RESEARCH ARTICLE

Microbial community succession and bacterial diversity in soils during 77 000 years of ecosystem development

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Abstract

The origins of the biological complexity and the factors that regulate the development of community composition, diversity and richness in soil remain largely unknown. To gain a better understanding of how bacterial communities change during soil ecosystem development, their composition and diversity in soils that developed over *c.* 77 000 years of intermittent aeolian deposition were studied. 16S rRNA gene clone libraries and fatty acid methyl ester (FAME) analyses were used to assess the diversity and composition of the communities. The bacterial community composition changed with soil age, and the overall diversity, richness and evenness of the communities increased as the soil habitat matured. When analysed using a multivariate Bray–Curtis ordination technique, the distribution of ribotypes showed an orderly pattern of bacterial community development that was clearly associated with soil and ecosystem development. Similarly, changes in the composition of the FAMEs across the chronosequence were associated with biomarkers for fungi, actinomycetes and Gram-positive bacteria. The development of the soil ecosystem promoted the development of distinctive microbial communities that were reminiscent of successional processes often evoked to describe change during the development of plant communities in terrestrial ecosystems.

Introduction

The richness and diversity of prokaryotes in soil is staggeringly large (Torsvik *et al.*, 1990; Borneman *et al.*, 1996; Borneman & Triplett, 1997). While a range of variables correlate with soil diversity (Dalmastri *et al.*, 1999; Sessitsch *et al.*, 2001; Fierer & Jackson, 2006), the origins of the biological complexity and the factors that ultimately regulate the development of community composition, diversity and richness remain largely unknown. It is widely hypothesized that this enormous diversity could be related to a complex and gradient rich chemical and physical environment in the soil (Zhou *et al.*, 2002; Ritz *et al.*, 2004). Indeed, a full understanding of communities and their function requires intimate knowledge of species–habitat relationships.

The link between the diversity and composition of microbial life in relation to habitat characteristics is not clearly understood. Most studies on microbial diversity have focused their efforts on the nature of microbial diversity in mature soils. An understanding of how these factors change during soil and ecosystem development would provide additional insights. For instance, recent studies have suggested that the diversity, composition and function of microbial communities in developing glacial forefields is dynamic over hundreds and thousands of years of soil and ecosystem development (Ohtonen *et al.*, 1999; Torres *et al.*, 2004; Tscherko *et al.*, 2004; Sigler & Zeyer, 2004; Nicol *et al.*, 2005). With soil development there is an accumulation of carbon for microbial growth and formation of gradient-rich weathered minerals and structural characteristics that increase the overall complexity of the soil environment (Nunan *et al.*, 2001; Zhou *et al.*, 2002). Previous studies of the progression of microbial communities in plant litters and soils have examined timescales ranging from days to several thousand years. By studying the progression of soil community structure and compositions through stages of soil habitat development over longer time scales, insights

can be gained into the factors that mediate the development of soil microbial communities and the origins of the enormous diversity of microorganisms in mature soils.

Here, a study to assess whether discernable patterns of microbial and bacterial community development co-occur in association with soil and ecosystem development is described. The authors hypothesize that soil and ecosystem development affects the composition and structure of prokaryotic communities. In addition, a concomitant increase in the diversity and richness of prokaryotic communities with soil and ecosystem development and soil ageing was expected. The microbial and bacterial communities were characterized using FAME and 16S rRNA gene clone libraries, respectively, in soils developed over *c.* 77 000 years of intermittent aeolian deposition. These chronosequences represent soils that are diagenetically similar but differ primarily as a function of age-related development (Markewich & Pavich, 1991). They offer a unique opportunity to study the composition of microbial communities in soils and ecosystems as a result of their natural development.

Materials and methods

Study area

The field sites are located in the subtropical Altamaha and Ohoopee River Valley dunes of southeast Georgia (31.92N, −82.11W). Inland dune fields are common along the northeast sides of southeast-trending streams in the southern Atlantic Coastal Plain and reside *c.* 100 m above sea level (Ivester *et al.*, 2001; Ivester & Leigh, 2003). Chains of parabolic dunes average *c.* 0.5 km in width and range from 3.7 to 14 m high at the crests. The study sites were chosen to provide intact and predominantly unperturbed set of soils with geochemically distinct horizons that could be used to assess the development of community and habitat features as a result of 'natural' as opposed to anthropogenic forces. These sites are unfavourable for intensive agriculture and have been minimally influenced by recent human activity.

Each soil dune is composed of a thick, well-drained sandy parent material. The drought-prone dune habitat described by low vegetative diversity is dominated by turkey oaks, longleaf pine, prickly pear cactus and various lichen and woody mint species. There is evidence of above ground plant succession throughout the chronosequence. For example, older sites are dominated by pines and younger ones are dominated by turkey oaks and associated species. Deposition of aeolian materials occurred intermittently over several thousands of years, and some of the better described dunes were sampled as represented in Table 1 (Ivester & Leigh, 2003). The depositional age of the youngest soil was close to 10 000 years. However, reworking of the dune surface and exposure of fresh material restarted the process of soil formation *c.* 5k years ago. For that reason, the age of that soil was classified as 5000 years and hereafter denoted as 5K.

Sampling

From the crest of each dune, soils were collected in May 2005 from the zone of major clay translocation (B_w-horizon), found typically between 25 and 45 cm depth. These deeper soils were chosen because the subsurface soil cambic type horizons represent zones where both organics and weatherable materials accumulate that are less prone to environmental and seasonal variations, and the heterogeneity of the soil matrix is substantially reduced. In addition, recent immigration by microorganisms was expected to play a smaller role in deeper soils. Although not a true component of the chronosequence, a sample of a recently deposited point bar sand was collected (<2 years) at a depth of 10–15 cm. This sample was used to simulate the source

Table 1. Site descriptions and depositional ages of the soil chronosequences

Site name	Age (BP)*	Clay+silt (%)	Soil organic matter (%)	pH	$Fe_2O_3+Al_2O_3$ (%)†	μg fatty acid (g^{-1} soil)	Euk/Prok ratio‖	Surface area ($m^2\ g^{-1}$)
Point bar‡	0	0.5	0.1	5.9	1.0	33	0.40	0
5K	4.9 (0.5)§	2.2	0.95	4.4	2.5	421	0.68	0.01
16K	16 (2.0)	1.7	2.2	4.3	2.7	195	0.71	0.05
38K	38 (5.0)	3.2 (0.5)	0.66 (0.15)	4.6	2.3 (0.4)	215 (20)	0.80 (0.05)	0.11 (0.03)
45K	45 (7.4)	4.1	0.76	4.6	2.2	133	1.2	0.3
77K	77 (6.6)	5.3	0.95	4.2	1.2	370	4.1	0.24

*Except for the point bar sample, ages are reported in thousands of years before AD 1950 (Ivester & Leigh, 2003).

†% of the following: SiO_2, TiO_2, Al_2O_3, Fe_2O_3, MnO, MgO, CaO, Na_2O, K_2O, P_2O_5.

‡The point bar sample is not a true component of the eolian dune chronosequences. It was sampled along with the dune soils in 2005, providing a reference for the types of material and communities that were deposited by the stream before dune and soil genesis.

§SDs when applicable are denoted in parentheses.

‖An index to describe the relative amount of eukaryote to prokaryote biomass was calculated using the mol% of the ratios of each of the following fatty acids: (18:2 ω6,9+18:1 ω9+20:0)/(i15:0+a15:0+i16:0+16:1 ω7+i16:0+i17:0+a17:0+18:1 ω7+cy19:0).

material of the dune before aeolian transport and deposition. The sampled depth represented the zone above the existing water table that was not exposed to environmental extremes found at the surface.

Soils were first augered to create a hole, and then subsampled with a spade after gently removing contaminating soil from the other zones. Samples were immediately placed into sterile Whirlpak® bags, and the process was repeated twice at 3 m intervals to accumulate 1 kg of soil. In addition, samples were collected at three separate locations in the 38K soil at 20 m intervals along a 60 m transect. The auger and the spade were wiped clean with 70% ethanol between each collection. After sampling, soils were immediately homogenized through repeated mixing and stirring. A 50 g subsample was placed into a sterile 60 mL polypropylene screw top vial and stored in a cooler packed with dry ice. Upon arrival in the laboratory, soils were thawed for 10–20 min. The extraneous roots and organic matter were removed, and the soil was stored at $-80\,^{\circ}C$ for 1–2 months before extraction of DNA and FAME. The remaining soil was transported in a cooler with ice and stored air-dry in the laboratory before analysis of organic matter, particle size and cation abundance.

Organic matter content was determined by mass loss after ignition (560 °C). Soil texture was assayed according to Wu *et al.* (1990). Total cations were measured by inductively coupled plasma spectrometry following a triple acid digestion of samples in HF–HCl–HNO_3. The surface area (adsorption of N_2) and pH (1 : 2 :: soil : 0.01 M $CaCl_2$ solution) of the soils were measured using standard soil laboratory techniques (Kemper & Rosenau, 1986).

Soil DNA extraction and preparation of 16S rRNA gene libraries

For each soil type, duplicate DNA extractions and library constructions were performed. DNA was extracted from 10 g of soil using PowerMax™ Soil DNA isolation kit (Mo Bio Laboratories Inc.) following the manufacturer's instructions, including shaking for 30 min in a water bath at 65 °C. The bacterial 16S rRNA genes were amplified in a 15-cycle PCR using primers 27F and 1492R and PuReTaq™ Ready-To-Go™ PCR beads (Amersham Biosciences). PCR products were immediately cloned in pCR2.1® TOPO® TA cloning vector (Invitrogen). Plates were incubated overnight at 37 °C and then stored at 4 °C for 24 h. White colonies were picked into 96-well culture blocks containing 1 mL of freezing medium [Luria–Bertani (LB) broth with 10% (v/v) anhydrous glycerol, kanamycin (12.5 $\mu g\,mL^{-1}$) and ampicillin (25 $\mu g\,mL^{-1}$)] $well^{-1}$. The blocks were incubated on a shaker at 200 r.p.m. at 37 °C for 12 h. A 150-L aliquot of the cultures was then transferred to sterile 96-well microtitre plates. The plates were sealed with aluminium seal tape, and both the blocks and plates were stored at $-80\,^{\circ}C$.

Sequencing and editing

The sequencing plates were shipped to SeqWright DNA Technologies (Houston, TX) on dry ice, where the cultures were regrown, the plasmids were isolated and the 16S rRNA genes were partially sequenced using the primer 27F. The complete 16S rRNA gene for selected clones was determined by further sequencing using the primer 1492R at the University of Georgia's sequencing facility (Athens, GA). Sequence chromatogram files were trimmed and edited with CHROMAS LITE version 2.0 (Technylesium). Sequence alignments were constructed using CLUSTALX and manually edited using GENEDOC v2.6.02 (Thompson *et al.*, 1997). All clones exhibiting $< 95\%$ sequence similarity to an existing GenBank sequence that occurred in only one library were examined as potential chimeras using the CHIMERA CHECK PROGRAM of the RDP database (Cole *et al.*, 2005). The edited sequences were submitted to GenBank with accession numbers EU043524–EU044592.

Library comparison and diversity estimates

After alignment, distance matrices were calculated using the Jukes–Cantor algorithm in the program DNADIST from the PHYLIP package. These distance matrices were then used to compare libraries utilizing the program LIBSHUFF v1.2 (Singleton *et al.*, 2001). The Bonferroni correction was used to correct for experiment-wise error when performing multiple LIBSHUFF comparisons.

Diversity measurements were used to compare the clone libraries independent of their phylogenetic composition. The operational taxonomic units (OTUs) were determined using the average neighbour algorithm in DOTUR (Schloss & Handelsman, 2005). The rarefaction analyses, richness, evenness, Shannon, Simpson's Reciprocal index and Chao1 estimates were calculated for OTUs formed at an evolutionary distance of 0.01 (or about 99% sequence similarity). To analyse the distribution of abundant taxa within libraries, groups were formed at a distance of ≤ 0.03.

Taxonomic assignment and phylogenetic analyses

The taxonomic assignment of sequences were made with RDP query (http://simo.marsci.uga.edu/public_db/rdp_query.htm) using similarity cutoff values of 80, 85, 91, 92, 95 and 100% for phylum, class, order, family, genus and species designations, respectively. Assignments at the phylum level were further tested with neighbour-joining trees constructed using Jukes–Cantor distances in MEGA v2.1 (Kumar *et al.*, 2001). The RDP query assignments of the

Acidobacteria and *Firmicutes* were substantially changed based upon these phylogenetic trees. Chi-Square tests were used to test for significant differences in taxonomic composition among the soil ages.

Fatty acid methyl-ester analysis

Fatty acid methyl-ester (FAME) extractions were conducted according to the method utilized by Grigera *et al.* (2006), with a few modifications. Briefly, 3 g of soil was placed in a 40 mL scintillation vial, followed by the addition of 15 mL of 0.2 M KOH (in methanol) to each sample and sonication for 1 h in a sonicating bath. Three millilitres of 1 M acetic acid was then added to neutralize the KOH, and the samples were vortexed for 1 min. Ten millilitres of hexane was added, and the samples were vortexed again for 1 min. Deionized water, 5 mL, was then added for better phase separation. The samples were vortexed a third time for 1 min and then centrifuged at 1000 ***g*** for 15 min. Five millilitres of the hexane layer was transferred into a 7-mL disposable glass tube using a pipette. The samples were completely dried under a gentle stream of 99.999% UHP N_2 gas. The remaining residue was then redissolved in 0.5 mL of hexane and placed in a 2 mL GC vial for analysis of FAME on a GC-MS using an INNOWAX capillary column (30 m, 0.25 ID, 0.25 μm film; Palo Alto, CA).

Multivariate statistical analyses

The OTUs defined as above were used for Bray-Curtis gradient analysis using distance described by the Bray-Curtis coefficient (McCune & Grace, 2002). For each soil class, the number of clones in each of the abundant OTUs was divided by the total number of clones (all 24 of the abundant OTUs) to derive the relative proportion of that OTU in each soil class. In a similar way, the mol% of 22 dominant fatty acids, accounting for $>$ 90% of the total, were analysed (Williams *et al.*, 2006). The reference point method developed by Bray-Curtis is a very common method for identifying relative similarity of communities during succession and effective for multivariate ecological data (Beals, 1984; McCune & Grace, 2002).

Results

Soil characteristics

Soil characteristics of the chronosequence were consistent with age related weathering. For example, there was a progressive accumulation of silt and clay with age (Table 1). Likewise, $Fe_2O_3+Al_2O_3$ accumulated during the early stages of soil development followed by a gradual decrease. However, except for the much higher levels in the 16K soils, organic matter content did not show a regular trend with soil age. Despite the increase in surface area, which was indicative of the number of small sized particles, with age, the surface area was still low even in the oldest soils. The low surface area may explain the absence of a relationship between clay and silt accumulation and organic matter content. Organic matter tends to accumulate during early stages of soil development and is often related to the presence of binding sites associated with clay and silt content. While the total mass of fatty acid showed no trend with developmental age, there was an increase in the amount of eukaryotic fatty acids, especially notable at the oldest soil age.

16S rRNA gene libraries

The bacterial diversity in the soils of five ages was evaluated by constructing duplicate 16S rRNA gene libraries for each soil type. LIBSHUFF, which is a statistical method to test if two or more libraries are derived from the same population, indicated that the compositions of the replicate libraries were not significantly different. Moreover, no significant differences were found in clone libraries extracted from replicate soil samples taken from the 38K site. Thus, the methods for extracting DNA and cloning were reproducible, and the libraries were pooled for most of the subsequent analyses.

In contrast, libraries from soils of different ages were very different by LIBSHUFF. When every pair-wise comparison was made between all five libraries, all differences were significant (experiment wise *P*-value ≤ 0.002). Further analyses were then performed to identify the specific nature of these differences.

Phylogenetic affiliation of 16S rRNA gene clones

The bacterial community composition of all five chronolibraries was diverse. Taxonomic assignments revealed that most sequences could be affiliated to 12 phyla or candidate divisions that were unevenly distributed throughout the chronolibraries. A search for similar sequences with the RDP database revealed that only 3% of the clones were closely related to type strains ($>$ 97% similarity). However, 32% of the clones exhibited $>$ 97% similarity to sequences from either nontype or uncultured clones, indicating the wide sampling of similar soil microbial communities by culture-independent methods.

Across all soils, *Acidobacteria* sequences were most abundant (54.5%), followed by *Proteobacteria* (19.2%), *Planctomycetes* (13.1%) and *Chloroflexi* (8.2%) (Fig. 1).

Among the *Proteobacteria,* the *Alphaproteobacteria* were the most abundant (10%) followed by the *Betaproteobacteria* (3.8%), *Gammaproteobacteria* (3.8%) and the *Deltaproteobacteria* (1.7%). In addition, small numbers of clones representing less abundant groups were found. The

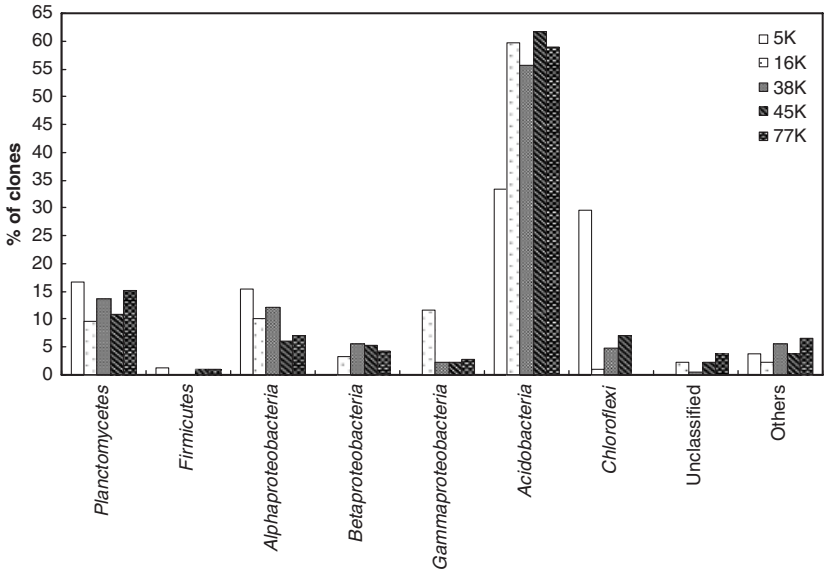

Fig. 1. Phylogenetic distribution of the 16S rRNA gene clone sequences. The percentage of clones from each soil type is presented. Phylogenetic assignments were based upon comparisons to sequences in the RDP database performed by RDP query using similarity cutoff values of 80% and 85% for phylum and class designations, respectively. The others group included sequences affiliated to the *Thermomicrobia*, *Gemmatimonadetes*, *Verrumicrobia*, *Nitrospira*, *Deltaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, WS3 and OP10.

Actinobacteria (10 clones) were distributed among the 5K, 38K and 77K soil libraries, the *Firmicutes* (six clones) were distributed among the 5K, 45K and 77K soil libraries, and the *Gemmatimonadetes* (three clones) were found only in the 38K soil libraries. Similarly, the *Bacteroidetes*, *Thermomicrobia*, *Nitrospira* and the WS3 division were only found in the 77K and 45K soil libraries. Overall, Chi-squared analysis suggested that the abundance of *Acidobacteria*, *Proteobacteria* and *Chloroflexi* varied significantly ($P \leq 0.01$) with soil age.

In contrast to the individual soils of the chronosequence, a more diverse [Evolutionary distance < 0.01: Simpson (1/D), 263; Shannon, 4.6; Chao 1, 502] and compositionally very different bacterial community was observed in the freshly deposited material (point bar). The majority of the sequences clustered within the *Betaproteobacteria* (21.4%), followed by the *Acidobacteria* (12.1%), *Gammaproteobacteria* (10.0%) and the *Bacteroidetes* (9.3%). Many of the minor taxa were the same as found in the chronosequence soils, however, sequences belonging to the *Thermomicrobia* and the *Gemmatimonadetes* were absent. In addition, three phylogenetic groups absent from the chronosequence soils were detected in the point bar soils: the *Cyanobacteria*, *Verrucomicrobia* and *Thermotoga*.

Although the *Acidobacteria* were well represented in all soil ages, their relative abundance was notably reduced in the 5K soil library (Fig. 1). Their wide distribution in soils suggest that they play important roles in soil community structure and function. Therefore, their phylogenetic relationships and distribution in the chronosequences was further investigated (Fig. 2). Although acidobacterial clones representing all seven of the subdivisions originally proposed by Hugenholtz *et al.* (1998) were found, the majority of the clones belonged to Groups 1–3 and 5 (Fig. 2). Out of the 483 acidobacterial clones observed in all the libraries, 142, 243, 56 and 21 clones were affiliated to the Groups 1, 2, 3 and 5, respectively. These clones were represented by only 44 OTUs (Fig. 2), many of which were observed > 20 times (Table 2). It is interesting to note that the majority of the Group 2 clones were derived from the 16, 38 and 45K soil libraries and none from the 5K soil library. While soils aged 16–77K were represented predominantly by sequences belonging to Groups 1–3, the sequences affiliated to Group 5 were nearly equally distributed amongst the older three soils.

Comprising the fourth most abundant group, the *Chloroflexi* were represented by 73 sequences that clustered into two clades (Fig. 3). The *Chloroflexi* have been described as consisting of perhaps eight candidate classes as delineated by Rappé & Giovannoni (2003). However, a recent study proposed the creation of seven new subdivisions (Costello & Schmidt, 2006). All *Chloroflexi* clones clustered within the novel subdivisions C05 and A07, with over 85% of the clones associated with the former group. Interestingly, although the *Chloroflexi* were abundant in the youngest soil (5K), they were nearly absent from the older soils. In contrast, both the *Gammaproteobacteria* and the *Betaproteobacteria* were absent from the 5K soil even though they were common in the older soils.

Diversity indices

In order to analyse the bacterial diversity represented in the chronosequences, clones were placed into OTUs with < 0.01 evolutionary distance or about 99% 16S rRNA sequence similarity. As measured by the Shannon index and Simpson's reciprocal index, the bacterial diversity

tended to increase with the soil developmental age (Table 3). This pattern was not altered using absolutely equal ($N = 158$; see Table 3) sample sizes for all chronolibraries and recalculating these indices (data not shown). The former index is more sensitive to changes in the abundance of the rare groups while the latter is heavily weighted by the dominants (Hill *et al.*, 2003). The increased diversity of older soils was also supported by rarefaction analysis. Although the rarefaction curves from all soils failed to plateau, the slopes were steeper for the older soils (Fig. 4) At the highest shared sample size (158 clones), the 95% confidence intervals only overlap for the 38K and 45K soils. Rarefaction analysis of individual taxa revealed that the *Acidobacteria* and the *Planctomycetes* were the most diverse (data not shown). In addition, diversity indices calculated for these phyla showed the same trend of increasing richness and diversity with soil age.

The failure of the rarefaction curves to plateau at OTUs defined at evolutionary distances of 0.01 (Fig. 4) or 0.03 indicated that these communities were incompletely sampled (data not shown). In fact, for most libraries only 30–50% of the number of OTUs predicted by the Chao1 estimator to be present in these soils were actually observed (Table 3). Thus, a large amount of the bacterial diversity still remains to be sampled from these soils. Furthermore, the Chao1 nonparametric richness estimator provides an alternative to undersampled libraries as it is considered valid before all phylotypes have been recovered because the estimator approaches a stable maximum before rarefaction curves plateau (Kemp & Aller, 2004). Although there is some overlap in the 95% confidence interval of the Chao1 estimator between soils close in age, no overlap is observed for a greater time span, further supporting the significance of the difference.

The evenness values (E) showed similar age-related trends to that of diversity and richness (Table 3). Increases in the abundance of a few dominant OTUs had a strong effect on E values. For example, the six most abundant OTUs accounted for 44% of the clones in the 5K soil while they accounted for only 17% of the clones in the 77K soil. In the same two libraries, E increased from 0.87 to 0.94. Similarly, the older soils also possessed more singleton OTUs that were rare and appeared only once (data not shown). In contrast, some OTUs were very abundant. For instance, 223 sequences or 25% of all sequences represented 24 of the most abundant OTUs (Table 2). Many of these OTUs were associated with

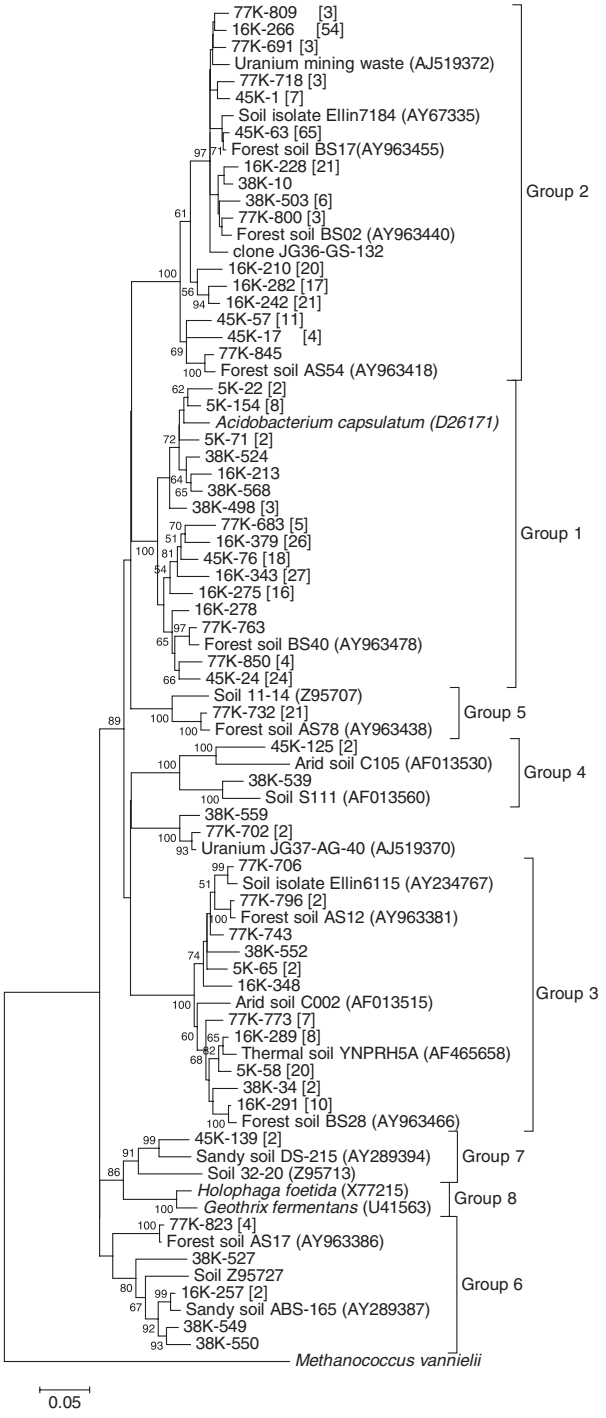

Fig. 2. Phylogeny of representative *Acidobacteria* clones from Altamaha dune soils of southeast Georgia. A representative clone (*c.* 700 bases) for each OTU was used to construct the phylogeny. OTUs were formed at an evolutionary distance of 0.03 (or about 97% sequence similarity). Clones are designated by a number and the letter 'K' (= 1000) to denote the soil depositional age followed by the clone number. When more than one clone was observed, the number was included within parenthesis following the clone designations. Classification of the subdivisions is according to Hugenholtz *et al.* (1998). Neighbour-joining trees were constructed using Jukes–Cantor algorithm in Mega v2.1 with 100 bootstrap replicates (only values ≥50% are given). The reference sequences are shown by species or clone name followed by the GenBank accession number in parentheses.

Table 2. Distribution and phylogenetic affiliation of the most abundant OTUs

*N**	Clone name†	RDP query phylogenetic group‡	% similarity	5K	16K	38K	45K	77K
24	5K-1	Unclassified (AY395405)	90.6	24				
14	5K-3	Unclassified (AY395405)	90.2	14				
20	5K-58	*Acidobacteria* (AY326550)	97.9	17	3			
17	5K-50	*Planctomycetes* (AF465657)	96.7	16			1	
25	45K-190	Unclassified (AY395405)	98.1	1	1	12	11	
10	5K-90	*Alphaproteobacteria* (DQ451506)	97.6	8			2	
12	16K-362	*Nostocoida limicola* (AF244749)	95.9	4	7	1		
16	16K-275	*Acidobacteria* (DQ451440)	96.9	1	4		6	5
65	45K-63	*Acidobacteria* (AY963455)	98.3	1	3	27	19	15
36	5K-4	*Alphaproteobacteria* (AY724041)	98.0	10	8	11	1	6
26	16K-379	*Acidobacteria* (AY963368)	95.7	7	6	2	8	3
20	16K-210	*Aciodobacteria* (AY326550)	95.8	7	8	2	2	1
54	16K-266	*Acidobacteria* (DQ451442)	97.9		20	20	9	5
21	16K-228	*Acidobacteria* (AY913273)	98.1		13	2	2	4
21	16K-242	*Acidobacteria* (AY913374)	98.3		9	1	4	7
18	16K-195	*Betaproteobacteria* (AY963445)	98.1		4	4	3	7
17	16K-282	*Acidobacteria* (DQ451510)	97.9		11	2	2	2
27	16K-343	*Acidobacteria* (AY043842)	98.3		12	4		11
10	16K-291	Unclassified (AY963466)	97.6		4		1	5
10	16K-194	*Gammaproteobacteria* (DQ068850)	99.7		10			
11	45K-57	*Acidobacteria* (AF529322)	94.7			1	10	
21	77K-732	*Acidobacteria* (AY963438)	96.2			6	6	9
18	45K-76	Unclassified (AY963415)	95.6			1	16	1
11	77K-802	*Planctomycetes* (AY963397)	92.8			3	3	5

*The data in the table is representative. Number of clones in an OTU $\geq$97% sequence similarity.

†Representative clone for each OTU.

‡Phylogenetic assignment of closest GenBank sequence (accession number in parentheses). Except for 16K-362, the closest matches were all uncultured clones.

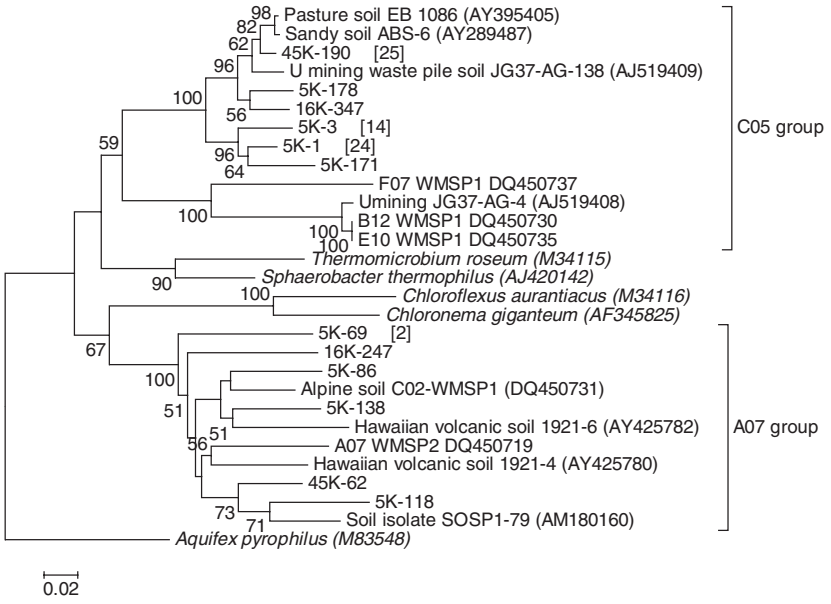

Fig. 3. Phylogeny of representative *Chloroflexi* clones from the dune soils of southeast Georgia. Classification of the subdivisions is according to Costello & Schmidt (2006). Other notations are as described for Fig. 2.

only a few soil ages. For instance, two OTUs which were unclassified according to RDP query but considered related to *Chloroflexi* (5K-1 and 5K-3, Fig. 3) by phylogenetic analysis, and the A*cidobacteria* (5K-58), and *Planctomycetes* (5K-50), were highly enriched in the youngest soil. Conversely, five abundant OTUs closely related to the

Table 3. Diversity indices for the chronolibraries

Diversity Index*	5K	16K	38K	45K	77K	5-77K
N†	158	177	182	186	183	887
S‡	56	87	102	107	125	354
1/D§	24	64	56	73	143	136
Shannon (H)	3.50	4.19	4.24	4.38	4.64	5.34
H/H_{max}	0.69	0.81	0.82	0.84	0.89	0.79
Evenness (E)	0.87	0.94	0.92	0.94	0.96	0.91
Chao1‖	111	125	292	223	378	696
95% COI	78–194	105–167	200–470	169–325	264–587	588–853

*Calculations were based on OTUs formed using DOTUR (Schloss & Handelsman, 2005) at an evolutionary distance of < 0.01.
†Number of clones in the library.
‡Number of OTUs.
§Simpson's reciprocal index.
‖Chao1 = $S+n_1^2/2n_2$, where n_2 is the number of clones that occur twice.

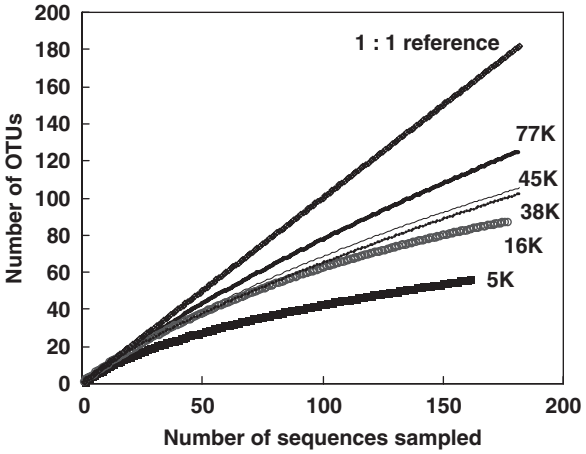

Fig. 4. Rarefaction curves of chronolibraries with OTUs defined at an evolutionary distance of 0.01. 95% confidence intervals were omitted for clarity of presentation. Diagonal line represents the 1 : 1 relationship where each screened clone is unique.

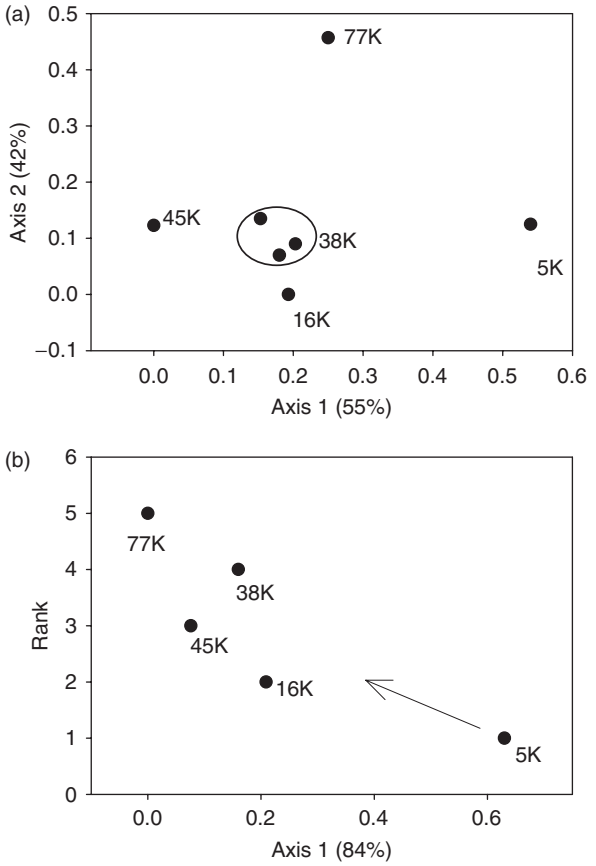

Fig. 5. Bray-Curtis ordination plot of the age-related (K = 1000 years) structural change in soil microbial communities. (a) Groupings used to separate communities were based on the mol% of 22 fatty acids. Percentages denote the amount of variability associated with each axis. Three replicates from the 38K soil are circled. (b) Groupings used to separate communities were based on the relative proportion of each of the 24 most abundant OTUs. The OTUs were formed using the average neighbour algorithm in DOTUR at a distance of 0.03. The arrow shows the apparent direction of community development with soil age. Each symbol represents one sample.

Acidobacteria (16K-266, 16K-228, 16K-242 and 16K-282) and *Betaproteobacteria* (16K-195) were specifically absent from the 5K soils. Similarly, two OTUs represented by clones 45K-57 and 45K-76 were present in high numbers in the 45K library. In contrast, OTUs such as two *Acidobacteria* (16K-379 and 16K-210) and one *Alphaproteobacteria* (5K-4, the closest uncultured relative of *Rhodoplanes elegans*) were evenly distributed within all the soils.

Multivariate analysis of the soil microbial communities

The mol% distribution of the FAMEs was strongly influenced by soil age (Fig. 5a). The spread of the data was dominated by Axis 1, which was highly correlated with 16 : 10me and 16i ($r > 0.80$) or FAMEs often associated with Gram-positive type bacteria. Axis 2 was largely defined by differences in FAMEs often associated with fungi and actinomycetes (18 : 1 ω9, 18 : 2 ω6,9 and 10me18 : 0, $r > 0.80$). The multivariate analysis of the most abundant OTUs in the rRNA gene libraries, described using a one-dimensional solution, also found a strong relationship between the bacterial community composition and soil age (Fig. 5b) and strongly reflected the patterns easily observable in Table 2. The differences between the youngest and the four oldest soils accounted for the majority of the variability between the bacterial communities. Moreover, important successive differences between the communities are noted throughout the chronosequence.

Discussion

By all of the measures examined, the bacterial community composition, diversity and richness changed with soil and ecosystem development. The changes in underlying OTU abundance are consistent with a progressive and orderly pattern of community development not unlike the concept of species succession that is often evoked to explain community dynamics in aboveground terrestrial plant communities (McIntosh & Odum, 1969). Indeed, it is interesting that the largest change in the bacterial community composition occurred during the transition from early (5K) to later (16–77K) successional stages. A parallel transition occurs in the surface vegetation, from more open sclerophyllous-like vegetation (*Quercus laevis* – Turkey oak, *Serenoa repens* – Saw palmetto) in the 5K dunes to dominance by mixed pine forests (*Pinus echinata* – Shortleaf pine, *Pinus palustrus* – Longleaf pine, *Quercus marilandica* – Blackjack oak) in the older dunes (Bozeman, 1964). It is an intriguing idea that plant–microbial feedbacks previously documented on short time scales could also influence the composition of microbial and plant communities during longer time periods associated with ecosystem and soil development (Klironomos, 2002; Garbeva *et al*., 2004).

One of the objectives of this study was to describe how bacterial communities change during soil and ecosystem development. With this in mind, the authors also studied the bacterial community composition and diversity from a sample of point bar sand freshly deposited (<2 years) on the banks of the Altamaha river. Because the dunes are formed by the wind blown deposition of the river sands, the composition of these point bar communities could have provided insight into the types of microorganisms that first colonized the dunes. The composition of the point bar sand communities was very different from those in the dune soils. They also exhibited substantially higher bacterial diversity and richness. The large number of sedimentary and bacterial inputs to the river along its 400 mile path can probably best explain the high diversity of bacteria described in the point bar sand. Microbial communities associated with these sedimentary river deposits may not be stable, however. While the roles that the communities associated with the point-bar play during the early stages of dune formation are not known, it is clear that they have little in common with the communities associated with the soil horizons formed in the dunes during ecosystem development.

Although the functional and metabolic roles of the *Acidobacteria* in soil are poorly understood, this group is now recognized as one of the most abundant bacterial taxa in many soils (Hugenholtz *et al*., 1998; Barns *et al*., 1999). Part of the reason that the role of the group is so poorly described is that only three representatives, *Acidobacterium capsulatum* (Group 1), *Holophaga foetida* and *Geothrix fermentans* (Group 8), have been cultured. Compared with the broader literature, dominance of the *Acidobacteria* in the soils was observed (Janssen, 2006). Other studies have also noted a high representation of this phylum in arid soils, thermal Yellowstone soil, and dry-mesic evergreen broad-leaved forest soils (Dunbar *et al*., 1999; Norris *et al*., 2002; Chan *et al*., 2006). Indeed, sequences related to the two largest groups, 45K-63 and 16K-266 with 65 and 54 clones, respectively, are also represented in these soils. Given their sandy nature and extremely low water-holding capacity, the Altamaha soils are undoubtedly drought-prone and are exposed to prolonged extremes in water stress. Perhaps it is the degree of water stress that can help explain why the populations appear to be most similarly related to those found in the arid and thermal Yellowstone type soils.

The Altamaha soils showed greater dominance by the *Planctomycetes* (*c*.12%) as compared with the average of 2% for other soil communities (Janssen, 2006). Interestingly, the soils also showed similarities to the *Planctomycetes* found in thermal Yellowstone soil (Norris *et al*., 2002) as represented by the clone 5K-50 with the largest OTU size among the *Planctomycetes* with 17 clones. The knowledge of *Planctomycetes* is limited to only a few characterized species, but these organisms possess a range of unusual characteristics and diverse metabolic capacity (Ward *et al*., 2006). It has been hypothesized that *Planctomycetes* communities are sensitive to soil history and disturbance, so that the high abundance of this group may reflect native conditions inherent to these soils (Buckley *et al*., 2006).

Very different taxonomic groups were present in the youngest 5K soil and the older soils. For instance, *Chloroflexi* was very abundant and the *Betaproteobacteria* and *Gammaproteobacteria* were completely absent from the 5K soils. In addition, acidobacterial clones retrieved from the 5K soil only clustered within subdivisions 1–3, while older soils contained representatives of seven of the eight monophyletic subdivisions identified by Hugenholtz *et al*. (1998). It is hypothesized that young and immature soils harbour communities with lower diversity and greater dominance by a few OTUs than older and more mature soils. Perhaps, this community structure could be a consequence of the initial colonization process that, similar to aboveground terrestrial ecosystems, might be characterized by a dynamic state that transitions to a completely new community with a different composition in older communities.

The results also support the hypotheses that the accumulation of chemically and physically heterogeneous habitat features and spatial isolation could play a role in community composition and diversity (Zhou *et al*., 2002). While surface area, a proxy for spatial isolation, follows the same trend as the increase in diversity and richness with soil age and development, the causal basis for this correlation is not known. Based upon the amount of prokaryotic fatty acids in

soils and assuming that a typical prokaryotic cell possesses a cross section of $10^{-12}\,m^2$ and $2.5 \times 10^{-14}\,g$ of fatty acids (Neidhardt & Umbarger, 1996), the percentage of the surface area that could be occupied by a monolayer of prokaryotic cells drops from 100% in the 5K soils to 10% in the 16K soils and 1% in the 38–77K soils. These calculations illustrate the possibility of a profound change in the spatial environment of cells as these soils mature, where changes in diversity and composition of the bacterial community are directly attributed to changes in surface area or the effect of spatial isolation. However, the surface area of the chronosequence soils is much less than typical soils, which have surface areas that range from 10 to $100\,m^2\,g^{-1}$. Compared with other studies on bacterial diversity in soils using similar methods, the richness (number of unique sequences/number of clones) of the older soils in the Altahama chronosequence is very similar to loam forest soils (Upchurch *et al.*, in press). These results suggest that while low surface area could play an important role in affecting community composition in coarse textured immature soils, it is unlikely to play a direct role in finely textured mature soils.

FAME analysis was also used to assess changes in microbial communities with developmental soil age. Unlike the clone libraries, fatty acid analysis provides a much lower resolution for differentiating the abundance of specific community members. In contrast, it provided a glimpse at the broader scope of community change that included eukaryotes. The fatty acid that was predominantly responsible for the spread of the data in the Bray-Curtis ordination (Axis 2) was a typical eukaryotic and often times a fungal biomarker (18:2 ω6,9). The increasing abundance of this fatty acid compared with the bacterial biomarkers with soil age suggests that other important changes in community structure are occurring simultaneously with changes in the bacterial communities. Fungi dominate the eukaryotic biomass in most soils, and the increase observed here is similar to the ecosystem succession documented in glacial moraines (Ohtonen *et al.*, 1999). However, a retrogressive shift in soil organic matter and fungal biomass has been documented once an ecosystem aggrades and moves into a decline phase (Williamson *et al.*, 2005). It remains to be seen, however, whether the large increase in relative fungal abundance co-occur with changes in the composition of the fungal community.

The most complete assessment of how the diversity and richness of soil bacterial communities varies with developmental stage of an ecosystem have been reported. While there remains little doubt that soils are good reservoirs of microorganisms, there is still an incomplete understanding of which emergent soil and ecosystem properties promote high degrees of microbial diversity. Nevertheless, the authors have contributed to this understanding by showing that development of the soil ecosystem can foster greater diversity and promote the development of distinctive microbial communities that are reminiscent of successional processes often evoked to describe change during the development of plant communities in terrestrial ecosystems. In a world dominated by managed and largely disturbed soil systems, this work suggests that a better understanding of how soils naturally vary in composition may shed further light on the issue of how soils harbour diverse microbial systems. Further studies that can more fully understand how heterogeneity of soil communities vary through both space and time will be valuable in helping to decipher why soils harbour such high levels of microbial diversity.

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