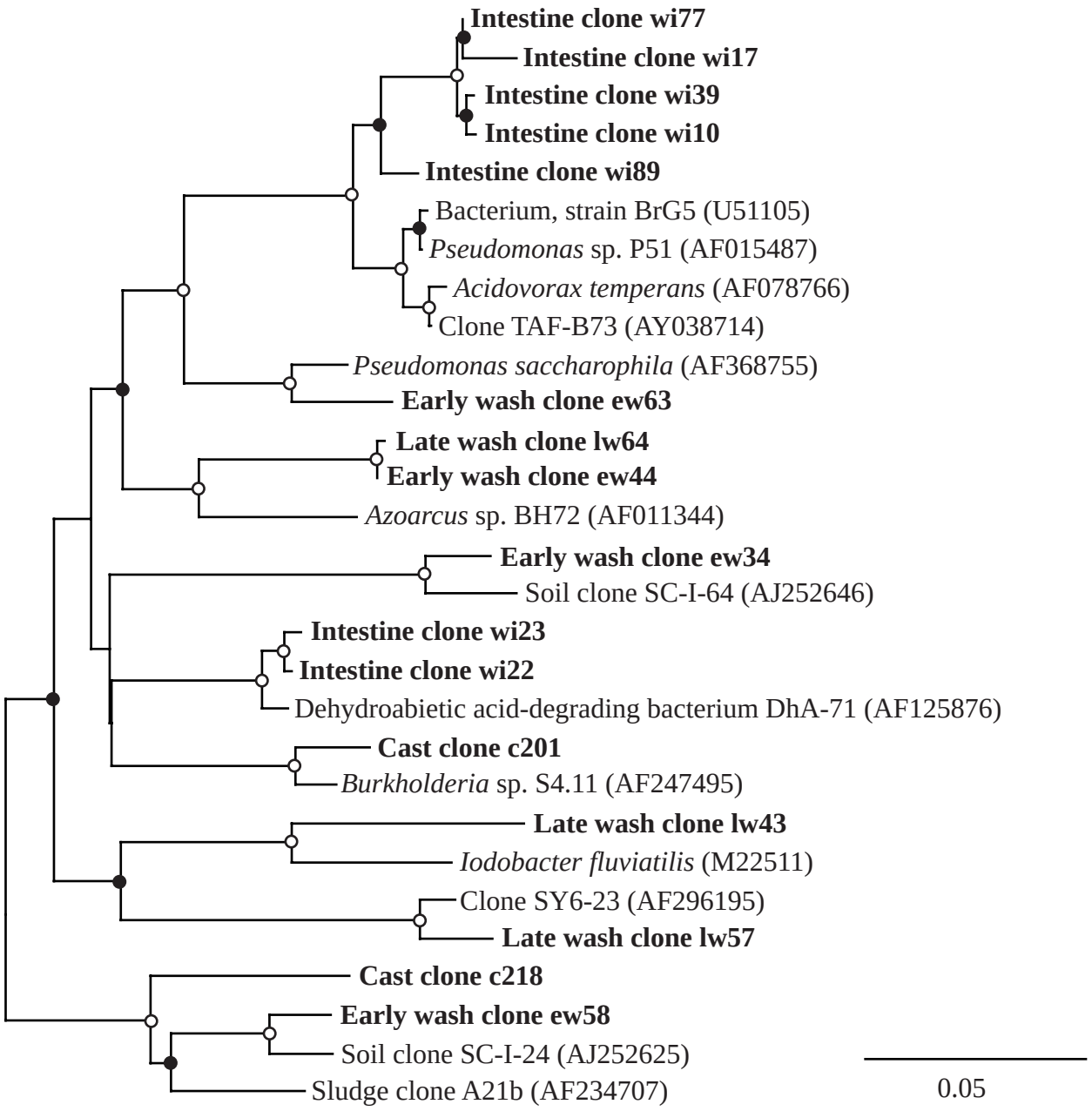
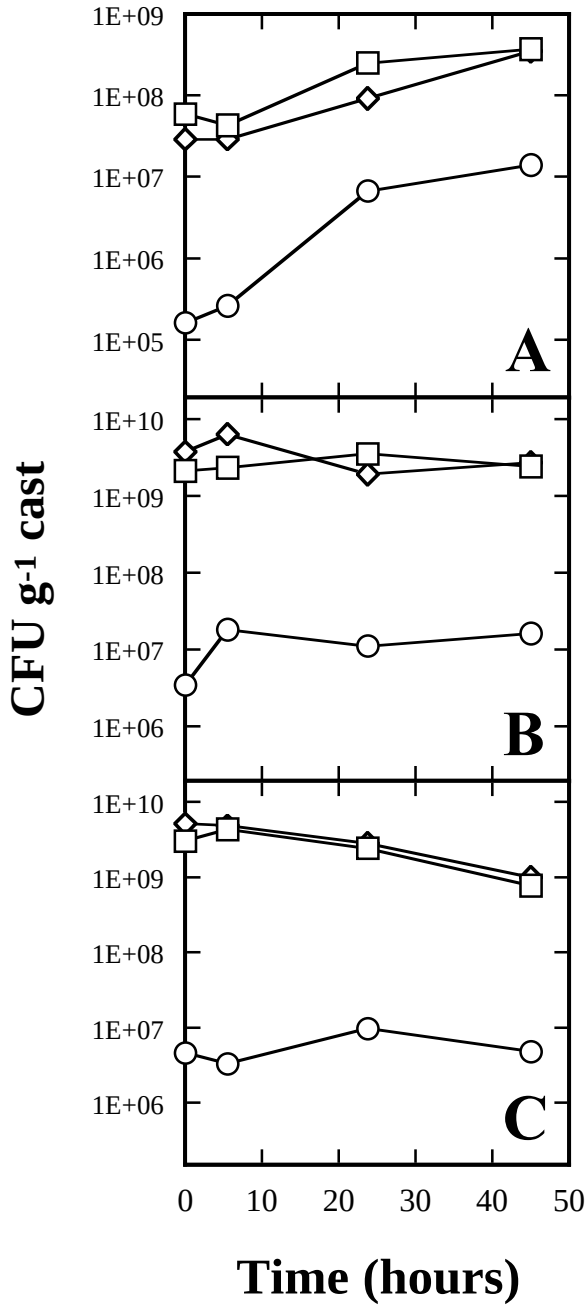

Intestine clone wi77
Intestine clone wi17
Intestine clone wi39
Intestine clone wi10
Intestine clone wi89
Bacterium, strain BrG5 (U51105)
Pseudomonas sp. P51 (AF015487)
Acidovorax temperans (AF078766)
Clone TAF-B73 (AY038714)
Pseudomonas saccharophila (AF368755)
Early wash clone ew63
Late wash clone lw64
Early wash clone ew44
Azoarcus sp. BH72 (AF011344)
Early wash clone ew34
Soil clone SC-I-64 (AJ252646)
Intestine clone wi23
Intestine clone wi22
Dehydroabietic acid-degrading bacterium DhA-71 (AF125876)
Cast clone c201
Burkholderia sp. S4.11 (AF247495)
Late wash clone lw43
Iodobacter fluviatilis (M22511)
Clone SY6-23 (AF296195)
Late wash clone lw57
Cast clone c218
Early wash clone ew58
Soil clone SC-I-24 (AJ252625)
Sludge clone A21b (AF234707)
0.05

Figure 4.5. Graphs showing the number of CFUs per gram (wet weight) of collected cast samples. Time is in units of hours after deposition. The media types are; squares (❒) - nutrient broth (NB); diamonds (✧) - enriched *Cytophaga* agar (ECA); and circles (❍) - Gould's S1 agar. Casts from (A) worm 1, (B) worm 2, and (C) worm 3.


1E+09
1E+08
1E+07
1E+06
1E+05
A
1E+10
1E+09
1E+08
1E+07
1E+06
B
1E+10
1E+09
1E+08
1E+07
1E+06
C
CFU g-1 cast
0
10
20
30
40
50
Time (hours)

CHAPTER V

Solirubrobacter paulii GEN. NOV., SP. NOV., A MESOPHILIC BACTERIUM WITHIN THE *Rubrobacteridae* RELATED TO COMMON SOIL CLONES

Abstract

A novel bacterium, strain B33D1, isolated from the burrow of an earthworm in agricultural soil, was characterized taxonomically and phylogenetically. Strain B33D1 was a Gram-positive, aerobic, rod of medium length that grew in long chains on a common laboratory medium. The optimal growth temperature was 30°C and the temperature range was 19 - 38°C. The optimal growth pH was 6 - 6.5, and the range was 6 - 7.5. The organism grew well on a variety of sugars and was capable of utilizing a few amino acids as a sole carbon source. Phylogenetically, strain B33D1 possessed 86% 16S rRNA sequence similarity to *Rubrobacter xylanophilus*. A number of other clones derived from soil samples were more closely related (up to 93% sequence similarity). These results placed strain B33D1 within the *Rubrobacteridae* subclass of the Actinobacteria phylum. The novel organism *Solirubrobacter paulii* gen. nov., sp. nov. is proposed, with strain B33D1 as the type strain.

Introduction

Members of the phylum Actinobacteria are widespread in soils throughout the world. In recent years, a number of 16S rRNA genes have been cloned from terrestrial samples that possessed sequence similarity to two species within a deep branch of the Actinobacteria, *Rubrobacter xylanophilus* (Carreto et al., 1996) and *Rubrobacter radiotolerans* (originally *Arthrobacter radiotolerans*; Yoshinaka et al., 1973; Suzuki et al., 1988). Both of these species were notable for their radiation tolerance, rare pigmentation, and thermophily. In contrast to the extreme environments that were the source material for the existing *Rubrobacter* species, a large number of the 16S rRNA genes have been cloneed from moderate, terrestrial environments, suggesting a wider range of habitat for the group (Furlong et al., 2002; Holmes et al., 2000; McCaig et al., 1999; Rheims et al., 1996; Ueda et al., 1995).

In this work, we characterize an organism which bears high sequence similarity to a subgroup within the *Rubrobacteridae* which had previously only been represented by environmental clone sequences.

Methods and Materials

Isolation. Strain B33D1 was isolated from a burrow of the epigeic earthworm *Lumbricus rubellus* in an agricultural soil by Furlong et al. (2002) on a plate composed of 50% Difco Nutrient Broth medium (50% NB; pH 7.0, ~23°C). Isolates were allowed to grow for two weeks before colonies were picked. A single pink colony was selected from a dilution series and maintained on 50% NB. The organism was stored at -70°C in a 15% glycerol stock solution.

Growth conditions. Unless otherwise indicated, the optimization of growth conditions for B33D1 was carried out in undiluted Difco Nutrient Broth (NB). The temperature range was determined using a temperature gradient incubator (Scientific Industries, Inc., Bohemia, NY). The pH range of B33D1 was determined by buffering NB with 25 mM of MES (pH 5.5, 6.0, and 6.5), HEPES (pH 7.0, 7.5, and 8.0), EPPS (pH 8.5), or CHES (pH 9.0, 9.5, 10.0). Growth in various salt conditions was testing by adding 0-10% NaCl to NB. Growth under anaerobic conditions was tested by sparging NB with N_2 gas for 30 minutes to remove O_2 and incubating cultures in Balch tubes (Balch et al, 1979). To test growth under microaerophilic conditions, 1% (v/v) of air was added to a N_2-sparged NB tube. The effect of increased CO_2 concentration on B33D1 was tested by adding 1-5% (v/v) of CO_2 to the headspace of DNB media in Balch tubes.

Biochemical properties. The use of various compounds as sole carbon sources was tested using a minimal media with the carbon source added to a final concentration of 0.2% (w/v). The minimal media consisted of 1 mM K_2HPO_4, 2 mM NH_4NO_3, 1 mM $MgSO_4$, 1% v/v trace mineral solution (Whitman et al., 1986), and 1.0 % iron solution (Whitman et al., 1986) adjusted to a pH of 7.0. In some tests (for example the carbon

utilization of lignin-associated compounds), the concentration of the iron solution produced a precipitate, and the lower concentration of 0.1% was used. Tubes were incubated at 30 °C for 2 weeks, and growth results were recorded.

The oxidase reaction of B33D1 was tested by applying a few drops of BBL™ Oxidase test reagent (Becton and Dickinson and Co., Cockeysville, MD) to cells on a piece of filter paper. Other tests were performed as described by Furlong et al. (2002).

Fatty acid analysis. A sample of B33D1 was analyzed by the Center for Biomarker Analysis at the University of Tennessee to determine the phospholipid fatty acid (PLFA) content of the organism. The cell paste was extracted with the single-phase chloroform-methanol-buffer system of Bligh & Dyer (1954) as modified by White et al. (1979). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (Guckert et al., 1985). The polar lipids were transesterified to the fatty acid methyl esters by a mild alkaline methanolysis (Guckert et al., 1985). The fatty acid methyl esters were then analyzed by capillary gas chromatography with flame ionization detection on a Hewlett-Packard 5890 Series 2 chromatograph with a 50 m non-polar column (0.2 mm I.D., 0.11 μm film thickness). The injector and detector were maintained at 270^{o}C and 290^{o}C, respectively. The column temperature was programmed from 60^{o}C for 2 minutes, then ramped at 10^{o}C per minute to 150^{o}C, then ramped to 312^{o}C at 3^{o}C per minute. Preliminary peak identification was by comparison of retention times with known standards. Detailed identification of peaks was by gas chromatography/mass spectroscopy of selected samples using a Hewlett-Packard 5890 series 2 gas chromatograph interfaced to a Hewlett –Packard 5971 mass-selective detector using the same column and temperature program as previously

described. Mass spectra were determined by electron impact at 70 eV. Methyl nonadecanonate (c19:0) was used as the internal standard, and the PLFA expressed as equivalent peak response to the internal standard.

Microscopy. The cellular morphology of B33D1 was observed by phase-contrast microscopy, negative staining with negrosin dye, as well as electron microscopy. A Nikon TE 300 inverted microscope was used to observe the cellular morphology of B33D1. Digital photomicrographs were taken through the IP Lab Spectrum software package (version 3.4.5) using an attached Princeton Instruments MicroMax high resolution, cooled CCD camera. Samples for scanning electron microscopy (SEM) were prepared according to the method of Hahn et al. (1998) except that 4% glutaraldehyde was used to fix the cells. A Leo 982 field emission SEM was used to examine the cells. To test for the presence of a capsule, an India ink suspension as well as a standard capsule stain were used (Benson, 1990). The Gram reaction of B33D1 was determined by a common staining technique and observed using light microscopy (Benson, 1990).

Phylogeny. The nearly complete 16S rRNA gene of B33D1 was obtained as part of an earlier study (Furlong et al., 2002). The nearly full length sequence was obtained by amplification of the genomic DNA with primers 27f and 1392r (Furlong et al., 2002). Sequencing reactions were performed using a Big Dye sequencing kit (Perkin-Elmer) with primers 27f, 1392r, 533f, 519r, and 907r (Lane, 1991). The sequencing reaction products were run on an ABI 377 Automated Sequencer (Perkin-Elmer). The nearly complete 16S rDNA sequence of B33D1 can be found in GenBank with the accession number AY039806. To construct the phylogenetic trees, the sequence was first aligned with reference organisms and environmental clone sequences using the PILEUP program

included in the GCG software package (Genetics Computer Group). The evolutionary distances between aligned sequences were determined using the Jukes-Cantor algorithm in the DNADIST program of the PHYLIP software package (Felsenstein 1989). The tree topology was determined using the FITCH program, and the tree robustness was testing using 100 replicate trees as generated by SEQBOOT within the PHYLIP set of programs. The nearest neighbors of B33D1 were determined by FASTA searches (Pearson and Lipman, 1988) of GenBank.

Mol % G+C. The mol % G+C percentage of B33D1 was determined by the method of Mesbah et al., (1989). Five samples of DNA were run, and the average of the samples was reported.

Results and Discussion

Cellular and colonial properties. Strain B33D1 was a rod, approximately 1.4 μm x 0.7 μm, although much longer cells were occassionally observed during the growth of the cells (Figure 5.1). Older cultures were generally composed of uniformly shorter rods. In liquid culture, cells were often observed with an indentation near the center of the cell, presumably in preparation for cellular division. Cells grew in long chains that wrapped around each other producing large aggregates in liquid culture. No capsule was observed. Cells of strain B33D1 stained Gram-positive. No motility was observed, and no spores were apparent by phase contrast microscopy.

Colonies of strain B33D1 grown on agar of Difco Nutrient Broth were round, convex, with entire edges, and usually pink in color. Plates incubated at higher temperatures (≥ 28°C) often produced less pigment initially, although the deep pink color appeared over time. Upon streaking, growth of strain B33D1 was sparse and few well isolated colonies were formed. Spread plating B33D1 on solid agar surfaces often resulted in only a few or no colonies. However, B33D1 grew well in liquid culture or embedded in a soft agar of NB. This apparent difficulty of the organism to grow on the surface of an agar plate may help explain why it had not been previously isolated.

B33D1 appeared similar in color to the other *Rubrobacter* isolates. Similarly to these organisms, the pigments were not easily extracted using traditional methods. However a small amount of pigment was recovered by extensive incubation of a cell pellet in methanol. A visible light spectrum of these extractable compounds in 100% methanol possessed absorption maxima at 466, 493, and 526 nm. These maxima were

similar to those reported for pigments extracted from *Rubrobacter radiotolerans* (Saito et al., 1994).

Nutritional characteristics. A variety of compounds were tested as sole carbon and energy sources for strain B33D1. Unfortunately, strain B33D1 grew to very low cellular density in liquid culture even in a rich medium (generally around 0.1 OD at 600 nm). Because of this, weakly positive growth in a minimal medium was sometimes difficult to ascertain. Growth was observed for a variety of sugars including fructose, galactose, glucose, lactose, mannose, sorbitol, sucrose, and xylose. Growth was not observed on cellobiose and mannitol. Strain B33D1 utilized the organic acid pyruvate, appeared to grow weakly on acetate, but did not grow on citrate, malate, or succinate. Casamino acids provided good growth; but when tested individually, only the amino acids alanine, arginine, and lysine were found to support growth. Glycerol was the only alcohol tested that could support the growth of B33D1. Other alcohols which produced negative results were methanol, ethanol, 1-propanol, 2-propanol, butanol, isobutyl alcohol, and iso-amyl alcohol. Because B33D1 was isolated from soil, a variety of products of lignin degradation were tested as possible carbon sources. Of the compounds tested, only chlorogenic acid gave positive growth. Anthranilic acid, benzoic acid, catechol, protocatechuic acid, *p*-courmaric acid, gentisic acid, ferulic acid, *p*-hydroxybenzoic acid, syringic acid, and vanillic acid did not produce observable growth after two weeks.

B33D1 utilized ammonium as a sole nitrogen source and could grow on Casamino acids as a sole carbon and nitrogen source. It did not reduce nitrate or produce urease.

Other growth conditions. A variety of pH and temperature conditions were tested. Strain B33D1 grew optimally at 28-30°C, but could grow between 19 and 38°C. B33D1

could grow at a pH between 6 and 7.5 with an optimum around 6 to 6.5. B33D1 did not grow in the absence of oxygen but grew when 1% (v/v) atmospheric gas was added to the headspace. Because the concentration of CO_2 is often higher in soil than in the atmosphere, the growth of B33D1 under these conditions was tested. However, neither the growth rate nor yield were enhanced by CO_2 additions from 1-5% (v/v). While *Rubrobacter* strains can grow in media with increased salt concentrations (Ferreira et al., 1999; Carreto et al., 1996; Suzuki et al., 1988), B33D1 did not grow when as little as 1% NaCl was added to the growth media.

Biochemical and chemotaxonomic properties. B33D1 was catalase positive and oxidase negative. Casein and Tween 80 were not hydrolyzed. B33D1 was sensitive to polymyxin, ampicillin, tetracycline, and streptomycin antibiotics. No hemolysis was observed on blood agar.

The fatty acid profile of B33D1 revealed no unusual compounds. The major phospholipid fatty acids (PFLAs) were i16:0 (54%) and 18:1ω9c (36%). Trace amounts of 16:0 (4%), 19:1ω12c (2%), 16:1ω7c (1%), and br17:1 (1%) were also detected. No polyunsaturated PFLAs were found. Related isolates in the *Rubrobacteria* genus contain only small amounts, if any, of the abundant PFLAs from B33D1 (Carreto et al., 1996).

The G+C content of the genomic DNA of B33D1 was 71.8 % ± 0.2 (mean ± SD; n = 5), which was slightly higher than the 67.6 % value reported for *Rubrobacter xylanophilus* (Carreto et al., 1996) but not unexpected given the high G+C content of the Actniobacteria phylum.

Phylogeny. Based on 16S rDNA sequence analyses, strain B33D1 grouped within the *Rubrobacteridae* subclass of the Actinobacteria phylum, more specifically within

subgroup 2 as defined by Holmes et al. (2000; Figure 5.2). No other isolates have been reported from this particular subgroup, although a number of environmental 16S rDNA clones have been recovered. The most closely related sequence in GenBank by FASTA analysis was an uncultivated clone from a thermal soil (93% identity over 1364 bases), while the closest characterized organism was *Rubrobacter xylanophilus* (86% over 1372 bases). The other clonal sequences in subgroup 2 originated from terrestrial or sediment sources similarly to B33D1. Given the apparent ubiquitous distribution of this group in soil samples in various parts of the world, there is little reason to presume an association of this organism with earthworms, even though it was originally isolated from earthworm burrow soil.

Description of *Solirubrobacter* gen. nov.

Solirubrobacter (So.li.ru.bro.bac'ter. L.n. solum soil; L.adj. ruber red; M.L.n. *bacter* the masculine equivalent of the GR. neut. n. *bakterion* a rod; M.L. masc. n. *Solirubrobacter* a soil red rod)

Colonies are round, convex, and pink in color. Cells are Gram-positive, rods of medium length, and grow in chains. Non-motile. Spores are not formed. Aerobic and mesophilic. Catalase positive and oxidase negative. The major fatty acids are i16:0 (54%) and 18:1ω9c (36%). Grow well on common sugars and a few amino acids as a sole carbon source. Phylogenetically within the *Rubrobacteridae* subclass of the Actinobacteria. The type species is *Solirubrobacter paulii*.

Description of *Solirubrobacter paulii* gen. nov., sp. nov.

(pau.li'i. M.L.gen.n. paulii of Paul; named for the prominent soil scientist Eldor A. Paul). Cells are rods 1.4 μm x 0.7 μm, grow in long chains. The G + C content of the type

strain B33D1 is 72%. The type strain grows at at a pH of 6.0 - 7.5 and a temperture between 19 - 38°C, with an optimum at pH 6.5 and 28 - 30°C. Fructose, galactose, glucose, lactose, mannose, sorbitol, sucrose, xylose, pyruvate, acetate, casamino acids, alanine, arginine, lysine, glycerol, and chlorogenic acid support growth. The organism does not grow on cellobiose, mannitol, citrate, malate, succinate, methanol, ethanol, 1-propanol, 2-propanol, butanol, isobutyl alcohol, iso-amyl alcohol, anthranilic acid, benzoic acid, catechol, protocatechuic acid, p-courmaric acid, gentisic acid, ferulic acid, p-hydroxybenzoic acid, syringic acid, and vanillic acid. The type strain is B33D1 (ATCC - not yet submitted; DSMZ - not yet submitted).

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Figure 5.1. Photomicrographs of B33D1. Both images are cells from liquid cultures. (A) Phase contrast image of an aggregate of cells. Scale bar = 5 μm. (B) SEM of an aggregate. Scale bar = 1 μm.

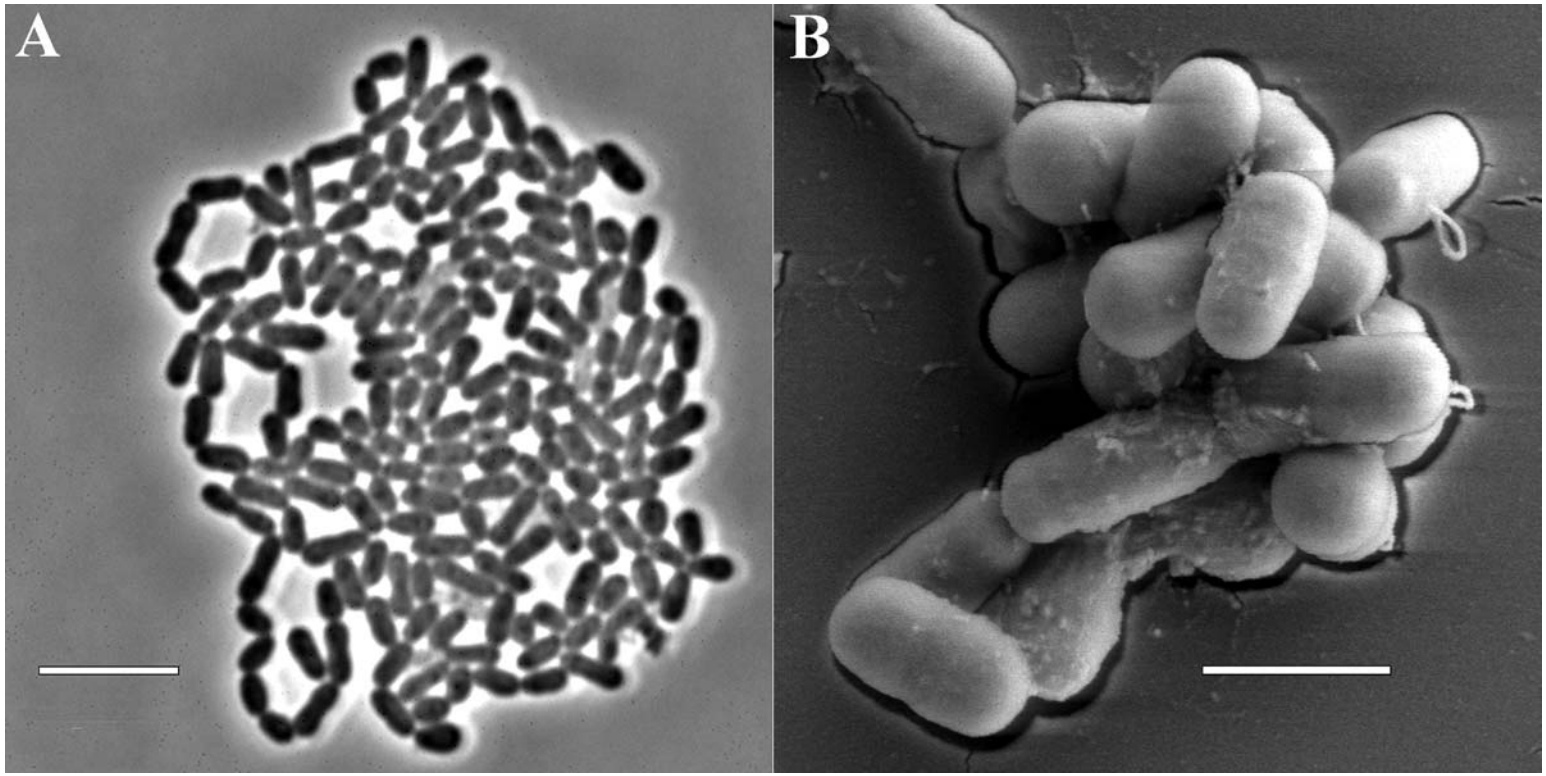
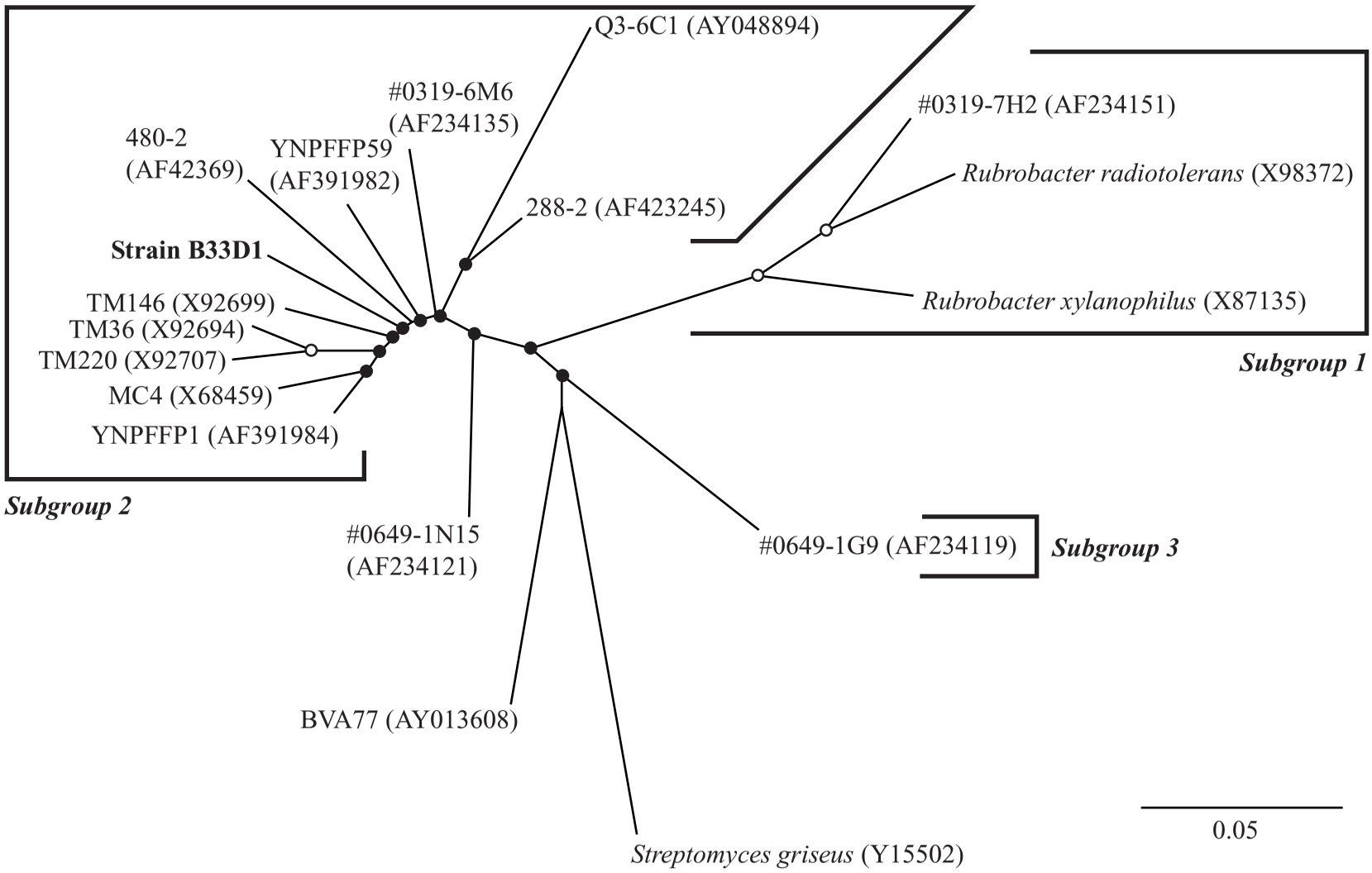

A
B

Figure 5.2. Phylogenetic tree of the 16S rRNA genes of B33D1 and the closest clonal and cultured relatives. Subgroup designations follow the nomenclature of Holmes et al. (2000). Isolates are written in italics and strain B33D1 is in bold. Closed (●) and open (❍) circles indicate bootstrap support of ≥ 50 and ≥ 95, respectively. GenBank accession numbers are in parenthesis following each name. Only clonal sequences with nearly complete 16S rDNA sequence information were used in this tree. Clones beginning with TM are from a peat bog (Rheims et al., 1996). Clones #649-1G9, #0319-7H2, #0649-1N15, and #0319-6M6 were obtained from an Australian arid soil (Holmes et al., 2000). Sequence YNPFFP1 was from a thermal soil (unpublished). Sequences beginning with MC were clones from a subtropical Australian soil (Liesack and Stackebrandt, 1992). Clones 480-2 and 288-2 were from soil (unpublished). BVA77 and Q3-6C1 were from a landfill (Röling et al., 2001) and rhizosphere soil (unpublished), respectively. The tree was based on 1306 bases of aligned sequence. The scale bar represents Jukes-Cantor evolutionary distance.


Q3-6C1 (AY048894)
#0319-6M6
(AF234135)
480-2
(AF42369)
YNPFFP59
(AF391982)
288-2 (AF423245)
Strain B33D1
TM146 (X92699)
TM36 (X92694)
TM220 (X92707)
MC4 (X68459)
YNPFFP1 (AF391984)
Subgroup 2
#0319-7H2 (AF234151)
Rubrobacter radiotolerans (X98372)
Rubrobacter xylanophilus (X87135)
Subgroup 1
#0649-1N15
(AF234121)
#0649-1G9 (AF234119)
Subgroup 3
BVA77 (AY013608)
Streptomyces griseus (Y15502)
0.05

CHAPTER VI

CONCLUSIONS

These collected works described the prokaryotic communities associated with the epigeic earthworm, *Lumbricus rubellus*; described a method to compare libraries of 16S rRNA genes; and characterized a novel bacterial genus. Prior to this work, few studies utilized molecular techniques to examine any earthworm species in great detail, and none of them retrieved information that would allow the identification of prokaryotes at a resolution more specific than phylum. Therefore, what was known about earthworm associated bacterial communities was heavily biased by that low percentage of the prokaryotic world that was culturable on the various media used.

Previous works with culture-based techniques did not document a significant difference in the microbial communities of the earthworm and surrounding soil. In most cases, organisms isolated from the casts and intestines were similar to those isolated from the soil. Our studies of earthworm casts suggested that several taxa might be influenced by passage through the earthworm intestine. In order to test this hypothesis, a novel method for comparing libraries of 16S rRNA genes (LIBSHUFF) was created. The analysis was used to determine that the microbial population of the cast was significantly different than the surrounding soil in four taxa. However, no sign of a uniquely earthworm-associated taxon was found.

Investigations of washed earthworm intestines revealed that significant numbers of prokaryotes remained associated with the intestine even after vigorous washings. In addition, a number of taxa that appeared to be associated with the digestive tract were identified through 16S rRNA gene clone libraries. Several of these taxa had not been previously observed in clone libraries constructed from soils, casts, or the material removed from the intestine. The LIBSHUFF analysis was again used to determine the

relationships between the constructed clone libraries. Earthworms from three seasons were screened for the presence of the intestine-associated taxa, and some of the taxa were found in multiple seasons. While the data did not conclusively support a stable earthworm intestinal community, the indications are that such an indigenous population does exist.

A novel organism isolated from Horseshoe Bend agricultural soil was characterized. The organism represented a novel genus, *Solirubrobacter*, which was phylogenetically related to a deep subclass within the Actinobacteria phylum. The data showed that *Solirubrobacter* did not readily grow on a solid, rich medium; grew to a low density in liquid culture; and had a growth temperature optimum higher than what might be expected for a soil organism. These factors may help explain why other members of this subclass, which are highly represented in terrestrial clone libraries (including those presented in this work), had not been cultured previously.