

# Molecular and Culture-Based Analyses of Prokaryotic Communities from an Agricultural Soil and the Burrows and Casts of the Earthworm *Lumbricus rubellus*

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The microbial populations in no-till agricultural soil and casts of the earthworm *Lumbricus rubellus* were examined by culturing and molecular methods. Clone libraries of the 16S rRNA genes were prepared from DNA isolated directly from the soil and earthworm casts. Although no single phylum dominated the soil library of 95 clones, the largest numbers of clones were from *Acidobacteria* (14%), *Cytophagales* (13%), *Chloroflexi* (8%), and  $\gamma$ -*Proteobacteria* (8%). While the cast clone library of 102 clones was similar to the soil library, the abundances of several taxa were different. Representatives of the *Pseudomonas* genus as well as the *Actinobacteria* and *Firmicutes* increased in number, and one group of unclassified organisms found in the soil library was absent in the cast library. Likewise, soil and cast archaeal 16S rRNA gene libraries were similar, although the abundances of some groups were different. Two hundred and thirty aerobic bacteria were also isolated on general heterotrophic media from casts, burrows, and soil. The cast isolates were both phenotypically and genotypically different from the soil isolates. The cast isolates were more likely to reduce nitrate, grow on acetate and Casamino Acids, and utilize fewer sugars than the soil isolates. On the basis of their ribotypes, the cast isolates were dominated by *Aeromonas* spp. (28%), which were not found in the soil isolates, and other  $\gamma$ -*Proteobacteria* (49%). In contrast, the soil isolates were mostly *Actinobacteria* (53%), *Firmicutes* (16%), and  $\gamma$ -*Proteobacteria* (19%). Isolates obtained from the sides of earthworm burrows were not different from the soil isolates. Diversity indices for the collections of isolates as well as rRNA gene libraries indicated that the species richness and evenness were decreased in the casts from their levels in the soil. These results were consistent with a model where a large portion of the microbial population in soil passes through the gastrointestinal tract of the earthworm unchanged while representatives of some phyla increase in abundance.

Earthworm activity profoundly affects the physical and chemical composition of soil. Earthworm burrowing creates pore spaces, thus increasing water conductivity and benefitting root growth (19, 44). Earthworms break down large soil particles and leaf litter, which increases the availability of organic matter for microbial degradation (18). They promote macroaggregate formation by depositing casts rich in glycoproteins, polysaccharides, bacteria, and clay (12, 18, 25, 75). Earthworms also affect soil profile formation by mixing litter layers with surface soil layers, which acts to deepen humus layers within soil (18, 44).

The microbial community of soil may be profoundly affected by the presence of earthworms. Studies of various earthworm species have generally shown an increase in microbial numbers or activity either during or after passage through the gut and in the drilosphere (2, 12, 13, 20, 42, 54, 57, 66, 75, 78, 83), although corresponding increases in microbial biomass were not always observed (12, 65). Karsten and Drake (38) found more anaerobes and cellobiose utilizers in the earthworm alimentary canal than in soil. Specific phylogenetic groups of organisms have also been found in higher numbers in earthworm guts, casts, or burrows, such as *Aeromonas hydrophila* in *Eisenia foetida* (76), fluorescent pseudomonads in *Lumbricus terrestris*

(13), and *Actinobacteria* in *Lumbricus rubellus* (41). The differences in microbial activity, bacterial numbers, and certain bacterial species between the earthworm alimentary canal or burrow and bulk soil indirectly support the hypothesis that the bacterial community structures of these habitats are different from those of the soil.

In this study we present the combined results of culture and culture-independent studies on earthworm casts, burrows, and the surrounding agricultural soil over a 3-year period. The phenotypic properties of 204 isolates were examined as well as the partial 16S ribosomal DNA (rDNA) sequences of the isolates and of over 200 clones constructed from earthworm casts and bulk soil. Of considerable interest was determining if the cast and soil populations were significantly different and whether the clone libraries would contain sequences similar to the isolates'.

## MATERIALS AND METHODS

**Sample site.** All samples were obtained from no-tilled agricultural plots at the Horseshoe Bend Long Term Environmental Research site, which is located in Athens, Georgia. This site is located on a river floodplain on Hiwassee series soil (fine loamy, siliceous, thermic, and Rhodic Kanhapludult). Clover and rye have been the winter cover crops in the plots since 1978 (3, 30, 31, 72). Maize (*Zea mays* L.) was the summer crop in 1996 and 1997. Kenaf (*Hibiscus cannabinus*) and cotton (*Gossypium hirsutum*) were planted during the summers of 1998 and 1999, respectively. Additional soil properties can be found in Beare et al. (3).

**Sample collection for isolates.** In April and May 1996 a shovel was used to expose 15 soil shelves, approximately 1 m long and 10 cm deep. Fifty-five soil samples were taken at various depths within the top 5 cm of these shelves. In May 1999 three soil shelves were made and 15 soil samples were taken in the same

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manner. Sampling was performed by horizontally inserting a sterile Pasteur pipette 5 mm into the soil. Care was taken to avoid earthworm burrows and roots. These samples were defined as "bulk" soil. Upon removal from the soil, the tip of the pipette containing the soil sample was broken off into a microcentrifuge tube containing 0.5 ml of 0.85% (wt/vol) NaCl and crushed with a sterile inoculating stick. In a separate experiment the wet weight of 50 samples was measured, and the average wet weight was 5.2 mg (standard deviation = 1.2 mg). Using the same methods, 16 samples were also taken in March and April of 1996 from the sides of burrows found in the soil shelves or on the soil surface. All soil and burrow samples were taken 3 or more days following the last rainfall. Samples were also taken from the fresh casts of 29 and 12 *L. rubellus* worms in April 1997 and May 1999, respectively. These worms were either collected from the soil surface after the application of an electrical current to the soil (64) or from burrows exposed after excavation of soil shelves. The worms were rinsed with sterile water and placed in sterile petri plates containing a small amount of water. The plates were checked every 2 h. When a cast was observed, it was processed as described above for the soil samples.

**Sample collection for clone libraries.** Soil and earthworm samples were collected in April 1999 from a no-till plot (main plot, subplot 7) which was overgrown with clover. Soil samples were collected from five randomly chosen sites from a depth of 5 to 10 cm below the litter layer and were put immediately on ice. The samples were stored at  $-20^{\circ}\text{C}$  until processed. Two *L. 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 4 h of sampling, combined into a single sample, and frozen at  $-20^{\circ}\text{C}$ .

**Bacterial isolation.** Each soil and cast sample was vortexed for 30 s in 0.85% (wt/vol) NaCl to disperse bacteria and was serially diluted to a factor of  $10^{-9}$  in two types of media. The dilution and growth media were MMSA (1 mM  $\text{K}_2\text{HPO}_4$ , 2 mM  $\text{NH}_4\text{NO}_3$ , 1 mM  $\text{MgSO}_4$ , 1% [vol/vol] trace minerals [82], 1% [vol/vol] iron solution [82], 10% [vol/vol] soil extract, and 10 mM sodium acetate) at a pH of 7.0 and 50% (vol/vol) Difco nutrient broth (DNB) (Difco manual, Difco Laboratories, Detroit, Mich.). The soil extract was prepared by the method of Stackebrandt and Prauser (71), except that it was sterilized by filtration (0.2  $\mu\text{m}$  pore size) rather than by autoclaving. Each soil, burrow, and cast sample taken in 1996 and 1997 was diluted and grown in MMSA and DNB. The samples taken in May 1999 were diluted and grown only in MMSA. All samples were incubated in the dark at  $25^{\circ}\text{C}$ . After 2 weeks, the highest dilutions showing growth in each medium were streaked onto solid medium of the same composition (MMSA or DNB with 1.5% [wt/vol] agar) for isolation of pure cultures. A representative of each colony type on each plate was then subcultured in 0.5 ml of DNB. In 1999, however, only one colony type per sample was selected. After turbidity became visible, 0.5 ml of sterile 15% (vol/vol) glycerol was added and the culture was stored at  $-70^{\circ}\text{C}$ .

**Phenotypic characterization of isolates.** Each isolate was cultured from frozen stocks on Difco nutrient agar (Difco manual) plates, and the colonial morphology was recorded. The presence of catalase was determined by a standard procedure (70). Each isolate was then subcultured in undiluted DNB. The cellular morphology was observed when turbidity became visible. Motility was tested using Difco Motility Test medium and was scored according to the Difco procedure (Difco manual). Substrate utilization was tested in 5.0 ml of minimal MMSA, prepared without acetate and soil extract. Potential substrates (cellulose, acetate, lactose, Casamino Acids, mannose, and cellobiose) were tested at a concentration of 0.2% (wt/vol). Test media were inoculated with 10  $\mu\text{l}$  of culture and incubated for 14 days at room temperature. If the absorbance at 600 nm increased from 0.00 to  $\geq 0.15$ , the culture was scored as positive for growth. Difco nitrate reduction medium was inoculated and incubated similarly. It was scored according to the Difco procedure (Difco manual). Partial nitrate reduction meant that nitrite was detected after 2 weeks. Complete nitrate reduction meant that neither nitrite nor nitrate remained after that time. The solid media that were used included Difco urea agar, Difco MacConkey medium, Difco blood agar, Difco Simmon's citrate, casein decomposition agar (21), and Tween hydrolysis agar (1). The isolates were patched onto each plate and incubated at room temperature for 14 days. The media from Difco were scored according to their procedures (Difco manual). The Tween hydrolysis agar and the casein decomposition agar were scored according to standard procedures (1, 21). For antibiotic resistance, each isolate was spread onto a nutrient agar plate (Difco). Antibiotic discs (Difco) were dispensed onto the plate, and the results were scored according to the Difco procedure (Difco manual).

**Statistics.** The statistical analysis of the isolates was performed with SAS (63). A chi-square test was used to determine if there was a significant difference in phenotypic properties and phylogenetic distribution between the isolate libraries

from the three sources. When the  $P$  was  $< 0.05$ , scaled deviance values were analyzed using the SAS genmod procedure to determine if the differences were due to the sample source (cast versus soil versus burrow) or the sampling protocol.

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

To determine if the distribution of  $D_i$  was consistent with a random model, a data set was generated by randomly assigning test results to "isolates" using the same ratio of positive to negative results as obtained for the original isolates. A new matrix of  $D_i$  values was then created, and the distribution of  $D_i$  was determined.

To determine if the phenotypes of the isolates collected in 1996 or 1997 or the control isolates were the same as those of the isolates collected in 1999 or the test isolates, lowest- $D_i$  (LDi) analysis was performed. A matrix of  $D_i$  values was constructed in Excel comparing the phenotypes of the test isolates to those of the control isolates from MMSA. The lowest  $D_i$  value for each test isolate and the mean of the lowest  $D_i$  value were then determined. To determine the expectation of the mean, a matrix of  $D_i$  values was first constructed in Excel comparing the phenotypes within the control isolates. After removal of  $D_i$  values for comparisons of the isolates to themselves, the LDi for each control isolate was determined. The means of the lowest  $D_i$  values of 100 random selections equal in number to those of the test isolates were then calculated.

**DNA extraction for clone libraries.** Total DNA was extracted from the soil and cast samples by a modification of the protocols of Tsai and Olson (79) and Moré et al. (52). Soil, 1 g (wet weight), from each of the five sites was 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 (0.15 M NaCl, 0.1 M disodium EDTA [pH 8.0], and lysozyme [1.5 mg/ml]). The samples were vortexed and placed in a shaking  $37^{\circ}\text{C}$  water bath for 1 h, with additional vortexing every 15 min. The soil slurries were aliquoted into 500- $\mu\text{l}$  portions in microfuge tubes. To each of the soil aliquots and the tube containing the cast material was added 400  $\mu\text{l}$  of a bead beating solution (0.1 M NaCl, 0.5 M Tris Cl [pH 8.0], and 10% sodium dodecyl sulfate), and approximately 0.1 g of 0.1-mm-diameter glass beads. These tubes were then placed into a TurboMix adapter (Scientific Products) for a Vortex Genie 2 (Fisher Scientific) and treated for 3 min at maximum speed. The tubes were centrifuged for 3 min, 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 min. 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  $-20^{\circ}\text{C}$ . After the precipitation, the tubes were centrifuged for 10 min. A dark brown pellet was visible in each tube. The pellets were vacuum dried and resuspended in 500  $\mu\text{l}$  of distilled water ( $\text{dH}_2\text{O}$ ). These preparations were treated with RNase (final concentration, 10  $\mu\text{g}/500 \mu\text{l}$ ) at  $37^{\circ}\text{C}$  for 1 h followed by passage through a Wizard DNA Clean-Up Kit (Promega). The quality and concentration of DNA were confirmed via gel electrophoresis on a 1% agarose gel.

**PCR amplification of 16S rRNA genes.** 16S rDNA was amplified from soil- and cast-extracted DNA for construction of bacterial and archaeal clone libraries. Each PCR consisted of 1 Ready-to-Go PCR bead (Amersham Pharmacia), 2  $\mu\text{l}$  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  $\mu\text{l}$  of reverse primer (1392r universal [5'-ACG GGC GGT GTG TRC-3']), 1 or 2  $\mu\text{l}$  of sample DNA (4 to 20 ng), enough  $\text{dH}_2\text{O}$  to bring the reaction to 25  $\mu\text{l}$ , and 30  $\mu\text{l}$  of mineral oil. Each PCR was 25 cycles of  $94^{\circ}\text{C}$  for 1 min,  $58^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1.75 min for denaturation, annealing, and extension steps, respectively, on a Thermolyne Temp-tronic thermocycler (Du buque, Iowa). 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.

The 16S rDNA of the 204 isolates was also PCR amplified using Ready-To-Go

PCR Beads and the same bacterial primers as the clone libraries. Isolates were grown on Difco nutrient agar plates, and a single colony of each isolate was resuspended and washed two times in 100  $\mu$ l of sterile dH<sub>2</sub>O. Each cell suspension was added to a reaction mixture at a concentration of 8% (vol/vol) to act as the template DNA, and each reaction mixture was heated for 5 min at 94°C. The reactions were denatured, extended, and annealed for 1 min at 94°C, 2 min at 72°C, and 1 min at 61°C for 30 cycles. Isolates which did not produce a PCR product with whole-cell cultures were treated with the same protocol used to extract DNA for the clone libraries (without the use of a Clean-Up kit), and 100 ng of the resultant DNA was used in a PCR. PCR products of the isolates were purified using Prep-A-Gene DNA Purification Systems (Bio-Rad).

**Construction of clone libraries.** Clone libraries of archaeal and bacterial PCR products were constructed using a TA Cloning Kit (Invitrogen) with plasmid pCR2.1. Successful transformants were inoculated into Luria-Bertani 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  $\mu$ l of purified plasmid, 2  $\mu$ l of buffer H (Promega), 1  $\mu$ l of *Eco*RI (Promega), and 18  $\mu$ l of dH<sub>2</sub>O and was incubated at 37°C for 1 h. 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  $\mu$ l of PCR product, 1  $\mu$ l of 10 $\times$  Multicore buffer (Promega), 1  $\mu$ l of *Pvu*II (Promega), 1  $\mu$ l of *Bgl*I (Promega), and 4  $\mu$ l of dH<sub>2</sub>O. The reaction was incubated at 37°C for 1 h, and the entire reaction mixture was 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 and isolates were sequenced using the primer 27f, while archaeal clones were sequenced with the primer 21f. 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. Isolate sequences containing greater than five ambiguous bases per 500 bp were cloned into the vector pCR 2.1 (Invitrogen), and the plasmid was resequenced.

**Analysis of 16S rDNA data.** The closest database relatives of all sequences were obtained using all available sequence information by FastA searches of GenBank (56). Duplicate sequences ( $\geq 99\%$  sequence similarity) were grouped into an operational taxonomic unit (OTU), and only one sequence was used for further analysis. The low similarity of many of the clone sequences to known organisms' sequences 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. Alignments of sequences and reference organisms were created using the PILEUP program of the GCG software package (26). Only sequence data corresponding to *Escherichia coli* bases 81 to 459 were used for the phylogenetic trees. Trees were constructed using the PHYLIP software package (22). 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. Phylogenetic trees specific for phyla and candidate divisions were constructed to insure support for the positioning of clone sequences. Sequences which did not associate with any of the formally described lineages using these methods 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 analyzed with the CHIMERA\_CHECK program at the Ribosomal Database Project II (RDP-II [47]) using the default settings. Potential chimeric sequences were then reanalyzed excluding Ribosomal Database Project sequences shorter than 1,000 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 were considered chimeric and were excluded from the study.

**Statistical analyses of the libraries.** Rarefaction was used to evaluate the OTU richness of the isolate libraries (35, 40). Because the sample sizes differed for the three collections, the diversity indices of the OTUs were calculated using normalized sample sizes. The sample size of soil isolates was reduced to 31 and 74 by random deletion for the comparison of diversity measurements to the burrow and cast collections, respectively. For both the isolates and clones, evenness was calculated to describe the distribution of abundance of isolates (59). Shannon diversity, a general diversity measurement, was used to evaluate OTU richness and evenness (67). The distribution of coverage (28) at various evolutionary distances (based on 16S rDNA data [D]) or dissimilarity values (based on phenotypic data [D<sub>i</sub>]) was calculated for the soil, cast, and burrow isolates. Coverage was calculated using Good's formula (28). LIBSHUFF analyses were used to compare libraries from different sources (68).

**Accession numbers.** Isolate sequences were deposited in GenBank with accession numbers AY039379 to AY039509 and AY039806. Clone sequences were deposited with the accession numbers AY037556 to AY037762.

## RESULTS

**Construction of bacterial libraries.** Totals of 100 and 104 bacterial 16S rDNA clones were sequenced from DNA extracted from Horseshoe Bend 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 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, the explanation for the disproportionate amplification of *Firmicutes* sequences was not obvious. 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 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 (52).

**Phylogeny of clone libraries.** Sequences with  $\geq 99\%$  sequence similarity over the region of the 16S rRNA gene used for phylogenetic analyses were combined into OTUs. The soil library contained 86 OTUs (out of 95 clones), which were classified into 11 phyla (or candidate divisions) and 9 clades which have not been formally described (undescribed groups). No single phylum dominated the soil library, but the largest number of clones were from *Acidobacteria* (14%), *Cytophagales* (13%), *Chloroflexi* (8%), and  $\beta$ -*Proteobacteria* (8%). The cast library contained 81 OTUs (out of 102 clones), which were grouped into 10 phyla (or candidate divisions) and 2 undescribed clades. 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  $\gamma$ -*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 using our methods (Table 1) and were classified into undescribed groups A through I. Group A

was the largest and was found only in soil. It possessed an average 87% sequence similarity to a clone obtained from an environment contaminated with pentachlorophenol (4). Because these clones, which possessed 85 to 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 to 89% similarity) with an average sequence similarity of 91% to clones from rhizosphere (48) and arid soil (43). Group C contained three soil clones (88 to 89% similarity) which possessed an average sequence similarity of 86% to hot spring clones (34). Group D contained two clones with an average similarity of 86% to each other and clones from an arid soil (17) 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 (81) and Antarctic ice (29). 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 (27). Groups G, H, and I each consisted of a single soil clone with sequence similarity to *Acidosphaera rubrifaciens* (74%), a subsurface clone (80% [10]), and a deep-sea bacterium (89% [46]), respectively.

While the abundances of many of the taxa in the soil and cast libraries were similar, the abundance of other taxa changed dramatically. For instance, the  $\gamma$ -*Proteobacteria* increased from 4% of the soil library to 22% of the cast library (Table 1). The majority of cast clones in this group were closely related to a number of *Pseudomonas* spp. (Fig. 1). Although numerically less abundant, closely related soil clones were also observed. The *Firmicutes* clones also increased from 4% in the soil to 12% in the casts (Table 1), and most of the clones were phylogenetically related to *Bacillus* spp. and similar genera (Fig. 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 1). While individual clones were associated with a number of different genera within the phylum (Fig. 3), the largest group of nine clones (two soil clones and seven cast clones) were related to the *Rubrobacteria*, which have been previously found in a number of clone libraries from soil (Fig. 4 and references 7, 33, 45, 49, and 61). A relative of *Rubrobacter* was also isolated from burrow soil in this study (see below).

**Library comparisons.** The hypothesis that the soil and cast clone libraries were not significantly different was tested by the LIBSHUFF method (68). 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 (Fig. 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 shuffles showed differences between the libraries only when  $D$  was  $\leq 0.02$ . 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 below). These conclusions were supported by the phylogenetic trees in which cast sequences often grouped near soil sequences but were rarely identical (Fig. 1 to

4). In fact, the average  $D$  ( $\pm$  standard deviation) from a cast clone to its closest relative in the soil libraries was only  $0.09 \pm 0.07$ , compared to a distance of  $0.18 \pm 0.14$  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 (Fig. 5B). This result indicated that some sequences in the soil library had no close relatives in the cast library. Differences between the libraries were especially apparent when  $D$  was between 0.03 and 0.05 and well as when  $D \geq 0.14$  (Fig. 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 1 suggested that the increased abundance of the *Actinobacteria*, *Firmicutes*, and  $\gamma$ -*Proteobacteria* in the cast library as well as the presence of six sequences in the 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 statistically significantly different (see comparison 2 [Table 2]). However, removal of any three of these four groups from the analysis still yielded  $P$  values that were  $< 0.05$  (data not shown). Thus, all four taxa combined were responsible for the differences between the soil and cast bacterial libraries. In contrast, the differences in the abundance of  $\alpha$ -*Proteobacteria* did not appear significant.

**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 libraries contained limited diversity. Therefore, 27 clones from the soil samples and 18 clones from the cast sample were screened by restriction fragment length polymorphism (RFLP) analysis. Five patterns (designated types A to E) were observed, and partial sequencing of representative clones indicated that they constituted a single archaeal lineage which had been detected previously in soil (Fig. 6A and reference 6). The RFLP types were not distributed evenly between the soil and cast clones (Fig. 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 than the soil clones. 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.

**Phylogeny of the isolates.** An initial phylogenetic analysis of the 230 isolates obtained from soil and earthworm casts and burrows assigned the isolates to major bacterial groups (Table 1). Sequences belonging to the *Actinobacteria* were most abundant in the original soil (54%) and burrow (52%) collections, while sequences belonging to the  $\gamma$ -*Proteobacteria* group comprised 77% of the collection of original cast isolates (Table 1). A chi-square analysis of the soil and burrow collections indicated that their phylogenetic distributions were not different ( $P > 0.05$ ). In contrast, the distributions of the cast and soil isolates were significantly different ( $P < 0.05$ ).

TABLE 1. Distribution of clone and isolate sequences in phylogenetic groups

| Phylogenetic group             | No. of clones from each library <sup>a</sup> |    |    |    |    |      |    | % Of total library (clones) |      | % Of total library (isolates) from: |               |              |             |              |
|--------------------------------|----------------------------------------------|----|----|----|----|------|----|-----------------------------|------|-------------------------------------|---------------|--------------|-------------|--------------|
|                                | Agricultural soil                            |    |    |    |    | Cast |    | Pooled clone reactions      |      | Soil (1996)                         | Burrow (1996) | Casts (1997) | Soil (1999) | Casts (1999) |
|                                | 1                                            | 2  | 3  | 4  | 5  | 1    | 2  | Soil                        | Cast |                                     |               |              |             |              |
| <i>Acidobacteria</i>           | 3                                            | 2  | 3  | 4  | 1  | 3    | 7  | 13.7                        | 9.8  |                                     |               |              |             |              |
| <i>Actinobacteria</i>          | 1                                            | 1  |    | 2  | 1  | 10   | 7  | 5.3                         | 16.7 | 53.5                                | 51.6          | 12.2         | 57.1        | 75.0         |
| Candidate division TM7         | 1                                            |    | 1  |    |    | 3    | 3  | 2.1                         | 5.9  |                                     |               |              |             |              |
| <i>Cytophagales</i>            | 3                                            | 2  | 3  | 1  | 1  | 8    | 5  | 10.5                        | 12.7 |                                     |               | 1.4          |             |              |
| <i>Fibrobacter</i>             |                                              |    | 1  | 2  | 1  | 1    |    | 4.2                         | 1.0  |                                     |               |              |             |              |
| <i>Firmicutes</i>              |                                              | 4  |    |    |    | 11   | 1  | 4.2                         | 11.8 | 15.2                                | 19.4          | 4.1          | 21.4        | 16.7         |
| <i>Chloroflexi</i>             | 2                                            | 1  | 3  | 2  |    |      | 2  | 8.4                         | 2.0  |                                     |               |              |             |              |
| <i>Nitrospira</i>              |                                              |    | 2  | 1  | 2  |      | 1  | 5.3                         | 1.0  |                                     |               |              |             |              |
| <i>Planctomycetes</i>          |                                              |    | 1  | 2  |    |      |    | 3.2                         |      |                                     |               |              |             |              |
| <i>Proteobacteria</i>          |                                              |    |    |    |    |      |    |                             |      |                                     |               |              |             |              |
| α-                             | 1                                            | 1  |    |    | 2  | 8    | 2  | 4.2                         | 9.8  | 5.1                                 | 6.5           | 1.4          | 7.1         |              |
| β-                             | 1                                            | 3  | 3  | 1  |    | 1    | 2  | 8.4                         | 2.9  | 5.1                                 | 9.7           | 4.1          | 7.1         | 8.3          |
| γ-                             | 1                                            | 1  | 1  |    | 1  | 15   | 7  | 4.2                         | 21.6 | 21.2                                | 12.9          | 77.0         | 7.1         |              |
| δ-                             |                                              | 2  | 2  | 1  |    | 1    |    | 5.3                         | 1.0  |                                     |               |              |             |              |
| <i>Verrucomicrobia</i>         |                                              | 2  |    |    |    | 1    |    | 2.1                         | 1.0  |                                     |               |              |             |              |
| Undescribed group <sup>b</sup> |                                              |    |    |    |    |      |    |                             |      |                                     |               |              |             |              |
| A                              | 3                                            | 1  |    | 2  |    |      |    | 6.3                         |      |                                     |               |              |             |              |
| B                              |                                              |    |    | 1  |    | 1    | 1  | 1.1                         | 2.0  |                                     |               |              |             |              |
| C                              | 1                                            | 1  |    | 1  |    |      |    | 3.2                         |      |                                     |               |              |             |              |
| D                              |                                              | 1  |    |    |    | 1    |    | 1.1                         | 1.0  |                                     |               |              |             |              |
| E                              |                                              | 1  |    |    | 1  |      |    | 2.1                         |      |                                     |               |              |             |              |
| F                              |                                              | 2  |    |    | 1  |      |    | 2.1                         |      |                                     |               |              |             |              |
| G                              |                                              | 1  |    |    |    |      |    | 1.1                         |      |                                     |               |              |             |              |
| H                              |                                              | 1  |    |    |    |      |    | 1.1                         |      |                                     |               |              |             |              |
| I                              |                                              |    |    | 1  |    |      |    | 1.1                         |      |                                     |               |              |             |              |
| Total sample size ( <i>n</i> ) | 17                                           | 27 | 20 | 21 | 10 | 64   | 38 | 95                          | 102  | 99                                  | 31            | 74           | 14          | 12           |

<sup>a</sup> Each column represents the number of clones from a particular PCR. Each soil PCR was derived from a separate sample, while the cast PCRs originated from the same DNA extracted from pooled cast samples. All clone sequences are from 1999.

<sup>b</sup> Clones which did not associate with a known phylum. Descriptions of each group are provided in the text.

Most of the *Actinobacteria* isolates grouped within the order *Actinomycetales* and the suborders *Corynebacterinaea*, *Streptomycineae*, and *Micrococcineae* (Fig. 3). The soil, burrow, and cast isolates were evenly distributed throughout these divisions. It was not unexpected to find representatives of these groups, since *Actinomycetales* are the bacteria most commonly cultured from soil (55).

In the *γ-Proteobacteria*, the isolates grouped with five families: *Moraxellaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Aeromonadaceae*, and *Enterobacteriaceae* (Fig. 1). Most of the soil and burrow *γ-Proteobacteria* isolates (10 and 3 isolates, respectively) were associated with the family *Pseudomonadaceae*. The remaining *γ-Proteobacteria* soil isolates grouped with *Moraxellaceae* (three isolates), *Xanthomonadaceae* (six isolates), and *Enterobacteriaceae* (two isolates). Most of the *γ-Proteobacteria* cast isolates were associated with *Aeromonadaceae* (21 isolates). The remaining cast isolates were found in families that contained soil isolates, including *Pseudomonadaceae* (16 isolates), *Xanthomonadaceae* (2 isolates), and *Enterobacteriaceae* (18 isolates) groups. The large percentage (28%) of the cast isolates associated with *Aeromonadaceae* was unexpected. *Aeromonas* spp. are typically associated with fish and aquatic habitats. Although they have never been isolated from soil, they have been previously identified as a dominant isolate in the casts of the earthworm *E. foetida* (76) and have been associated with the gut of *Pheretima* sp. (77).

The *Firmicutes* isolates all grouped within the class *Bacilli* (Fig. 2). The majority of the soil, cast, and burrow isolates were associated with *Bacillus* spp. Since *Bacillus* spp. are commonly found in soil, this result was not surprising. Some of the cast and soil isolates grouped with the *Paenibacillus* spp., and some of the soil isolates were associated with the *Brevibacillus* and *Staphylococcus* spp.

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

**LIBSHUFF comparisons of the isolates and clones.** The LIBSHUFF comparisons of the isolates suggested that soil and burrow isolates were derived from similar populations but that the cast isolates were from a population different from that of the soil isolates (comparisons 5 and 6 [Table 2]). Analysis of the coverage curves constructed from the comparison of the soil isolates and cast isolates indicated a significant increase in the heterologous coverage at *D* = 0.13 (data not shown), which appeared to be due to the presence of a large number of

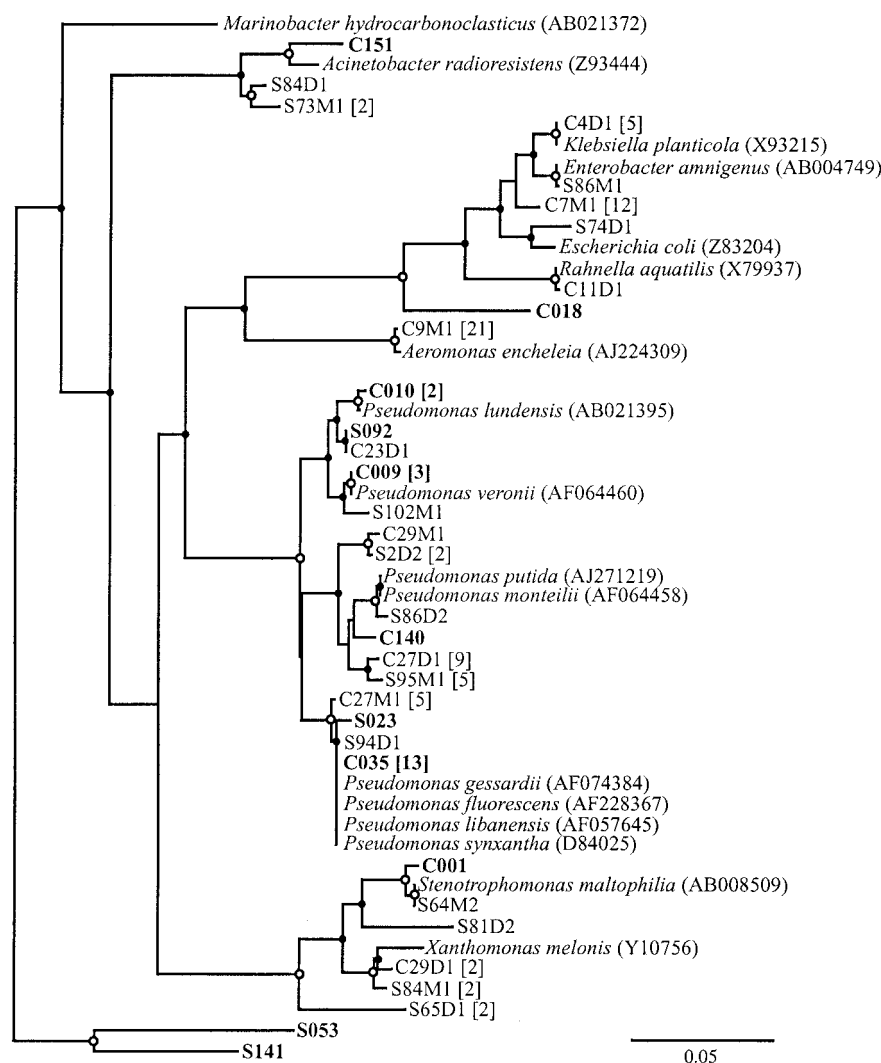


FIG. 1. Phylogenetic tree of the  $\gamma$ -Proteobacteria clones and isolates from Horseshoe Bend. Sequence names are coded. For the clones, the names are in boldface where the letter C or S before the number indicates cast or soil, respectively, e.g., C102. For the isolates from 1996 and 1997, the names include the letter C, S, or B, followed by a unique number; the letter M or D; and the number 1 or 2. The first letter indicates the habitat: cast, soil, or burrow, respectively. The first number is the sample number. The second letter indicates the isolation medium: MMSA or DNB, respectively. The last number indicates whether the isolate was the first or second isolate from the particular sample, e.g., S84D1. For the isolates from 1999, the names include the letters NC or NS for cast or soil isolates, respectively, and a unique number, e.g., NC8. Brackets after sequence names denote the total number of sequences in the OTU when that number was greater than 1. Accession numbers appear in parentheses for all reference sequences. Open circles (○) denote bootstrap support of  $\geq 95\%$ , and closed circles (●) denote bootstrap support of  $\geq 50\%$ . The scale bar represents the Jukes-Cantor evolutionary distance.

*Aeromonas* isolates from the cast but not from soil. The possibility that this taxon alone was responsible for the differences in the isolate libraries was eliminated by performing an additional LIBSHUFF analysis after removing the *Aeromonas* sequences from the data set (comparison 7 [Table 2]). The differences between the cast and soil isolate libraries remained significant, especially at low values of  $D$  (data not shown). More simply stated, it appeared that all the genera found in the cast isolates (except *Aeromonas*) were also found in the soil isolates; however, the species within those genera were different.

LIBSHUFF comparisons of the clone and isolate libraries were significantly different (comparisons 3 and 4 [Table 2]).

Given that the clone libraries contained many more taxa than the isolates, it was not surprising that the isolate library provided poor heterologous coverage of the clone library. However, the clone library might have been expected to provide good heterologous coverage of the isolate libraries. Presumably, this did not occur because of the low representation of the taxa abundant among the isolates in the clone libraries and the fact that the isolates and clone libraries were collected in different years. It is worth noting that previous studies utilizing both molecular and cultivation methods have produced similar findings (5, 10, 17, 50, 58, 69, 73).

**Diversity indices.** Various diversity indices were calculated for all libraries (Table 3). The diversity and evenness indices

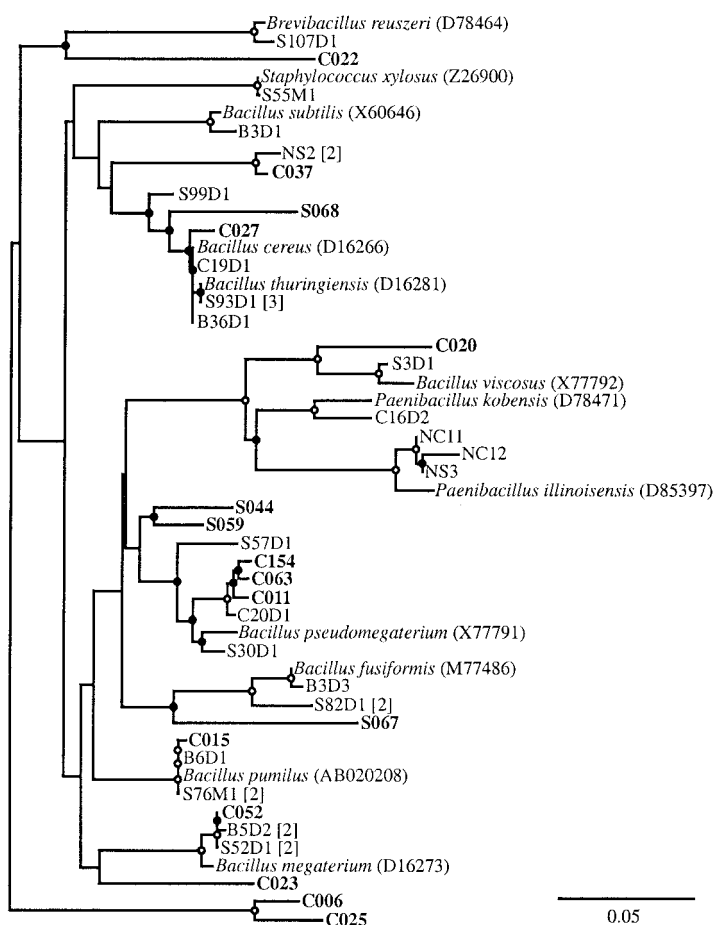


FIG. 2. Phylogenetic tree of the Horseshoe Bend clones and isolates associated with the *Firmicutes* phylum. Notation is as described for Fig. 1.

approached the maximum for both the soil and cast clone libraries, although the values for the soil library were always higher. The higher diversity of the soil clone library was reflected in the phylogenetic distribution. The soil library contained representatives of more phyla and other deep phylogenetic groups than did the cast clones (Table 1). Coverage values were low for both clone libraries, 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's, which was consistent with the somewhat lower diversity in the cast library. Additionally, the large number of nearly identical sequences representing the genus *Pseudomonas* (Fig. 1) was also consistent with the higher overall coverage of the cast library.

Although the diversity was much less, similar trends were also found for the isolate libraries. First, rarefaction was used to compare the OTU richness within each group of isolates at standardized sample sizes (data not shown). The original soil isolate collection contained about twice as many OTUs as the original cast isolate collection at sample sizes greater than 35 and just as many as the burrow collection at all sample sizes. The diversity and evenness indices for the soil isolates were close to the maximum possible values and higher than the values for the cast isolates (Table 3). Similarly, coverage by the

soil isolates was lower. The 80% coverage of the cast isolates indicated that the majority of the cast community culturable with the methods used here was collected. At  $D = 0.05$ , the coverage was  $> 90\%$  (data not shown), indicating that most of the deeper phylogenetic groups obtainable by these methods were also isolated. In contrast, the diversity and evenness indices for the soil and burrow isolates were identical (Table 3). By these criteria, those libraries appeared the same.

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

The phenotypic properties of soil, burrow, and cast isolates collected in 1996 and 1997 were different (Table 4). A two-tailed chi-square analysis identified tests that possessed  $P$  values below 0.05 and might be statistically significantly different between sample sources. These phenotypic tests were further analyzed using scaled deviance to determine if the differences were due to the sample source or sampling procedure. In these analyses, the results of 12 of the 17 phenotypic tests varied

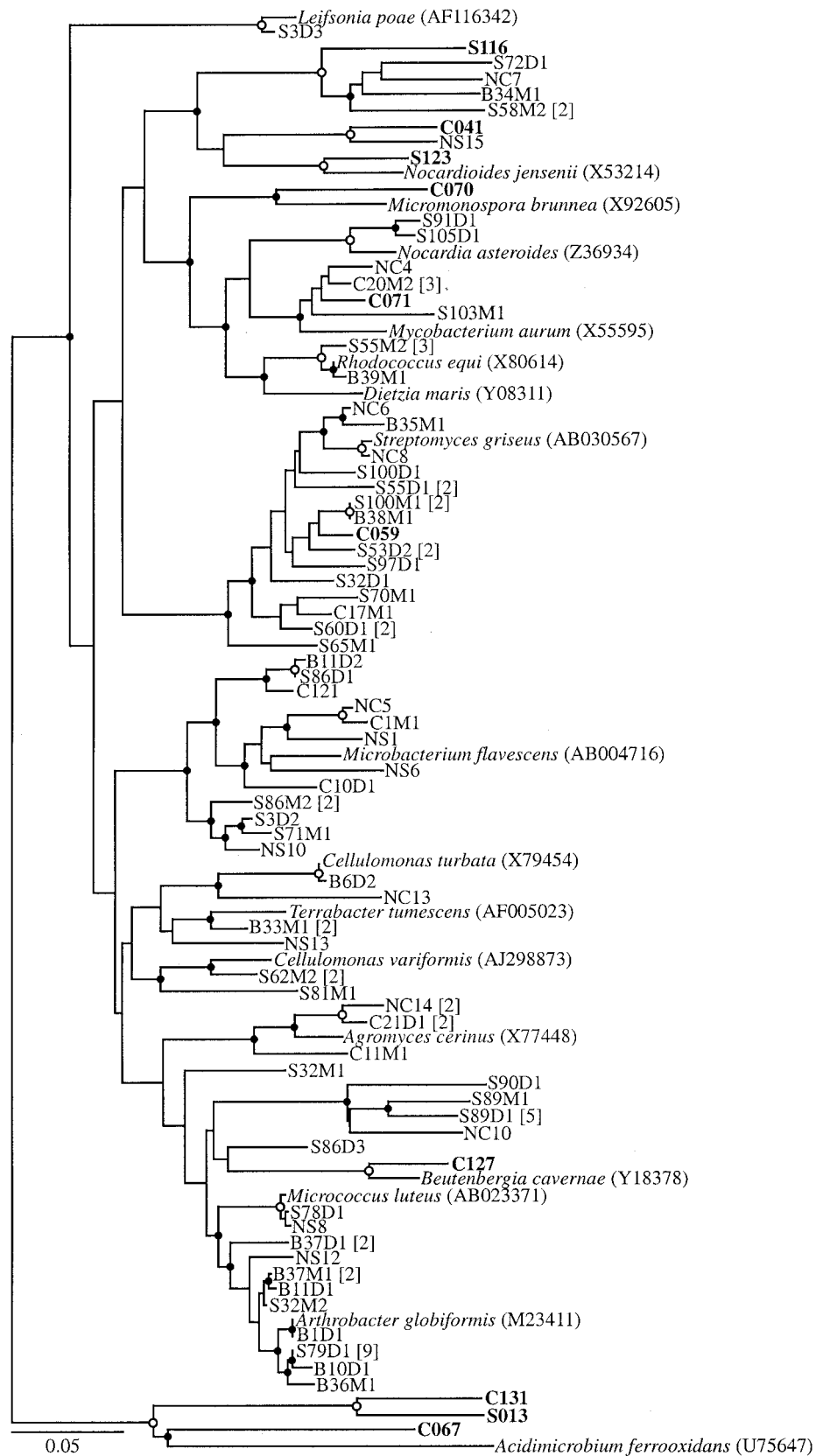


FIG. 3. Phylogenetic tree of the Horseshoe Bend clones and isolates associated with the *Actinobacteria* phylum except *Rubrobacter*. Notation is as described for Fig. 1.

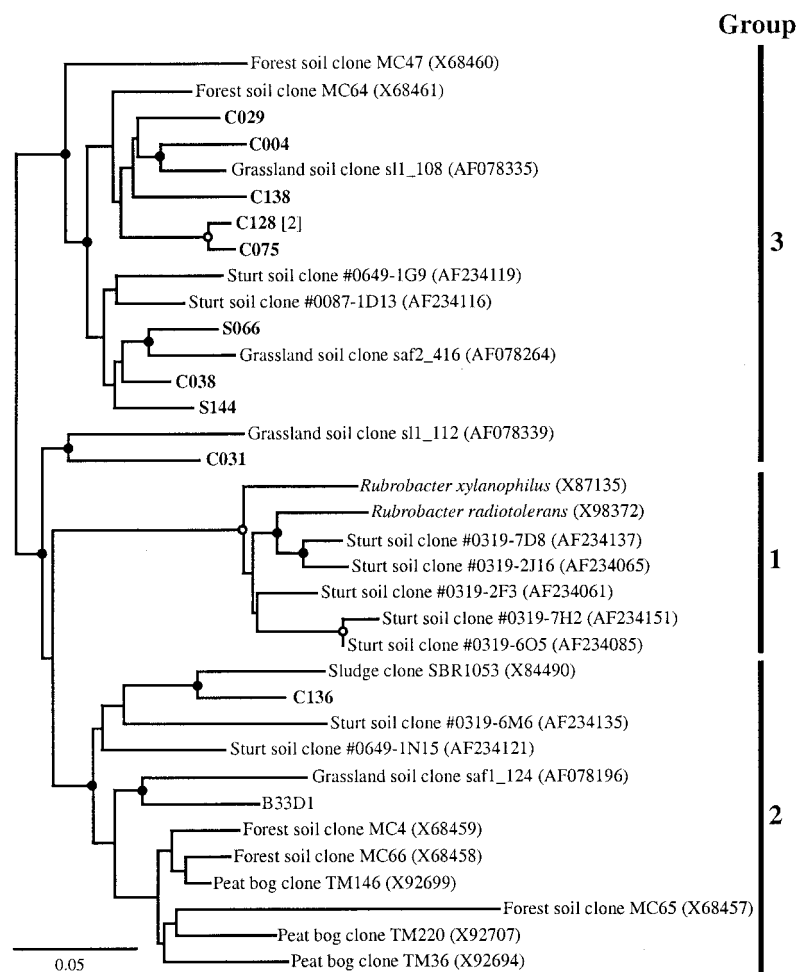


FIG. 4. Phylogenetic tree of the Horseshoe Bend clones and isolates associated with the *Rubrobacteria*. Groups denoted on the right side of the tree follow the nomenclature of Holmes et al. (33). Other notation is as described for Fig. 1.

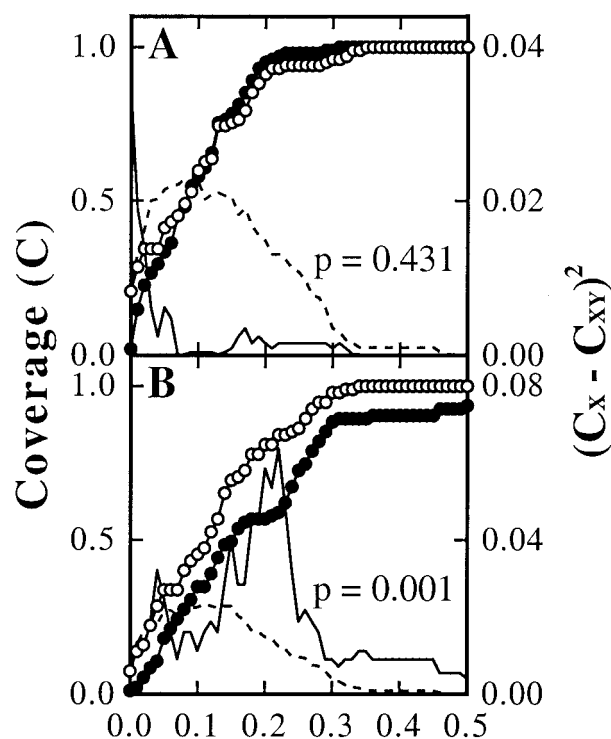
statistically significantly with the sample source. The only tests which did not vary statistically significantly with the sample source were those for hemolysis, ampicillin, and tetracycline resistance; casein hydrolysis; and urea degradation. Pairwise phenotypic differences between cast and burrow isolates, cast and soil isolates, and burrow and soil isolates were also analyzed using a chi-square analysis. Of the tests that varied significantly with the sample source, the cast isolates were significantly different ( $P \leq 0.01$ ) from the soil and burrow isolates in 11 and 12 tests, respectively (data not shown). In contrast, only the cellular morphology and lactose utilization were different ( $P \leq 0.01$ ) between the soil and burrow isolates. Thus, the phenotypic differences between the three groups of isolates were largely due to differences between the cast isolates and the soil and burrow isolates.

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

amino acids and acetate. A trivial explanation for this observation is that the original media used to enrich the isolates lacked sugars and that saccharolytic organisms were selected against. Nevertheless, sugars were utilized by about one-third of the soil and burrow isolates, so this cannot be the full explanation.

Since nitrate and nitrite are more abundant in casts than in surrounding soil (44) and since  $N_2O$  is emitted from the alimentary canal of *L. rubellus* (37), bacteria capable of reducing nitrate were expected to be abundant in casts. A high percentage of the cast isolates, 77%, were capable of reducing nitrate. The samples studied were obtained from no-till soil, which contains lower levels of nitrate (14, 16, 51, 74) and more denitrifiers (16) than does adjacent tilled soil. Likewise, no-tilled soil contains five times more earthworms than tilled soil (32). Therefore, the higher number of denitrifying bacteria in the no-till soil might come from the alimentary canal flora of the earthworms.

The phenotypic properties of the isolates from burrows and soil were very similar. Because urea is excreted through the surface of the earthworm body (18, 44), bacteria that degrade urea were expected to be more abundant in the burrow than



## Evolutionary Distance (D)

FIG. 5. LIBSHUFF comparison of HSB soil 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); (B) comparison of the HSB soil clones (X) to the HSB cast clones (Y).

the soil and casts. However, no differences in the proportion of urea-degrading isolates were found between the sample sources.

**Phenotypic dissimilarity of the isolates.** As a measure of the diversity within each group of isolates,  $D_i$ s were calculated. Isolates with a  $D_i$  of 0 had the same responses on all of the phenotypic tests and were defined as possessing the same phenotype. For the isolates from 1996 and 1997, the 99 soil isolates possessed 90 different phenotypes, the 31 burrow isolates possessed 30 phenotypes, and the 74 cast isolates possessed 56 phenotypes. This corresponded to coverages of 14.4, 6.5, and 40.5% for soil, burrow, and cast phenotypes, respectively, and indicated that the sources contained about 630, 460, and 140 phenotypes of bacteria culturable by our methods, respectively. Even though not all of the culturable phenotypes were represented within the collection of isolates, the coverage of culturable phenons with a  $D_i$  of  $\leq 3$  was nearly 100% by both the soil and cast isolates (data not shown). Thus, the collections of isolates provided a good representation of the phenotypic diversity of the organisms culturable by our sampling protocols at the time of isolation.

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

Within each group of isolates, those from the casts were phenotypically more similar to each other than were the isolates from the soil or burrows. For instance, the mean  $D_i$  ( $\pm$  standard deviation) for the cast isolates was  $5.2 \pm 2.4$ . In contrast, the comparable values for the soil and burrow isolates were  $7.2 \pm 2.6$  and  $6.9 \pm 2.5$ , respectively. When the same analysis was performed at standard sample sizes ( $n = 74$  and  $29$  for the soil and cast, respectively) the mean  $D_i \pm$  standard deviation did not change greatly. The lower mean  $D_i$  for the cast isolates suggested that their phenotypes were more similar to each other than were the burrow or soil phenotypes.

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

**Correlation of phenotypic and rDNA relatedness.** The analysis of both of the 16S rRNA genes and the phenotypic data showed that the burrow and soil isolates were more diverse than the cast isolates. However, the phenotypic data or  $D_i$  was only weakly correlated with the rRNA evolutionary distance or  $D$ . The correlation coefficient  $r$  was only 0.49, which was statistically significant only because of the large number of comparisons ( $n = 2,775$ ). Thus, the phenotypic dissimilarity ( $D_i$ ) was a poor predictor of phylogenetic relationships ( $D$ ). This conclusion was further supported by the observation that the distribution of  $D_i$  for the cast and soil isolates was very close to that expected if the test results were assigned to the isolates according to a random model (data not shown). For instance, the means and standard deviations of  $D_i$  for the casts and soil isolates in a random model would be  $5.3 \pm 1.9$  and  $7.3 \pm 2.0$ , respectively, or very close to the observed values. This result suggests that the phenotypic characters were not linked, as might be expected if the  $D_i$  comparisons contained significant phylogenetic information.

**Community stability.** Although the cast and soil isolates were both collected in the spring, the soil isolates were taken 1 year prior to the cast isolates. Therefore, it was possible that the observed differences between the soil and cast isolates were due to sampling time. To address this concern, 14 soil and 12 cast isolates were collected on the same day in May 1999. In this experiment, the isolation was performed on MMSA, and only a single colony type was chosen from each enrichment. However, all other aspects of the sampling protocol were identical to that used for the original isolates. These isolates were characterized by 16S rRNA gene sequencing (Table 1) and by the phenotypic tests used previously (Table 4).

Because the mean  $D_i$  and chi-square analyses were sensitive to sample size (data not shown), the new collections were compared to the original isolates by the LDi mean method.

TABLE 2. LIBSHUFF comparisons of isolate and clone libraries

| Comparison no. | Homologous (X) coverage data     |           |          | Heterologous (Y) coverage data   |          |
|----------------|----------------------------------|-----------|----------|----------------------------------|----------|
|                | Library                          | Sample yr | <i>n</i> | Library                          | <i>P</i> |
| 1              | Soil clones                      | 1999      | 95       | Cast clones                      | 0.001    |
|                | Cast clones                      | 1999      | 102      | Soil clones                      | 0.431    |
| 2              | Soil clone subset <sup>a</sup>   | 1999      | 76       | Cast clone subset <sup>a</sup>   | 0.144    |
|                | Cast clone subset <sup>a</sup>   | 1999      | 51       | Soil clone subset <sup>a</sup>   | 0.421    |
| 3              | Soil clones                      | 1999      | 95       | Soil isolates                    | 0.001    |
|                | Soil isolates                    | 1996      | 99       | Soil clones                      | 0.001    |
| 4              | Cast clones                      | 1999      | 102      | Cast isolates                    | 0.042    |
|                | Cast isolates                    | 1997      | 74       | Cast clones                      | 0.001    |
| 5              | Soil isolates                    | 1996      | 99       | Burrow isolates                  | 0.192    |
|                | Burrow isolates                  | 1996      | 31       | Soil isolates                    | 0.551    |
| 6              | Soil isolates                    | 1996      | 99       | Cast isolates                    | 0.001    |
|                | Cast isolates                    | 1997      | 74       | Soil isolates                    | 0.001    |
| 7              | Soil isolates                    | 1996      | 99       | Cast isolate subset <sup>b</sup> | 0.001    |
|                | Cast isolate subset <sup>b</sup> | 1997      | 53       | Soil isolates                    | 0.005    |

<sup>a</sup> Does not contain clones within *Actinobacteria*, *Firmicutes*,  $\gamma$ -*Proteobacteria*, and undescribed group A.

<sup>b</sup> Does not contain isolates belonging to the *Aeromonas* lineage.

First, the expected LDi mean was calculated from the data on the original isolates collected in 1996 and 1997. If the bacterial populations were the same in 1999, then the LDi mean of the new isolates should be within the range of the expected values. The LDi mean ( $\pm$  standard deviation) for the new soil isolates was  $2.3 \pm 0.4$ . This value was close to the expected LDi mean ( $\pm$  standard deviation of the mean) of  $2.1 \pm 0.3$  and indicated that the phenotypes of the culturable soil bacteria had not

changed. This conclusion was unchanged if the analysis was performed with only the original isolates from MMSA. In contrast, the expected LDi mean for the cast isolates was  $1.4 \pm 0.3$ , which was much less than  $3.3 \pm 0.4$ , the value observed. Therefore, the phenotypes of the culturable cast bacteria had changed. When compared to the original soil isolates, the LDi mean for the new cast isolates was  $2.8 \pm 0.4$ , indicating that the cast isolates were also different from the soil isolates of both

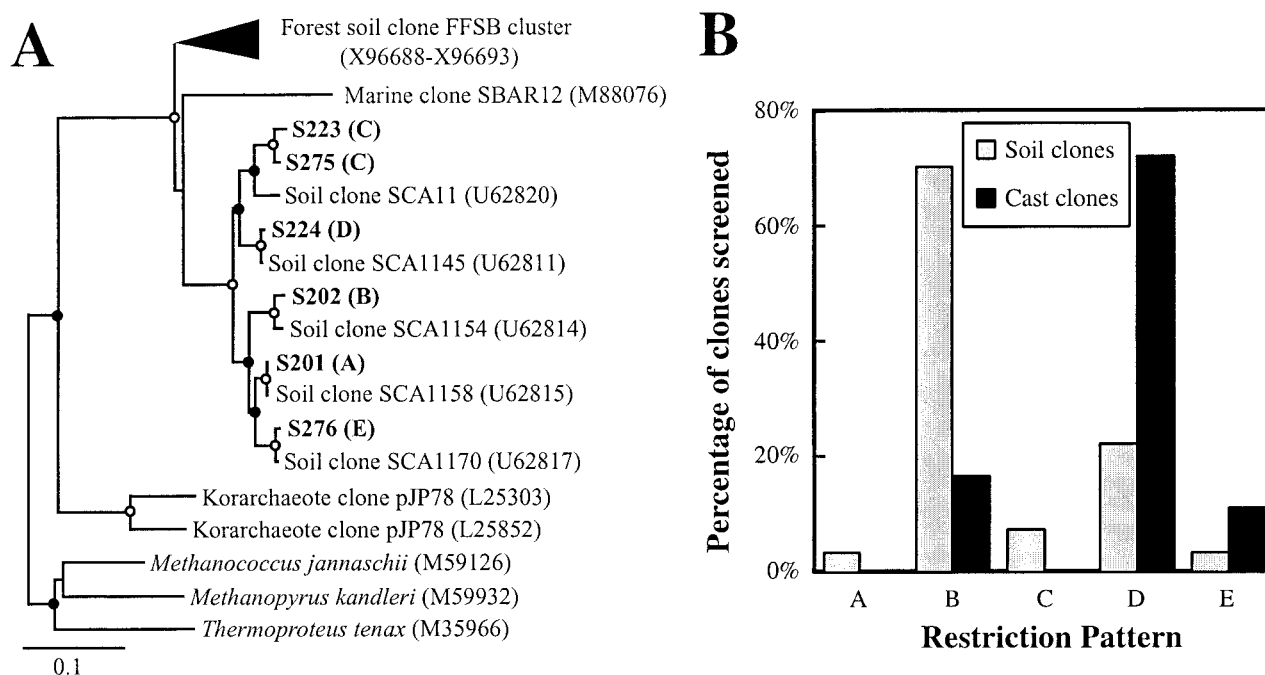


FIG. 6. (A) Phylogenetic tree of the soil archaeal clones from Horseshoe Bend. RFLP patterns are in parentheses after clone names. Other notation is as described for Fig. 1. (B) Distribution of RFLP patterns of archaeal clones in soil and cast clone libraries.

TABLE 3. Diversity indices for clone and isolate libraries based on 16S rDNA sequences<sup>a</sup>

| Type of data   | Clone library data |                          |                      | Isolate library data     |             |                      |                          |               |                      |
|----------------|--------------------|--------------------------|----------------------|--------------------------|-------------|----------------------|--------------------------|---------------|----------------------|
|                | Soil (1999)        | Cast <sup>b</sup> (1999) | Maximum <sup>c</sup> | Soil <sup>b</sup> (1996) | Cast (1997) | Maximum <sup>c</sup> | Soil <sup>b</sup> (1996) | Burrow (1996) | Maximum <sup>c</sup> |
| Shannon-Weaver | 4.40               | 4.11                     | 4.61                 | 3.65                     | 2.47        | 4.30                 | 3.21                     | 3.21          | 3.43                 |
| Evenness       | 2.27               | 2.19                     | 2.33                 | 2.18                     | 1.81        | 2.30                 | 2.27                     | 2.27          | 2.30                 |
| Coverage       | 0.14               | 0.20                     | 1.00                 | 0.57                     | 0.80        | 1.00                 | 0.32                     | 0.32          | 1.00                 |

<sup>a</sup> All calculations are based on an OTU of  $\geq 99\%$  sequence similarity over positions 81 to 459. The sample sizes for the clone library, soil-cast isolate, and soil-burrow isolate comparisons were 95, 74, and 31, respectively.

<sup>b</sup> The size of these libraries was randomly reduced to allow direct comparison to a smaller library. Values shown for the cast clone library are an average of 10 random reductions.

<sup>c</sup> Maximum value for each index. The minimum values for each index were 0.

1996 and 1999. Therefore, while the phenotypes of the population of culturable bacteria from soil appeared to be stable, those of the casts changed over the period examined. This conclusion was also supported by the distribution of the ribotypes in two sets of samples. For the soil isolates, the distribution of ribotypes was essentially the same (Table 1). In contrast, the ribotypes of the cast isolates varied greatly. In the 1997 sample, 72% of the isolates were *γ-Proteobacteria*. In 1999, this phylum was not found and 57% of the isolates were *Actinobacteria*. A change in crop may have caused the change in phenotype of the cast population. In 1997, maize (*Z. mays* L.) was planted during the growth season, and corn litter was observed in the sample site. In 1998, kenaf (*H. cannabinus*) was planted and that litter was observed at the sampling site in 1999.

## DISCUSSION

The comparisons of the cast and soil libraries are consistent with the model that most of the prokaryotic community in casts is derived from soil with the amplification of only a few prokaryotic groups. *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 (15). 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 prokaryotes 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. 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. This interpretation is consistent with lower diversity indices for the clone library and the isolate collection from the casts than from soil. The lower phenotypic diversity observed in the cast isolates is also consistent with the lower genotypic diversity.

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 dramatic difference between the cast and soil libraries. This phenomenon has been observed previously in *L. terrestris*, in which members of the *γ-Proteobacteria* increased nearly 20-fold between the foregut and the cast (23). Of the *γ-Proteobacteria* 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, 1-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 (57) 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 (53), which could be important given that the alimentary canal of the earthworm contains higher levels of moisture, organic carbon, and total nitrogen than does soil (12, 38). Additionally, many pseudomonads are also resistant to antibiotics produced by *Actinobacteria* (53), 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.

Members of the *Actinobacteria* and *Firmicutes* phyla also appeared in greater numbers in the cast library than in the soil library. *Actinobacteria* have been commonly isolated from earthworms, and the genera *Streptomyces* and *Micromonospora* have been associated with *L. rubellus* (11, 41, 54). While a number of isolates in this study associated with the *Streptomyces* genus, only two cast clones were closely related to either of these genera. While no single organism dominated the *Actinobacteria* cast clones, the *Rubrobacteria* were the most represented group. 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

TABLE 4. Phenotypic characterization of bulk soil, burrow, and cast isolates

| Test or characteristic    | % Occurrence in isolates (yr) (n) |                  |                    |                  |                  | chi-square $P^a$   | Scaled deviance <sup>a</sup> |
|---------------------------|-----------------------------------|------------------|--------------------|------------------|------------------|--------------------|------------------------------|
|                           | Soil (1996) (99)                  | Soil (1999) (14) | Burrow (1996) (31) | Cast (1997) (74) | Cast (1999) (12) |                    |                              |
| Cell morphology           |                                   |                  |                    |                  |                  | 0.024 <sup>b</sup> |                              |
| Cocci                     | 9                                 | 36               | 0                  | 3                | 25               |                    | 16                           |
| Small rod                 | 13                                | 14               | 36                 | 11               | 8                |                    | 21                           |
| Rod                       | 46                                | 36               | 36                 | 58               | 25               |                    | 20                           |
| Large rod                 | 33                                | 14               | 29                 | 28               | 42               |                    | 38                           |
| Catalase                  |                                   |                  |                    |                  |                  | 0.024              | 29                           |
| Production                | 90                                | 79               | 94                 | 77               | 83               |                    |                              |
| Hemolysis                 |                                   |                  |                    |                  |                  | 0.480 <sup>b</sup> |                              |
| Alpha-                    | 30                                | 14               | 16                 | 23               | 8                |                    |                              |
| Beta-                     | 7                                 | 7                | 10                 | 12               | 8                |                    |                              |
| Hydrolysis                |                                   |                  |                    |                  |                  |                    |                              |
| Casein                    | 22                                | 43               | 32                 | 32               | 17               | 0.286              |                              |
| Urea                      | 44                                | 21               | 58                 | 27               | 25               | 0.007              | 39 <sup>c</sup>              |
| Tween 80                  | 18                                | 29               | 13                 | 36               | 50               | 0.006              | 14                           |
| Antibiotic resistance     |                                   |                  |                    |                  |                  |                    |                              |
| Ampicillin                | 32                                | 14               | 29                 | 49               | 42               | 0.043              | 35 <sup>c</sup>              |
| Tetracycline              | 22                                | 0                | 0                  | 0                | 0                | 0.339              |                              |
| Polymyxin                 | 29                                | 7                | 23                 | 7                | 25               | 0.002              | 27                           |
| Streptomycin              | 9                                 | 14               | 13                 | 0                | 25               | 0.014              | 32                           |
| Carbon utilization        |                                   |                  |                    |                  |                  |                    |                              |
| Acetate                   | 45                                | 29               | 39                 | 73               | 42               | 0.001              | 22                           |
| Lactose                   | 24                                | 7                | 52                 | 0                | 17               | 0.001              | 17                           |
| Casamino Acids            | 57                                | 36               | 65                 | 84               | 42               | 0.001              | 29                           |
| Mannose                   | 35                                | 21               | 42                 | 7                | 25               | 0.001              | 27                           |
| Cellobiose                | 23                                | 7                | 26                 | 1                | 17               | 0.001              | 25                           |
| MacConkey                 |                                   |                  |                    |                  |                  | 0.001 <sup>b</sup> |                              |
| Growth                    | 19                                | 7                | 23                 | 91               | 25               |                    | 46                           |
| Fermentation <sup>d</sup> | 10                                | 7                | 0                  | 18               | 17               |                    | 23                           |
| Nitrate reduction         |                                   |                  |                    |                  |                  | 0.001 <sup>b</sup> |                              |
| Partial                   | 28                                | 36               | 26                 | 73               | 42               |                    | 37                           |
| Complete                  | 0                                 | 0                | 0                  | 4                | 0                |                    | 5                            |

<sup>a</sup> The null hypothesis was as follows:  $\mu_{\text{bulk soil (1996)}} = \mu_{\text{burrow (1996)}} = \mu_{\text{cast (1997)}}$ . When the chi-square analysis was statistically significant ( $P < 0.05$ ), the genmod scaled deviance analysis was performed to confirm that the difference was not due to the sampling procedure.

<sup>b</sup> These tests had more than one response. For the chi-square analysis, the responses were grouped together to observe overall differences in the test for the three types of isolates. For the genmod scaled deviance analysis, each response was observed separately.

<sup>c</sup> These  $P$  values for these scaled deviances were not statistically significant at  $P = 0.05$ . The  $P$  values for all of the other scaled deviances were statistically significant at  $P < 0.05$ .

<sup>d</sup> Fermentation of lactose.

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, which are expected to be sensitive to antibiotics, in the cast library may have resulted from endospore germination but not growth. When Fisher et al. (24) 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 *E. 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 (60). 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 (6, 9, 36, 39, 62, 80). The archaeal sequences recovered in this study were similar to sequences found in other agricultural soils (6, 80). The sequenced representative of the dominant soil library RFLP type (B) was a close relative of archaeal clones SCA1154 and FIE16 from other soils (6, 80). 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 in 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 (6) 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 worthwhile to know if this group was associated with earthworms at other sites as well.

Because most of the isolates were obtained in different years from those in which the clone libraries were developed, it is not possible to make direct comparisons between the two. However, it is somewhat remarkable that the isolates were mostly representatives of the  $\gamma$ -*Proteobacteria*, *Actinobacteria*, and *Firmicutes* or the same taxa that were amplified in the bacterial cast libraries. Presumably, some phyla of soil prokaryotes are inherently more readily culturable on standard heterotrophic media, and this property correlates with the ability to respond to passage through the earthworm gut. However, the differences in phenotypes for the cast and soil isolates support the hypothesis that the taxa amplified in the cast are not identical to the culturable soil prokaryotes, even though they may be closely related. For instance, the phenotypes of the soil and cast isolates from 1999 were different, even though they represented the same phylogenetic groups.

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