Microbial Ecology

Spatial Stratification of Soil Bacterial Populations in Aggregates of Diverse Soils

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Abstract

Most soil microbial community studies to date have focused on homogenized bulk soil samples. However, it is likely that many important microbial processes occur in spatially segregated microenvironments in the soil leading to a microscale biogeography. This study attempts to localize specific microbial populations to different fractions or compartments within the soil matrix. Microbial populations associated with macroaggregates and inner- versus total-microaggregates of three diverse soils were characterized using culture-independent, molecular methods. Despite their relative paucity in most surveys of soil diversity, representatives of *Gemmatimonadetes* and *Actinobacteria* subdivision *Rubrobacteridae* were found to be highly abundant in inner-microaggregates of most soils analyzed. By contrast, clones affiliated with *Acidobacteria* were found to be relatively enriched in libraries derived from macroaggregate fractions of nearly all soils, but poorly represented in inner-microaggregate fractions. Based upon analysis of 16S rRNA, active community members within microaggregates of a Georgian Ultisol were comprised largely of *Gemmatimonadetes* and *Rubrobacteridae*, while within microaggregates of a Nebraska Mollisol, *Rubrobacteridae* and *Alphaproteobacteria* were the predominant active bacterial lineages. This work suggests that microaggregates represent a unique microenvironment that selects for specific microbial lineages across disparate soils.

Introduction

Understanding soil microbial diversity and the environmental processes it controls requires an in-depth understanding of how microbial diversity influences, and is influenced by, the environment. Such an understanding can only be attained by analysis at scales relevant to those at which processes influencing microbial diversity actually operate [29]. However, because of the biotic and abiotic complexity exhibited by most soils at nearly all scales, determining soil microbial diversity patterns remains a formidable challenge for soil microbiologists [54].

The vast majority of soil microbial analyses are conducted on bulk soil samples, and often composited bulk soil samples, which averages localized heterogeneity and provides little insight into the spatial origins of detected strains. Moreover, as several studies indicate that molecular approaches tend to be biased toward the more abundant organisms at the time of sampling [5, 20, 41], stratified sampling strategies, which restrict sample area to definable habitats, may increase numbers of detectable organisms in a given habitat [14], and increase the power of detecting differences by decreasing variability within a given sample [25]. Such approaches should also provide insights into the controlling factors and functions in different segments of the soil microbial community. Increased understanding of relationships between microbial community composition and soil microhabitats may facilitate the integration of biotic data with the copious soil physical and chemical information available in the literature, potentially providing for better understanding of soil functioning.

Soil consists of a complex physical framework that largely determines solute, substrate, and energy flows. Therefore, soil provides a heterogeneous habitat for microorganisms characterized by different substrate, nutrient, water, and oxygen concentrations, as well as pH and the size of pores available for microbial habitation [13, 17, 18, 42]. These structural aggregates are classified hierarchically in terms of size and relative stability [40, 55]. Macroaggregates (diameter > 250 μm) are formed

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by temporary associations of microaggregates, minerals, and particulate organic matter, predominantly through enmeshment by fungal hyphae and plant roots [39]. Water-stable microaggregates (diameter < 250 μm), generally the most stable soil secondary structures, typically form within macroaggregates through microbially mediated processes [39, 51] and are largely dependent upon persistent organic binding agents for structural stability [55]. Because soil structure is a dominant factor controlling microbial diversity and processes [59], analysis at the aggregate and subaggregate levels will be required to elucidate the principle biotic and abiotic controls on soil microbial diversity.

A number of studies have reported contrasting distributions of microbial biomass, specific species or populations, and functional attributes in different aggregate size classes [4, 6, 15, 28, 56]. A number of authors have suggested partitioning soil microorganisms into inner- and outer-aggregate fractions based on soil microsite physicochemical differences and presumed microbial functional differences [3, 16, 17], and a number of microscopy, molecular, and cultivation-based studies have explored microbial community structure in inner and outer macroaggregates [7, 17, 18, 37, 44, 45, 57]. However, despite the fundamental importance of microaggregates to soil structure and function, including the sequestration of organic matter [22, 52], microbial community structure within this size class has received very little study, particularly with regard to the outer- and inner-aggregate distribution of microbial populations.

Although habitats existing within microaggregates offer relatively stable water availability and protection against predation, they are typically described as oligotrophic environments, containing limited amounts of biologically recalcitrant organic matter having much slower turnover rates than are observed for soil as a whole [53]. Inner-microaggregate organisms, principally bacteria and archaea, would be expected, due to pore size constraints, to largely escape predation by soil fauna [57, 58] and perhaps exposure to fungal antibiotics. Due to pore size and diffusion constraints, inner-microaggregate organisms might also be expected to be of smaller size and have slower turnover rates than their outer-aggregate or macropore counterparts [24].

A previous study [36] provided evidence for spatial stratification of bacterial division- and subdivision-level lineages within soil microaggregates of two different soils, an Aridisol and a mine spoil (Entisol). The present study expands on that work to examine whether similar spatial stratification is observable across a broad range of geographically separated soil types, differing greatly in climate and soil forming factor influences. To accomplish this, a suite of three additional disparate soils were examined to: (1) directly compare results from microaggregates to the macroaggregate fraction of soil; (2) compare whole and inner microaggregate communities across the five soils; and (3) determine relationships between organismal presence and activity for inner-microaggregate communities based on phylogenetic analysis of both 16S rRNA (rRNA) and 16S rRNA genes (rDNA).

Materials and Methods

Sites and Soils

Georgia. Soil samples were collected from no-tillage agricultural plots at the Horseshoe Bend Long Term Environmental Research site (HSB) near Athens, GA (33°54′N, 83°24′W). The site has been under continuous summer grain crops (*Sorghum bicolor* or *Glycine max*) and winter crops (*Secale cerale* or *Trifolium incarnatum*) since 1978. Soil at the site is a fine loamy, siliceous, thermic Rhodic Kanhapludult. Annual precipitation (125 cm) is relatively evenly distributed throughout the year. Management history and site characteristics are described in detail by Hendrix [19].

Nebraska. This site is located at on the High Plains Agricultural Laboratory (HPAL), 8.3 km north of Sidney, NE (41°14′N, 103°00′W). Soil samples were collected from long-term, no-tillage plots under a wheat–fallow cropping system. Soil at the site is classified as a fine silty, mixed mesic Pachic Haplustoll. Average annual precipitation (38 cm) predominantly (75%) occurs between April and August. Management history and site characteristics are described by Lyon *et al.* [30].

Montana. Soil samples were collected from a gently sloping undisturbed grassland site (MGS) 10 km north of Missoula, MT (46°45′00″N, 114°07′30″W). The site has a semiarid climate, receiving on average 34–42 cm precipitation per year. Soil at the site is classified as a cobbly loam Argixeroll.

Soil samples from all sites were collected to a depth of 10 cm and placed on ice in sterile plastic bags in the field. All samples were frozen within 6 h of collection and stored at −20°C prior to fractionation and nucleic acid isolation.

Soil Fractionation. Field-moist soils were immersed in deionized H_2O on top of three-tiered, nested sieves of progressively finer mesh (2000, 250, and 53 μm). After an initial hydration period (5 min), large macroaggregates were gently pressed through the 2000-μm sieve while gently raising and lowering the sieves. The 2000-μm sieve was then removed, and the two remaining nested sieves slowly raised and lowered (3 cm) to separate stable microaggregates from macroaggregates (250–2000 μm) and silt and clay particles.

High-energy UV radiation was used to isolate the inner-microaggregate fraction. This procedure is based

on the ability of high-energy UV light to oxidize organic compounds. Because UV light does not penetrate soil mineral components to a great extent [49], organic matter, including microbes and their nucleic acids, within microaggregates are largely protected. Our photooxidation reactor system and procedure are as described in Mummey and Stahl [36], except that reactions were conducted at 4°C to minimize microbial growth and nucleic acid synthesis or degradation within aggregates during treatment. Therefore, between the time each sample was thawed and nucleic acids were extracted, samples were exposed to a maximum 26 h at 4°C.

After wet sieving and/or UV photooxidation, microaggregates were centrifuged (14,000 × *g*, 30 s) and the supernatant was removed with a sterile pipette prior to nucleic acid isolation.

Isolation of Nucleic Acids. To obtain both rRNA and genomic DNA for use as PCR templates, direct nucleic acid extraction from Nebraska and Georgia soil fractions was performed using a modification of the bead-beating method of Griffiths *et al.* [12]. To 2-mL microcentrifuge tubes containing 0.5 g glass beads (1 mm; Biospec Products, Bartleville, OK, USA), 0.5 g soil, 0.5 mL CTAB buffer (10% (w/v) CTAB in 0.7 M NaCl with 240 mM potassium phosphate buffer, pH 8.0), and 0.5 mL phenol–chloroform–isoamyl alcohol (25:24:1) were added. Samples were then homogenized on a Mini Beadbeater 8 (Biospec Products) for 2 min, placed on ice for 2 min, then homogenized for an additional 2 min as before. Separation of the solid phase was accomplished by centrifugation (16,000 × *g*) for 5 min at 4°C. The aqueous layer was removed and an equal volume chloroform–isoamyl alcohol (24:1) added and mixed by vortexing prior to a second round of centrifugation as before. The aqueous layer was removed and nucleic acids were precipitated with 0.2 volume 10 M sodium acetate (pH 5.2) and 0.6 volume isopropanol for 2 h at room temperature, followed by centrifugation (16,000 × *g*) for 10 min at 4°C. Nucleic acid pellets were then washed with 70% EtOH, air-dried, and resuspended in 50 μL TE buffer (pH 8). For RNA analyses, all solutions were treated with diethyl pyrocarbonate (Sigma, St. Louis, MO, USA) and all solid materials not certified RNase-free were baked overnight at 350°C.

Community genomic DNA extraction from Montana soil fractions was conducted using the UltraClean soil DNA kit (MoBio, Solano Beach, CA, USA). The manufacturer's recommended protocol was modified by incorporating a 4-min bead-beating homogenization and lysis step as described above.

PCR, Cloning, and Sequence Analysis. Prior to reverse transcription of rRNA from Georgia and Nebraska nucleic acid extracts, DNA was removed by digestion with RQ1 RNase-free DNase (Promega, Madison, WI, USA). An RNA–DNA extract from each sample (7 μL) was placed in an RNase-free microcentrifuge tube along with 2 μL RQ1 RNase-free DNase and 1 μL 10× reaction buffer and subsequently incubated at 37°C for 1.5 h with gentle mixing every 0.5 h. The efficacy of DNase treat- ment was determined by direct PCR (with no reverse transcription step) using methods described below. DNase-treated samples yielding no PCR amplification products were considered free of DNA contamination.

Reverse transcription of rRNA templates was conducted using SuperScript II H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and the manufacturer's recommended protocol.

PCR amplification of genomic DNA templates from the Georgia, Montana, and Nebraska sites and cDNA derived from Georgia and Nebraska soil fractions utilized the conserved general 16S rDNA primers 536f and 907r. PCR mixtures (50 μL) contained 1× PCR buffer, 20 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, and 2 U *Taq* polymerase (Roche, Indianapolis, IN, USA). PCR amplification began with a denaturing step of 95°C for 5 min, followed by 32 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min. This was followed by a final extension at 72°C for 3 min.

PCR products generated from rDNA or rRNA templates derived from Georgia, Nebraska, and Montana soil fractions were cloned into the pT7Blue-3 plasmid using the Perfectly Blunt cloning kit (Novagen, Madison, WI, USA). Clones were randomly selected for analysis. Plasmids were purified from overnight cultures using Qiagen miniprep kits (Qiagen, Valencia, CA, USA) according the manufacturer's specifications. The insert size of individual clones was confirmed by restriction fragment analysis employing the restriction enzyme *Eco*RI. Randomly selected clones having inserts of correct size were used as templates in DNA sequencing reactions using vector-specific primers T7 and U19 (Novagen) and subsequently sequenced on a Prism 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analyses. The partial rRNA gene sequences obtained were screened for the presence of chimeric artifacts based on secondary structure anomalies and using the CHIMERA_CHECK program (version 2.7) of the Ribosomal Database Project (RDP) [32]. Cloned sequences generated in this study were aligned with 16S rDNA reference sequences using the ARB database and software [31] and manually refined. Hypervariable regions of 16S rDNA alignments were excluded from similarity calculations using the bacterial mask of Lane [27].

Two methods were employed to generate tree topologies using the software package PAUP* [D.L. Swofford, Phylogenetic Analysis Using Parsimony (* and other methods), Version 4 (Sinauer Associates, Sunder-

land, MA, USA, 1998). They include an evolutionary distance (ED) method (neighbor joining with Kimura two-parameter correction with empirically determined base frequencies and empirically determined gamma distribution models of site-to-site rate variation) and maximum parsimony (MP) (default settings, heuristic search). The robustness of tree topologies was determined by bootstrap resampling (1000 repetitions).

Sequences derived from rDNA from macroaggregates, and whole and inner microaggregates are hereafter designated M_{rDNA}, Wm_{rDNA}, and Im_{rDNA}, respectively. The rRNA sequences derived from whole- and inner-microaggregate fractions of Nebraska or Georgia soils are hereafter designated Wm_{rRNA} and Im_{rRNA}, respectively. Between 18 and 21 sequences were analyzed for each clone library. The 230 sequences examined in this study were deposited in Genbank under accession numbers DQ412734 to DQ412943.

Results

Alphaproteobacteria were found to be more abundant in microaggregate fractions relative to macroaggregates in both Georgia and Montana soils (Fig. 1). Inner microaggregates yielded greater numbers of *Alphaproteobacteria*-affiliated clones than the other soil fractions in Georgia, Nebraska, and Montana soils. As was the case for Wyoming reclamation soils in our previous study [36], *Actinobacteria* representatives were relatively enriched in the microaggregate fractions of Montana and Georgia soils. Furthermore, similar to what was found in the Wyoming reclamation soils where *Rubrobacteridae* represented 47% of sequenced clones derived from inner microaggregates, the data indicate increased *Rubrobacteridae* representation in inner microaggregates of Montana and Georgia soils. For example, in Montana soils, no *Rubrobacteridae* clones were obtained from the M_{rDNA} library, whereas 19% and 22% of clones sequenced from Wm_{rDNA} and Im_{rDNA} libraries were affiliated with the *Rubrobacteridae*, respectively. Similarly, within Georgia M_{rDNA}, Wm_{rDNA}, and Im_{rDNA} clone libraries, 5%, 18%, and 26% of sequenced clones were determined to be affiliated with *Rubrobacteridae*, respectively (Fig. 1).

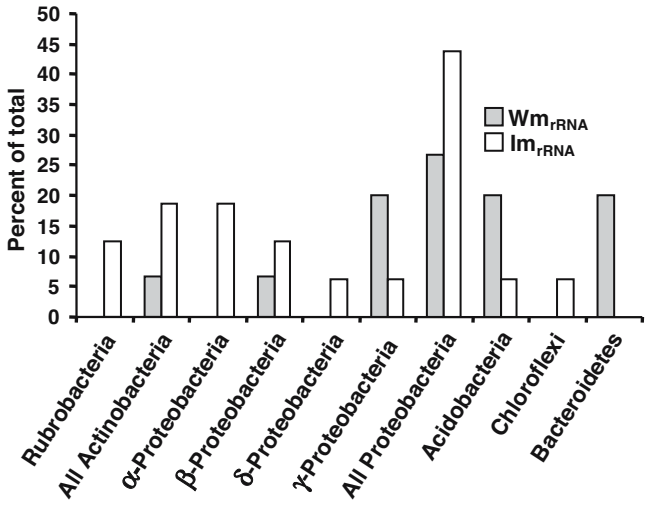

Figure 1. Frequency of major microbial groups in 16S rRNA clone libraries derived from Nebraska soil whole and inner microaggregates. Only groups having 5% or more representation within at least one soil fraction are included.

Clones affiliated with *Gemmatimonadetes* were overwhelmingly obtained from inner aggregate fractions. Although poorly represented in Wm_{rDNA} or Im_{rDNA} libraries, *Gemmatimonadetes* representatives constituted 10%, 18%, and 32% of sequenced clones from Im_{rDNA} libraries derived from Montana, Nebraska, and Georgia soils, respectively.

Acidobacteria were the most abundant division-level lineage in M_{rDNA} libraries derived from Montana and Georgia soils, comprising 35% of sequenced clones in each of these libraries. *Acidobacteria*, although to a lesser extent than in M_{rDNA} libraries, were also relatively well represented in Wm_{rDNA} libraries derived from Montana and Georgia soils, comprising 25% and 14% of sequenced clones, respectively. This is in contrast to Im_{rDNA} libraries, where *Acidobacteria* comprised only 10% of sequenced clones of the Montana soil, but were either absent, or comprised less than 5% of Im_{rDNA} of all other soils.

The rDNA-based results described above indicate only the presence and relative abundance of the corresponding populations in the soil fractions analyzed. To estimate the distribution of metabolically active cells of the populations of interest, clone libraries were also made from 16S cDNA derived from total RNA isolated form the Nebraska and Georgia soils. Because cells that are more active are assumed to have greater numbers of ribosomes than inactive cells [26, 42], 16S cDNA clones from active cells should be more abundant than those from inactive cells, when cell abundance (based on rDNA cloning frequency) is taken into account.

Numerous clones affiliated with *Alphaproteobacteria* were obtained from the Im_{rRNA} libraries derived from both Nebraska and Georgia soils (19% and 12%, respectively), suggesting that representatives of this bacterial lineage are active in the inner-microaggregate environment of these two very diverse soils (Figs. 1 and 2). The results also suggest that representatives of Rubrobacteria are active members of the inner microaggregate environment in both Georgia and Nebraska soils, comprising 16% and 12.5% of Im_{rRNA} clone libraries derived from these soils, respectively (Figs. 1 and 2). Although not present among clones obtained from Nebraska Wm_{rRNA} or Im_{rRNA} libraries, clones affiliated with *Gemmatimonadetes* were more abundant

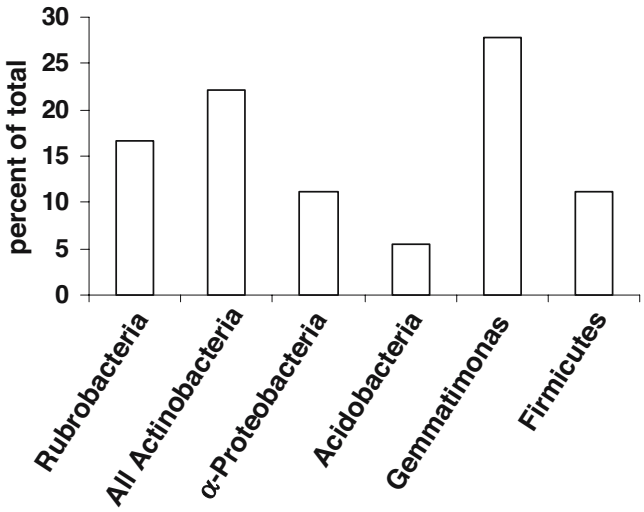

Figure 2. Frequency of major bacterial groups in 16S rRNA clone libraries derived from Georgia soil inner microaggregates. Only groups having 5% or more representation are included.

than any other bacterial lineage in the Georgia I_{rRNA} library, comprising 28% of all clones sequenced (Fig. 2).

Discussion

Soil secondary structural characteristics are thought to represent a salient control on soil microbial diversity. The objective of this study was to assess spatial stratification of microbial communities within the soil aggregate hierarchy. We analyzed 16S rRNA and 16S rRNA clone libraries derived from macroaggregates and whole and inner microaggregates of diverse soils varying greatly in weathering status and climate regime. Although relatively few clones were analyzed for each clone library, it is reasonable to assume that multiple clones affiliated with a given division- or subdivision-level lineage is indicative of the group being abundant within the sample from which the clone library was derived.

Alphaproteobacteria were relatively more abundant in whole- and, especially, inner-microaggregate fractions of both Montana and Georgia soils (Fig. 3). Interestingly, *Alphaproteobacteria* were also highly abundant in whole-microaggregate fractions of the Wyoming disturbed and undisturbed soils analyzed previously [36], suggesting that microaggregates tend to be enriched in this bacterial group.

Actinobacteria, as a whole, were primarily associated with inner-microaggregate fractions (Fig. 3). This is in contrast with the work of Hattori [18], who reported preferential localization of cultivable Gram-positive organisms in the outer part of soil aggregates. However, a large proportion of inner microaggregate *Actinobacteria* representatives were affiliated with the subdivision *Rubrobacteridae*, which has few cultured isolates and thus would likely have gone undetected in the Hattori study.

Rubrobacteridae represent a poorly described bacterial group that branches deeply from the *Actinobacteria* line of descent [21]. Judging from the results of a number of 16S rRNA-based surveys, *Rubrobacteridae* are present in a range of soils, including an acidic peat bog [33], a Scottish grassland [46], and arid Australian soils [21]. A recent study [34] reported 16S rDNA gene clones affiliated with the *Rubrobacteridae* dominated 16S rDNA gene clone libraries derived from a heavy metal-contaminated agricultural soil, whereas the results of Gremion *et al.* [11] suggest that *Rubrobacteridae* may make up a major portion of the metabolically active bacteria in both rhizosphere and bulk soils contaminated with heavy metals from septic tanks. Although mostly known from sequence data, a number of *Rubrobacteridae* soil representatives have recently been cultivated [23, 50], which will allow for phenotypic analysis.

The present study indicates greater *Rubrobacteridae* representation in inner microaggregate relative to other soil fractions analyzed. Despite the lack of *Rubrobacteridae* representation in 16S rDNA clone libraries derived from

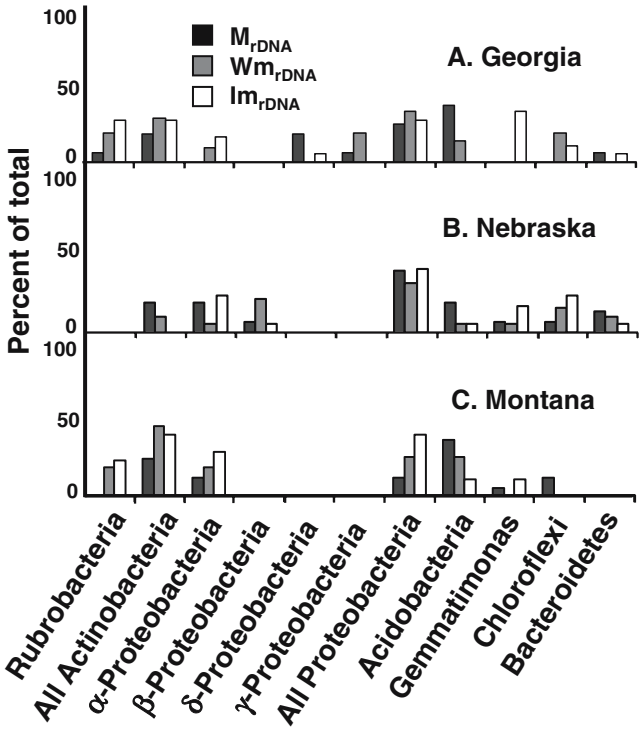

Figure 3. Frequency of major bacterial groups in rDNA clone libraries derived from Georgia, Nebraska, and Montana soil macroaggregates and whole and inner microaggregates. Only groups having 5% or more representation within at least one soil fraction are included.

any Nebraska soil fraction, clones affiliated with this bacterial group were highly abundant in the Nebraska Im_{rRNA} clone library (Fig. 2), suggesting that they comprise an active component of this environments microbial community. Although the reasons why interior regions of microaggregates obtained from such diverse soils are enriched in this relatively rare phylogenetic group are not clear, the apparent abundance of *Rubrobacteridae* representatives within microaggregates may have important implications for biotechnological applications. *Actinobacteria* are a primary source of antibiotics and other bioactive molecules currently seeing pharmacological use. The potential for pharmacologically useful secondary metabolite production in the *Rubrobacteridae* is likely high and knowledge of the growth environment is an important first step toward development of effective cultivation strategies.

Interestingly, *Rubrobacteridae* have also been shown to be abundant in earthworm casts of the Horseshoe Bend site in Georgia [10] and in association with earthworm intestines collected from the site [50]. Although much of the soil microstructure is initially destroyed in earthworm guts, new microaggregates are reformed during passage [2, 48], suggesting that the apparent enrichment of *Rubrobacteridae* representatives within microaggregates may be influenced by earthworm activity and microaggregate reformation dynamics. *Rubrobacteridae* enrichment was especially pronounced in inner microaggregates of reclamation soils analyzed in our previous study [36] (47% of sequenced clones). Interestingly, these soils had recently undergone extensive and drastic disturbance associated with mining and reclamation activities. Similar to what occurs during passage through earthworm guts, much of the soil structure would be expected to have been destroyed, followed by new microaggregate development. The apparent association of *Actinobacteria* with soil inner microaggregates, and especially inner microaggregates present under conditions likely conducive to new microaggregate formation, suggests that this bacterial group may play a role in microaggregate reformation dynamics and that future research should be conducted to elucidate these potential relationships.

The phylum *Gemmatimonadetes* is a very recently described bacterial group [60] that, on the basis of surveys of 16S rRNA genes, appear to be abundant in a number of soils [1, 10, 21, 35, 61]. To date, the phylum is represented by a single activated sludge isolate [60] and three soil isolates [23]. Although *Gemmatimonadetes* representatives were found in clone libraries derived from at least one fraction of all our study soils, the majority were from clone libraries derived from inner-microaggregate fractions (Fig. 3). For example, *Gemmatimonadetes* representation was especially large in I_{rDNA} and I_{rRNA} libraries derived from the Georgia soil (Figs. 2 and 3), whereas no *Gemmatimonadetes* clones were sequenced from Georgia M_{rDNA} or Wm_{rDNA} clone libraries. These results suggest that this phylum is not only abundant in inner microaggregates of this soil, but relatively active. Similarly, the Nebraska Im_{rDNA} library yielded relatively large numbers of clones affiliated with *Gemmatimonadetes* compared to M_{rDNA} and Wm_{rDNA} libraries derived from soil of this site.

Members of the phylum *Acidobacteria* are commonly detected in 16S rRNA gene surveys of soil ecosystems [1, 8, 10, 26, 47], and have been shown to be active members of soil microbial communities [9, 38]. Their widespread abundance in soils suggests ecological importance, although little phenotypic data are available and, hence, little is known about ecological function of any soil *Acidobacteria* representative. Although *Acidobacteria* representatives were found in nearly all clone libraries, our data suggests that *Acidobacteria* predominantly resided in macroaggregates and the outer fractions of microaggregates, possibly in coarse pores (Fig. 3). For example, *Acidobacteria* clones were highly abundant in the Georgia M_{rDNA} library, whereas only single clones were sequenced from Wm_{rDNA} and Im_{rRNA} libraries, and absent in the Im_{rDNA} library. Although somewhat less pronounced than in the Georgia soils, the same trend was found in all of the study soils, as well as in Wyoming reclamation soils in our previous study where *Acidobacteria* were represented by 27%, 14%, and 0% of sequenced clones in M_{rDNA}, Wm_{rDNA}, and Im_{rDNA} libraries, respectively. Interestingly, *Acidobacteria* representatives were much more abundant in the Nebraska Wm_{rRNA} library than in the Wm_{rDNA}, Im_{rDNA}, or Im_{rRNA} libraries. These results, in conjunction with the relatively high abundance of *Acidobacteria* representatives in the M_{rDNA} library derived from the same soil, suggest that not only are *Acidobacteria* predominantly outer-aggregate organisms, but that they represent a relatively active component of this environment's bacterial community.

More work will be required, potentially using soil washing techniques [43] in conjunction with molecular identification, to substantiate the supposition that *Acidobacteria* are predominantly loosely attached outer-aggregate bacteria. However, it is clear from our results that this phylum is poorly represented in inner-microaggregate fractions of the soils examined.

The methods utilized here provide the means to determine soil microbial community composition and activity patterns at aggregate and submicroaggregate scales. Armed with these approaches, it should now be possible to more closely approximate the distribution, abundance, and activity of functionally important microbial populations and groups within the soil matrix. Small-scale analyses, which provide information about how microbial populations, functional genes, and activities are distributed within the soil matrix,

will not only provide insights into how microbial diversity is controlled, but may allow for identification of the scale at which important ecological processes are regulated.

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References

1. Axelrood, PE, Chow, ML, Radomski, CC, McDermott, JM, Davies, J (2002) Molecular characterization of bacterial diversity from British Columbia forest soils subjected o disturbance. Can J Microbiol 48: 655–674
2. Barois, I, Villemin, G, Lavelle, P (1993) Transformation of the soil structure through *Pontoscolex coretrhrurus* (Oligochaeta) intestinal tract. Geoderma 56: 57–66
3. Chenu, C, Hassink, J, Bloem, J (2001) Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition. Biol Fertil Soils 34: 349–356
4. Chotte, JL, Jocteur, Monrozier, L, Villemin, G, Albrecht, A (1993) Soil microhabitat and the importance of fractionation method. In: Mulongoy, K, Merckx, R (Eds.) Soil Organic Matter Dynamics and Sustainability of Tropical Agriculture. Wiley, Leuwen
5. Darrell, DP, Shu-Mei, LI, Spadoni, CM, Drake, GR, Balkwill, DL, Fredrickson, JK, Brockman, FJ (1997) A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep substance sediment. FEMS Microbiol Ecol 23: 131–144
6. Dechesne, A, Pallud, C, Debouzie, D, Flandrois, JP, Vogel, TM, Gaudet, JP, Grundman, GL (2004) A novel method for characterizing the microscale 3D spatial distribution of bacteria in soil. Soil Biol Biochem 35: 1537–1546
7. Drazzkiewicz, M (1994) Distribution of microorganisms in soil aggregates: effect of aggregate size. Folia Microbiol 39: 276
8. Dunbar, J, Takala, S, Barns, SM, Davis, JA, Kuske, CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 64: 1662–1669
9. Felske, A, de Vos, WM, Akkermans, ADL (2000) Spatial distribution of 16S rRNA levels from uncultured acidobacteria in soil. Lett Appl Microbiol 31: 118–122
10. Furlong, MA, Singleton, DR, Coleman, DC, Whitman, WB (2002) Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Appl Environ Microbiol 68: 1265–1279
11. Gremion, F, Chatzinotas, A, Harms, H (2003) Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ Microbiol 4: 896–907
12. Griffiths, RI, Whiteley, AS, O'Donnell, AG, Bailey, MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl Environ Microbiol 66: 5488–5491
13. Grundmann, GL, Dechesne, A, Bartoli, F, Flandrois, JP, Chasse, JL, Kizungu, R (2001) Spatial modeling of nitrifier microhabitats in soil. Soil Sci Soc Am J 65: 1709–1716
14. Grundmann, GL (2004) Spatial scales of soil bacterial diversity—the size of a clone. FEMS Microbiol Ecol 48: 119–127
15. Gupta, VVRS, Germida, JJ (1988) Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biol Biochem 20: 777–786
16. Harris, PJ (1994) Consequences of the spatial distribution of microbial communities in soil. In: Ritz, K, Dighton, J, Giller, KE (Eds.) Beyond the Biomass. Compositional and Functional Analysis of Microbial Communities. Wiley, Chichester, pp 239–246
17. Hattori, T, Hattori, R (1976) The physical environment in soil microbiology: an attempt to extend principles of microbiology to soil microorganisms. CRC Crit Rev 4: 423–461
18. Hattori, T (1988) Soil aggregates as microhabitats of microorganisms. Rep Inst Agric Res Tohoku Univ 37: 23–26
19. Hendrix, PF (1997) Long-term patterns of plant production and soil carbon dynamics in a Georgia piedmont agroecosystem. In: Paul, EA, Paustian, K, Elliott, ET, Cole, CV (Eds.) Soil Organic Matter in Temperate Agroecosystems: Long Term Experiments in North America. CRC Press, New York
20. Holben, WE, Feris, KP, Kettunen, A, Apajalahti, JHA (2004) GC fractionation enhances microbial community diversity assessment and detection of minority populations of bacteria by denaturing gradient gel electrophoresis. Appl Environ Microbiol 70: 2263–2270
21. Holmes, AJ, Bowyer, J, Holley, MP, O'Donoghue, M, Montgomery, M, Gillings, MR (2000) Diverse, yet-to-be-cultured members of the Rubrobacter subdivision of the Actinobacteria are widespread in Australian arid soils. FEMS Microbiol Ecol 33: 111–120
22. Jastrow, JD, Boutton, TW, Miller, RM (1996) Carbon dynamics of aggregate-associated organic matter estimated by carbon-13 natural abundance. Soil Sci Soc Am J 60: 801–807
23. Joseph, SJ, Hugenholtz, P, Sangwan, P, Osborne, CA, Janssen, PH (2003) Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl Environ Microbiol 69: 7210–7215
24. Killman, K, Amato, M, Ladd, JN (1992) Effect of substrate location in soil and soil pore water regime on carbon turnover. Soil Biol Biochem 25: 57–62
25. Klironomos, JN, Rillig, MC, Allen, MF (1999) Designing belowground field experiments with the help of semi-variance and power analyses. Appl Soil Ecol 12: 227–238
26. Kuske, CR, Barnes, SM, Busch, JD (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographical regions. Appl Environ Microbiol 63: 3614–3621
27. Lane, DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt, E, Goodfellow, M (Eds.) Nucleic Acid Techniques in Bacterial Systematics. Wiley, New York, NY, pp 115–175
28. Lensi, R, Clays-Josserand, A, Monrozier, LJ (1995) Denitrifiers and denitrifying activity in size fractions of a mollisol under permanent pasture and continuous cultivation. Soil Biol Biochem 27: 61–69
29. Levin, S (1992) The problem of pattern and scale in ecology. Ecology 76: 1943–1967
30. Lyon, DJ, Monz, CA, Brown, RE, Metherell, AK (1997) Organic matter changes over two decades of winter wheat–fallow cropping in western Nebraska. In: Paul, EA, Paustian, K, Elliott, ET, Cole, CV (Eds.) Soil Organic Matter in Temperate Agroecosystems. CRC Press, New York
31. Ludwig, W, Strunk, O, Westram, R, Richter, L, Meier, H, Yadhukumar, Buchner, A, Lai, T, Steppi, S, Jobb, G, Förster, W, Brettske, I, Gerber, S, Ginhart, AW, Gross, O, Grumann, S, Hermann, S, Jost, R, König, A, Liss, T, Lüßmann, R, May, M,

Tiedje, JM, Reichel, B, Strehlow, R, Stamatakis, A, Stuckmann, N, Vilbig, A, Lenke, M, Ludwig, T, Bode, A, Schleifer, KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32: 1363–1371
32. Maidak, BL, Cole, JR, Lilburn, TG, Parker, CT, Saxman, PR, Farris, RJ, Garrity, JM, Olsen, GJ, Schmidt, TM, Tiedje, JM (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29: 173–174
33. McCaig, AE, Glover, LA, Prosser, JI (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl Environ Microbiol 65: 1721–1730
34. Moffett, BF, Nicholson, FA, Uwakwe, NC, Chambers, BJ, Harris, JA , Hill, TCJ (2003) Zinc contamination decreases the bacterial diversity of agricultural soil. FEMS Microbiol Ecol 43: 13–19
35. Mummey, DL, Stahl, PD (2003) Candidate division BD: phylogeny, distribution and abundance in soil ecosystems. Syst Appl Microbiol 26: 228–235
36. Mummey, DL, Stahl, PD (2004) Analysis of soil whole- and inner-microaggregate bacterial communities. Microb Ecol 48: 41–50
37. Nishio, N, Furusaka, C (1970) The distribution of nitrifying bacteria in soil aggregates. Soil Sci Plant Nutr 16: 24–29
38. Nogales, BE, Moore, RB, Abraham, WR, Timmis, KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. Environ Microbiol 5: 199–212
39. Oades, JM (1984) Soil organic matter and structural stability: mechanisms and implications for management. Plant Soil 76: 319–337
40. Oades, JM, Waters, AG (1991) Aggregate hierarchy in soils. Aust J Soil Res 29: 815–828
41. O'Donnell, AG, Godfellow, M, Hawksworth, DL (1995) Theoretical and practical aspects of the quantification of biodiversity among microorganisms. In: Hawksworth, DL (Ed.) Biodiversity, Measurement and Estimation. The Royal Society,pp 65–73
42. Pallud, C, Dechesne, A, Gaudet, JP, Debouzie, D, Grundmann, GL (2004) Modification of 2,4-dichlorophenoxyacetic acid degrader microhabitats during growth in soil columns. Appl Environ Microbiol 70: 2709–2716
43. Ranjard, L, Richaume, A, Jocteur Monrozier, L, Nazaret, S (1997) Response of soil bacteria to Hg(II) in relation to soil characteristics and cell location. FEMS Microbiol Ecol 24: 321–331
44. Ranjard, L, Nazaret, S, Gourbiere, F, Thioulouse, J, Linet, P, Richaume, A (2000) A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints. FEMS Microbiol Ecol 31: 107–115
45. Ranjard, L, Richaume, A (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 152: 707–716
46. Rheims, H, Felske, A, Seufert, S, Stackebrandt, E (1999) Molecular monitoring of an uncultured group of Actinobacteria in two terrestrial ecosystems. J Microbiol Methods 36: 65–75
47. Sessitsch, A, Weilharter, A, Gerzabek, MH, Kirchmann, H, Kandeler, E (2001) Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl Environ Microbiol 67: 4215–4224
48. Shipitalo, MJ, Protz, R (1988) Factors influencing the dispersibility of clay in worm casts. Soil Sci Soc Am J 52: 764–769
49. Skjemstad, JO, Janik, LJ, Head, MJ, McClure, SG (1993) High-energy ultraviolet photo-oxidation: a novel technique for studying physically protected organic matter in clay- and silt-sized aggregates. J Soil Sci 44: 485–499
50. Singleton, DR, Furlong, MA, Peacock, AD, White, DC, Coleman, WB, Whitman, WB (2003) *Solirubrobacter pauli* gen. nov., sp. nov., a mesophilic bacterium within the *Rubrobacteridae* related to common soil clones. Int J Syst Evol Microbiol 53: 485–490
51. Six, J, Elliott, ET, Paustian, K (2000) Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. Soil Biol Biochem 32: 2099–2103
52. Six, J, Conant, RT, Paul, EA, Paustian, K (2002) Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. Plant Soil 241: 155–176
53. Six, J, Feller, C, Denef, K, Ogle, SM, de Moraes Sa, JC, Albrecht, A (2002) Organic matter, biota and aggregation in temperate and tropical soils—effects of no-tillage. Agron Agric Environ 22: 755–775
54. Stahl, DA, Tiedje, JM (2002) Microbial ecology and genomics: a crossroads of opportunity. An American Academy of Microbiology Critical Issues Colloquia Report. American Academy of Microbiology, Washington DC
55. Tisdall, JM, Oades, JM (1982) Organic matter and water-stable aggregates in soils. J Soil Sci 33: 141–163
56. Van Gestral, M, Merckx, R, Vlassak, K (1996) Spatial distribution of microbial biomass in microaggregates of a silty-loam soil and their relation with the resistance of microorganisms to soil drying. Soil Biol Biochem 28: 503–510
57. Vargas, R, Hattori, T (1991) The distribution of protozoa within soil aggregates. J Gen Appl Microbiol 37: 515–518
58. Wright, DA, Killham, K, Glover, LA, Prosser, JI (1995) Role of pore-size location in determining bacterial-activity during predation by protozoa in soil. Appl Environ Microbiol 61: 3537–3543
59. Young, IM, Crawford, JW (2004) Interactions and self-organization in the soil–microbe complex. Science 304: 1634–1637
60. Zhang, H, Sekiguchi, Y, Hanada, S, Hugenholtz, P, Kim, H, Kamagata, Y, Nakamura, K (2003) *Gemmatimonas aurantiaca* gen. nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultivated representative of the new bacterial phylum *Gemmatimonadetes* phyl. nov. Int J Syst Evol Microbiol 53: 1155–1163
61. Zhou, J, Xia, B, Huang, H, Treves, DS, Hauser, LJ, Mural, RJ, Palumbo, AV, Tiedje, JM (2003) Bacterial phylogenetic diversity and a novel candidate division of two humid region, sandy surface soils. Soil Biol Biochem 35: 915–924