ELSEVIER

Soil Biology & Biochemistry 39 (2007) 2496–2507

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Characterization of developing microbial communities in Mount St. Helens pyroclastic substrate

A. Mark Ibekwe[a,*], Ann C. Kennedy[b], Jonathan J. Halvorson[c], Ching-Hong Yang[d]

[a]*USDA-ARS, US Salinity Laboratory, 450 West Big Springs Road, Riverside, CA 92507, USA*
[b]*USDA-ARS, 215 Johnson Hall, Washington State University, Pullman, WA 99164, USA*
[c]*USDA-ARS, Appalachian Farming Systems Research Center, Beavers, WV 25813, USA*
[d]*Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA*

Received 30 November 2006; received in revised form 3 May 2007; accepted 8 May 2007
Available online 11 June 2007

Abstract

The 1980 eruption of Mount St. Helens created a unique opportunity to study microbial communities in a developing soil ecosystem containing little total carbon (C) or total nitrogen (N). We collected surface samples (0–5 cm) from areas near Mount St. Helens National Volcanic Monument 17 years after the eruption. The samples were from bare soil with no plant development, soil under living prairie lupine (*Lupinus lepidus*) and dead prairie lupine in the pyroclastic plain near Spirit Lake, Washington. We also collected soil from a nearby forested area. Phospholipid fatty acids (PLFAs) from pyroclastic materials were analyzed to determine changes in soil microbial composition. Total bacterial DNA was also extracted from the soils and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes and DNA sequence analysis of cloned 16S rRNA gene libraries were used to determine the influence of plants on microbial development. Both principal components analysis (PCA) of PLFA fingerprints and non-metric multidimensional scaling (NMS) of DGGE fingerprints distinguished the four soils. Lupine plants influenced the PLFA and DGGE fingerprints depending on the distance of the samples from the plant. DGGE and PLFA profiles from the forest soil were significantly different ($P = 0.001$, based on Monte Carlo permutation test) from those of the bare soil and soil with live lupine. Bacterial clone libraries were constructed, and 800 clones were analyzed by amplified ribosomal DNA restriction analysis (ARDRA) and grouped into operational taxonomic units (OTUs). A total of 51, 77, 58, and 42 different OTUs were obtained from forest soil, soil with live and dead lupine, and bare soil, respectively. Phylogenetic analysis revealed that 62% of the 228 OTUs were classified as *Proteobacteria*, *Actinobacteria*, *Acidobacterium*, *Verrucomicrobia*, *Bacteroides*, *Cyanobacteria, Planctomycetes*, and candidate divisions TM7 and OP10. Members of *Proteobacteria* represented 29% of the OTUs. Thirty-eight percent of the OTUs could not be classified into known bacterial divisions. This study emphasized the role of prairie lupine in the establishment of pioneering microbial communities and the subsequent roles the biotic components played in improving the quality of pyroclastic soil.
Published by Elsevier Ltd.

Keywords: Community structure; Pioneering communities; Recultivation; Carbon (C); Nitrogen (N); Mount St. Helens; Phospholipid fatty acid (PLFA); Pyroclastic substrate; Denaturing gradient gel electrophoresis (DGGE)

1. Introduction

There was wide-spread devastation following the 1980 eruption of Mount St. Helens in southwest Washington State; however, soil ecosystem succession began soon after. Two species of lupines, broadleaf lupine (*Lupinus latifolius*), an upright deciduous species, and prairie lupine (*L. lepidus*), a prostrate wintergreen species (Braatne and Chapin, 1986; Braatne and Bliss, 1999) were among the initial colonists of pyroclastic deposits at Mount St. Helens. They were successful in the nutrient-poor volcanic substrates in part due to their symbiotic relationship with *Rhizobium*, allowing them to fix atmospheric N_2 (Halvorson et al., 1991a). Photosynthesis, litter input, and symbiotic nitrogen fixation by lupines contributed to the formation of

*Corresponding author. Tel.: +1 951 369 4828; fax: +1 951 342 4963.
E-mail address: aibekwe@ussl.ars.usda.gov (A. Mark Ibekwe).

0038-0717/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.soilbio.2007.05.010

highly localized soil-resource islands, characterized by higher concentrations of carbon and nitrogen that are believed to support larger and more diverse active populations of heterotrophic soil microorganisms (Halvorson et al., 1991b, 2005; Halvorson and Smith, 1995). As unique and important integrators of the C and N cycles, lupines growing in the most volcanically disturbed sites near Mount St. Helens influence plant succession, ecosystem development, and increase the fertility of the pyroclastic soils. We thus hypothesized that lupines would increase the complexity of soil microbial community structure in pyroclastic substrate.

While a number of studies have tracked plant development at Mount St. Helens (del Moral, 1988; Halpern and Harmon, 1983; Braatne, 1989; Morris and Wood, 1989), fewer have studied the details of microbial colonization and resumption of biogeochemical processes (Halvorson et al., 1991a, 2005; Allen et al., 2005; Parmenter, 2005). Unfortunately, some of these studies used culture-based techniques, which are limited because only a small percentage of all microorganisms thought to exist in soil can be cultured. Few have incorporated culture-independent techniques into their studies (Halvorson et al., 2005).

This report presents three culture-independent strategies to examine microbial community development in pyroclastic soil 17 years after volcanic eruption. The three methods used were phospholipid fatty acid (PLFA), 16S rRNA analysis of DGGE, and cloning and sequencing of total community DNA from soil. Phospholipid fatty acid analysis can be used to quantify community structure and biomass without relying upon cultivation of microorganisms. This approach produces descriptions of microbial communities based on functional groupings of fatty acid profiles; however, it does not have the capability to identify microorganisms at the species or strain levels. This concept has resulted in the identification and quantification of viable biomass and community structure in sediments (Balkwill et al., 1988; Rajendran et al., 1992; Ringelberg et al., 1988) and in agricultural soils (Zelles et al., 1992, 1995; Ibekwe and Kennedy, 1998; Ibekwe et al., 2002) as well as in forest and grassland soils (Pennanen, 2001; Leckie, 2005). Microbial community analysis using 16S rRNA genes can detect and identify community members with high specificity to the species and strain levels, and can detect and suggest phylogenetic affinities of uncultured organisms (Amann et al., 1995). Our objective was to investigate microbial community composition and structure in pyroclastic soils with living and dead plant material, and no plant material, as well as the adjacent forest soil.

2. Materials and methods

2.1. Site selection and sampling

Surface soil (0–5 cm) was collected from bare soil with no plant development, soil under living prairie lupine (*L. lepidus*), dead prairie lupine, as well as from adjacent forest soils near Spirit Lake, Washington, USA in September 1997. All soil samples, except forest soil, were collected from the Pumice Plain pyroclastic-flow deposits (46°12′N 122°11′W) at an elevation of about 1160 m near Spirit Lake. The substratum is rocky volcanic material deposited by numerous surface pyroclastic flows that occurred on the north face of Mount St. Helens. The first three soils consisted of a coarse pyroclastic material consisting largely of small plant material and sand. The average pH of the three pyroclastic soils was 5.90. The forest samples were collected from underneath live plants where volcanic ash did not kill vegetation and approximately 200 m from the forest edge at Bear Meadow (46°311′N 122°028′W). The soil from the forest site was characterized by high organic matter A soil horizon. The forest soils had a pH of 5.65. All soils were placed in plastic bags on ice in a cooler for transport to the laboratory, and were then frozen at −75 °C until analyzed. In the laboratory, soil samples that had been stored at −75 °C were taken from the freezer, sieved to pass a 4.0-mm screen for microbiological analysis, and then air dried at 25 °C for chemical analysis. Total C and N were determined by the dry combustion method at 1350 °C with a LECO CNS 2000 analyzer (LECO, St. Joseph, MI). Total C was measured by infrared spectrometry and total N was measured by thermal conductivity.

2.2. Phospholipid extraction, separation, and analysis

Soil samples (5 g, $n = 5$) were extracted by using the modified method of Bligh and Dyer as described by Petersen and Klug (1994). The total lipid extract was fractionated into glyco-, neutral, and polar lipids (Ibekwe and Kennedy, 1998). The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane. The PLFAs were separated, quantified and identified by Gas Chromatography-flame ionization detection (MacNaughton et al., 1999). Samples were run for 38 min, long enough for fatty acids with up to 28 carbons to elute from the column using the column and run protocol as described by Ibekwe and Kennedy (1998). The system consisted of a GC (HP6890-Hewlett Packard, Wilmington, DE) with a flame ionization detector and HP 3365 ChemStation software.

2.3. Fatty acid nomenclature

The suffixes "-c" for *cis* and "-t" for *trans* refer to geometric isomers. The prefixes "i-," "a-," and "me-" refer to iso-, anteisomethyl, and mid-chain methyl branching, respectively, with cyclopropyl rings indicated by "cy" (Kates, 1986).

2.4. DNA extraction, PCR conditions, and DGGE analysis

Total bacterial community DNA was extracted by placing about 500 mg of soil in Fast DNA spin kit for

soil (BIO 101, Vista, CA) containing lysing matrix. Isolation of total DNA was accomplished with a FastPrep DNA isolation kit according to the manufacturer's protocol (BIO 101). The PCR procedure was first performed using 20–80 ng of templates DNA for DGGE analysis with the primers, PRBA338f and PRUN518r, located at the V3 region of the 16S rRNA genes of bacterioplankton (Øvreås et al., 1997) to assess bacterial community diversity. The PCR mixtures for bacterial 16S rRNA sequence amplification contained 10 pmol of primers, 4 μg of bovine serum albumin, and sterile distilled water for a final volume of 25 μl. The PCR cycles used for amplification were as follows: 92 °C for 2 min followed by 30 cycles of 92 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min; and finally at 72 °C for 6 min. DGGE was performed with 8% (wt./vol.) acrylamide gels containing a linear chemical gradient ranging from 30% to 70% denaturant with 100% defined as 7 M urea and 40% formamide. Gels were run for 3 h at 200 V with a Dcode^{TM} Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after ethidium bromide staining by UV transillumination and photographed with a Polaroid camera. Major bands were excised for identification of bacterial species. Bands were placed into sterilized vials with 20 μl of sterilized distilled water and stored overnight at 4 °C to allow the DNA to passively diffuse out of the gel strips. Ten microliters of eluted DNA was used as the DNA template for bacterial primers. The sizes of the PCR products were checked on a 1.5% agarose gel, and the DNA was cloned into a pGEM-T Easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109. Isolation of plasmids from *E. coli* was performed using standard protocols from Qiagen plasmid mini kit (Valencia, CA). The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (ABI Perkin-Elmer, Boston, MA).

2.5. Amplification of 16S rRNA genes for phylogenetic analysis

16S rRNA genes were amplified from soil-extracted DNA for phylogenetic analysis of bacterial species. Each PCR consisted of One Ready-To-Go PCR bead (Amersham Pharmacia GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 2 μl of forward primer 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). Amplification of the 16S rRNA was done using the following steps: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 7 min. The 1500 bp PCR products were excised from gel, purified and cloned directly into a pGEM-T Easy vector (Promega, Madison, WI) as described above. Isolation of plasmids from *E. coli* was performed using standard protocols from the Qiagen plasmid mini kit (Valencia, CA). From the four soil samples, plasmid DNA was isolated from 200 colonies from each soil. Colonies were screened by *Eco*RI restriction endonuclease digestions for inserts and digested for 3 h at 37 °C. Digestion products were visualized on a 1.5% agarose gel to ensure the presence of inserts of the expected size. For each soil, all 200 colonies (clones) had the correct size of insert and were selected and amplified. Ribosomal DNA restriction analysis (ARDRA) was performed to assess different bacterial species from the different soils by digesting 5 μl of each sample (800 samples in total) for 3 h at 37 °C with 2.5 U of *HinfI* and *HhaI*, (New England Biolabs, Ipswich, MA) in 20-μl reaction mixtures. Restriction patterns were visualized on a 3% agarose gel in 1 × TAE buffer and stained with ethidium bromide (1% [wt./vol.]). Gel images were digitalized, and restriction patterns were analyzed using the Image master as previously stated (Ibekwe et al., 2001). Each different restriction pattern was defined as an operational taxonomical unit (OTU). DNA plasmids from one of each different representative OTU was commercially sequenced with an Applied Biosystems Prism 377 DNA sequencer using universal M13 forward and reverse primers. DNA sequences were edited manually to correct falsely identified bases and trimmed at both the 5′ and 3′ ends using the Chromas software (Technelysium Pty. Ltd, Helensvale, Australia). Only sequences with unambiguous reads of about 600 bp were used for further analysis.

2.6. Statistical analysis of PLFA profiles, DGGE bands and 16S rRNA gene sequence

Principal components analysis (PCA) in the PC-ORD version 5 (McCune and Mefford, 1999) was used to compare PLFA profiles among the four soils as well as show relationships among different samples that contain multiple variables (PLFAs). We used PCA because the PLFA data set approximated multivariate normality and variables have linear relation (Jolliffe, 1986). These are the major assumptions for using PCA. Data were then presented as a 2D plot for better understanding of the relationship.

DNA fingerprints obtained from the 16S rRNA banding patterns on DGGE gels were photographed and digitized using Image Master Labscan (Amersham-Pharmacia Biotech, Uppsala, Sweden). The lanes were normalized to contain the same amount of total signal after background subtraction which was carried out using the rolling disk mechanism. The gel images were straightened and aligned using Image Master 1D Elite 3.01 (Amersham-Pharmacia Biotech, Uppsala, Sweden) and analyzed to give a densitometric curve for each gel (Ibekwe et al., 2001). All analyses were compared to the software generated synthetic marker and were used as reference to align DGGE tracks and standardize internal distortions. Band positions were converted to Rf values between 0 and 1, and profile similarity was calculated by non-metric multidimensional

scaling (NMS) (McCune and Mefford, 1999) as an alternative method to PCA for sample ordination purposes. NMS is an iterative ordination method that is well suited to data that are non-normal or are on arbitrary, discontinuous, or otherwise questionable scales. For analysis of DGGE fingerprints by NMS, a binary matrix showing presence (1) and absence (0) of DGGE bands was used. This matrix was used for NMS without transformation. Subsequently, NMS analysis was applied to data to compare their ability to discriminate microbial communities from the four soils. We performed NMS analysis based on Sørensen's distance, and the "slow and thorough" autopilot mode of NMS in PC-ORD (McCune and Mefford, 1999) using randomized data for a Monte Carlo test of significance. Final stability of each run was evaluated by examining plots of stress (a measure of the dissimilarity between ordinations in the original *n*-dimensional space and in the reduced dimensional space) versus number of iterations.

The comparison of the different measured richness, evenness, and diversity was done by using a one-way analysis of variance, and Tukey HSD test for post hoc analysis (SAS, 1988). Richness (S) refers to the number of PLFAs or bands detected in a given soil sample. The PLFA or DGGE evenness (E), a measure of how evenly PLFAs or DGGE bands were distributed in a given soil sample, was calculated as $E = H/\ln(S)$. Diversity was calculated by using the Shannon index of diversity (H) to compare changes in diversity of microbial communities within all treatments at each time (Shannon and Weaver, 1963) by using the following function:

$$H = -\sum P_i \log P_i,$$

when $P_i = n_i/N$, n_i is the height of peak, and N is the sum of all peak heights in the curve.

For phylogenetic analysis, clone sequences were BLAST analyzed using a 16S rRNA database containing over 33,000 sequences (Cole et al., 2003). The predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences from the BLAST gene bank (ftp://ftp.ncbi/nih.gov/blast/executables/LATEST) and a FASTA-formatted file containing the predicted 16S rRNA sequences. Matrices of evolutionary distances were computed using the Phylip program with the Jukes–Cantor model (Jukes and Cantor, 1969). Phylogenetic trees were constructed and checked by bootstrap analysis (1000 data sets) using the program SEQBOOT. Bootstrap values represented the frequency of resampling that supported a specific branching pattern. For dendrogram construction, partial 16S rRNA sequences representing the most prevalent OTUs from the dominant phylum were aligned using CLUSTALX version 1·8 for Windows (Thompson et al., 1997). Also included in this alignment were the most similar 16S rRNA sequences to each OTU from the NCBI databases.

3. Results

3.1. Carbon and nitrogen accumulation in soil

Soil organic carbon is an important feature in the development of a microbial community in any soil environment. The adjacent forest soils had soil organic carbon levels close to 1% and soil nitrogen was 0.04%. The soil organic carbon levels of the pyroclastic soils were 0.6% under the dead lupines and 0.37% under the live lupines but were ~0.05% in the bare soil. Total soil carbon under forest, live or dead lupine plants was significantly higher than the concentration in bare soil ($P<0.05$; Fig. 1). More C and N under lupines suggest the influence of nutrient inputs from leaf litter and root inputs. Carbon in surface soil under lupines has been reported to be strongly and positively linearly correlated with nitrogen (Halvorson and Smith, 1995).

3.2. Microbial community analysis of the four soils by principal components analysis

PLFA profiles from the four soils were significantly different ($P = 0.001$, based on Monte Carlo permutation test of PC-ORD) from each other. PCA of PLFA profiles is presented in Fig. 2a. PCA was conducted with 25 fatty acids that formed major components in the samples. In our analysis, we computed the correlation between principal components and PLFAs for PC1 and PC2. In PCA, the eigenvectors determine the directions of maximum variability and the eigenvalues specify the variances. Based on this information, 25 PLFAs with the highest loadings on the PCA were used to construct the bi-plot using PC-ORD. Results of this analysis showed most samples of the forest soil clustered away from bare soils with only pyroclastic material (Fig. 2a) resulting in four clusters. The percentage of variance explained by PC1 was 37%, while PC2 explained 20% of the variance, for a cumulative total of

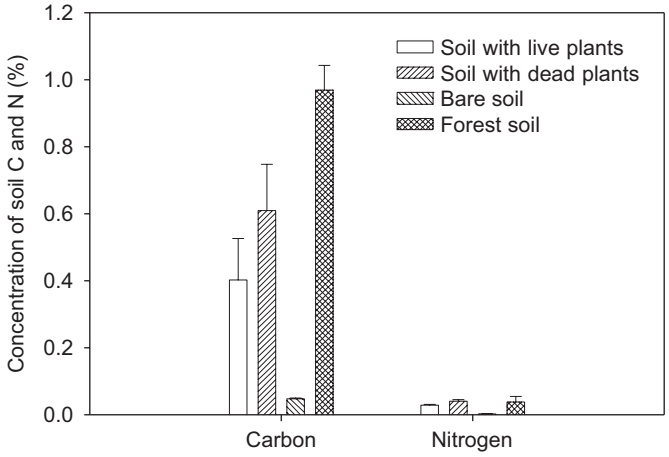

Fig. 1. Concentration of soil carbon and nitrogen in Mount St. Helens pyroclastic deposits. Standard errors were derived from means of five samples collected at 0–5 cm.

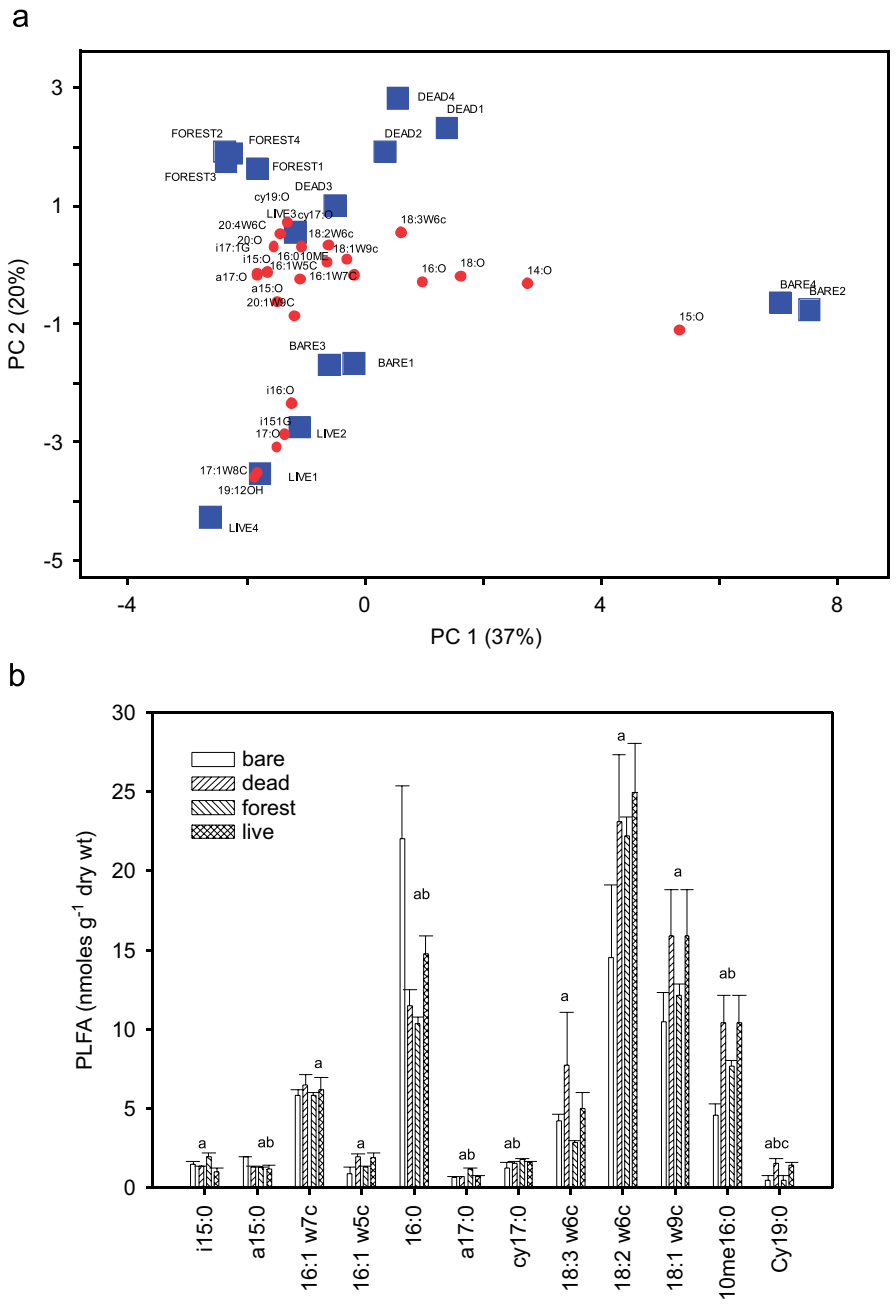

Fig. 2. Principal components analysis calculated from PLFA of pyroclastic deposits from bare soil without plants, from under live lupine plants, from soil under dead plant material, and from forest soil. Data are from soil samples (0–5 cm depth) collected in 1997. (a) A scatterplot of the PCA data using two ordination axes. Closed squares represent sample units (the four soils) and closed circles represent fatty acids. (b) Concentration of biomarker PLFA in pyroclastic materials from (a) indicating sample points. Bars represent standard errors of values from each soil.

57%. A scree plot analysis indicated that no significant information would be gained by evaluating the third principal component (PC3 = 15%). Most of the bare soils formed distinct clusters along PC1, while soil with live lupine, dead lupine, and forest soil clustered along PC2 (Fig. 2a). The specific fatty acids or species responsible for the differences among the four soils are plotted in Fig. 2a using the same ordination with sample units. Analysis by PC-ORD showed that straight-chain PLFA weighted more on PC1 (PC1 = 14:0, 15:0, 16:0, 18:0,) with correlation coefficient $\geqslant 0.50$ whereas PC2 was heavily weighted by branched, monounsaturated, and cyclopropane PLFAs (a15:0, i15:0, i16:0, 16:1ω7c, a17:0, i17:0, cy17:0, 18:1ω9c, cy19:0) with correlation coefficient $\leqslant 0.30$. Most of the fatty acids in PC2 are primarily of bacterial origin, indicating that microbial community development was aided by the presence of plants, whereas PC1 contained general biomarker fatty acids.

Analysis of PLFA from Fig. 2a showed marked significant variations in PLFA contents among sample soils. The content of individual PLFA peaks ranged from a minimum of $\leqslant 1$ nmol to a maximum of $\geqslant 25$ nmol per g

(dry weight) for the four soils (Fig. 2b). Biomass contents, as indicated by total PLFA, were significantly different for some soils ($P<0.05$). The biomarker fatty acids with the highest relative abundance in the samples were 16:0, 18:2ω6c and 18:1ω9c, and 10me16:0. The biomarker PLFAs along PC2 (i15:0, a15:0, i17:0, a17:0, 16:1ω7c, 16:1ω5c, cy17:0, cy19:0, 18:3ω6c) were also major components. Differences in concentrations were significant for six of the PLFA biomarker groups (Fig. 2b). In these soils, the polyunsaturated PLFAs, mostly 18:2ω6c, characteristic of fungi (Wilkinson, 1988), had the highest PLFA concentrations of all the markers. Hydroxy PLFA compositions (data not shown) in the four soils were lower than cyclopropane PLFA, and these PLFAs are characteristic of Gram-negative bacteria (Ratledge and Wilkinson, 1988). Branched PLFAs, characteristic of Gram-positive bacteria (White, 1983; Federle, 1986), were also higher in all the soils than the hydroxy group. However, there was a significant increase in PLFA for the actinomycetes (10me16:0) biomarker, as the soils with live and dead lupine plants showed significantly higher concentrations of this marker than the forest or bare soils (Fig. 2b).

3.3. Microbial community structure by DGGE

Examination of DNA band profile in the DGGE gel (Fig. 3a) by Image Master ID revealed that the communities consisted of complex banding patterns with a total of 147 bands (species) distributed in 118 electrophoretic positions. A breakdown of the band frequency showed

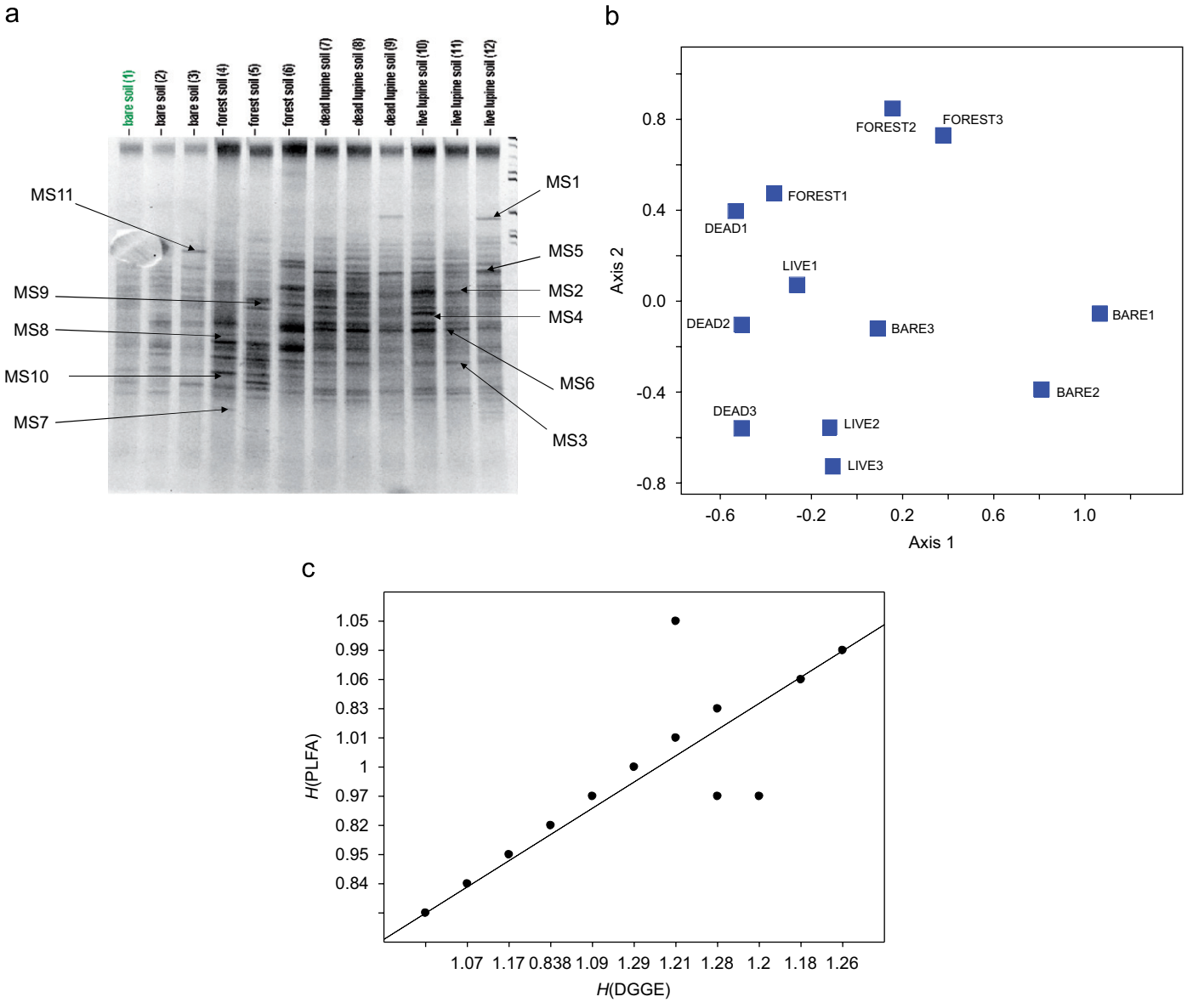

Fig. 3. DGGE patterns (a) produced from 16S rRNA analysis of replicate samples of soil from Mount St. Helen deposits. Amplified products were separated on a gradient gel of 30–70% denaturant. All labeled bands were excised from the gel, reamplified, and subjected to sequence analysis. The reamplified products were cloned and screened as described in the text. (b) The scatter plot shows an NMS ordination with closed squares representing sample units (the four soils). (c) Relationship between pyroclastic soil microbial diversity index (H) obtained by PLFA and DGGE.

that averages of 13, 19, 18, and 20 bands were detected from bare, forest, soil with dead lupine, and soil with live lupine, respectively. The DGGE profiles have bands in close proximity, but band analysis showed that few bands shared the same electrophoretic mobility, suggesting diverse bacterial species in the samples. For the direct comparison of microbial communities of all DGGE profiles in the four soils, NMS ordination was used to create a scatterplot to examine the relationship between species and sample units (Fig. 3b). DGGE profiles from the forest soil were significantly different ($P = 0.001$, based on Monte Carlo permutation test) from those of the bare soil and soil with live lupine. DGGE profiles from forest soils and soil with dead lupines were not significantly different from each other (Fig. 3b). The species diversity values (H') calculated from PLFA and DGGE data from the four soils were compared (Fig. 3c) by Pearson correlation. Pearson correlation was 0.92 with the outlier excluded, but 0.52 with outlier included resulting in a P value of 0.071. The outliers were included because they strongly influence the correlation matrix along the first axis of the PCA that contributed to the separation between the PC1 and PC2. This resulted in a stronger agreement between the PLFA and DGGE data.

Several diversity indices for PLFA and DGGE were used to determine effective methods for microbial community analysis (Table 1). The richness (or number of peaks) was not significantly different ($P = 0.65$) between the two methods of analysis. No significant differences were observed in evenness and diversity between the DGGE method and PLFA. When both richness and diversity indices were compared in different soils, there were significant differences between soils with live lupine compared to bare soil (Table 1). Both methods showed that soils with live lupine had a significantly more diverse microbial community than the bare soils.

The analysis of predominant bacterial species was carried out with soil samples collected in triplicate from soil with live lupine, dead lupine, bare, and forest soils. Bands selected for analysis are shown in Fig. 3a, while Table 2 shows the 11 prominent bands recovered from the DGGE gel. These dominant bands were excised and sequenced for band identification. The derived sequences from these bands confirmed seven of the sequences were 100% similar to sequences in the database, and four were 99% similar. Most of the bands were present in more than one soil except bands ms9 and ms10 that were present only in forest soils. These two bands, and bands ms5 and ms8, are from the *Firmicute* phylum that had previously been identified (Kop et al., 1984). The appearance of another band (ms2) in the soil with live and dead plants and forest soil was an indication of the influence of legumes on microbial development in the pyroclastic materials. This band showed a 100% relationship to an uncultured eubacterium derived from the rhizosphere of *Trifolium repens* (Mailley and Aragno, 1999).

Table 1
Diversity indices calculated from PCR-DGGE and PLFA data

Method	Soil	Richness	Evenness	Diversity
PLFA	Live lupine	20.0±1.70a	0.33±0.01a	1.01±0.24a
	Bare-no plants	13.6±3.70b	0.34±0.02a	0.87±0.04b
	Dead lupine	16.0±2.30ab	0.34±0.01a	0.95±0.07ab
	Forest	17.3±0.33ab	0.35±0.01a	0.99±0.01ab
PCR-DGGE	Live lupine	20.3±0.70a	0.42±0.00a	1.25±0.02a
	Bare-no plants	12.7±1.70b	0.35±0.04a	0.87±0.04b
	Dead lupine	18.3±1.30ab	0.42±0.00a	1.22±0.03ab
	Forest	18.6±1.60ab	0.41±0.01a	1.20±0.06ab

Values are means±standard errors ($n = 4$) for PLFA and ($n = 3$) for PCR-DGGE data.
Means with the same letters are not significantly different by Tukey's studentized test.

3.4. Phylogenetic analysis of bacterial diversity based on 16S rRNA

A total of 200 clones were analyzed from each soil to estimate the bacterial diversity in the four soils (Table 3). The sequence analysis grouped the clones into 228 OTUs. On the basis of the phylogenetic analysis, the 228 OTUs were affiliated with at least ten bacterial phyla, including unidentified bacterium. The most dominant group of bacteria was allocated to an unidentified bacterium followed by the phylum *Proteobacteria* (29% of the total number of clones) (Fig. 4a). The dominant organisms within the class were in the alpha, gamma, beta, and delta classes (Table 3). The third most dominant group of the library, represented by 28 OTUs (19%), was classified into the phylum *Actinobacteria* (Fig. 4b). The other groups in the library were, in order of abundance, within phyla *Acidobacteria* (Fig. 4c), *Verrucomicrobia*, *Cyanobacteria,* and *Planctomycetes*. Some OTUs affiliated with the candidate phyla OP10 and TM7 were also observed. Soil with live lupine has the most diverse population of bacteria, followed by soil with dead lupine (Table 3).

4. Discussion

Our results showed that microbial community structure characterized by PLFA was strongly influenced by plants resulting in the development of specific bacterial functional groups (Figs. 2a and b). There were significant differences between Gram-positive and Gram-negative bacterial indicators in the four soils. Samples taken directly from where plants had been growing showed significantly higher concentrations of biomarkers for Gram-negative bacteria, indicating the profound effect plants have on soil development and lipid profiles. Biomass from PLFA is a strong indicator of bacterial composition since it indicates active living microbial components. Biomass from phospholipid analysis has been found to be more representative of the microbial biomass than that obtained from the chloroform-fumigation method (Jordan et al., 1995). In our study, PLFA offers a powerful approach to demonstrate

Table 2
Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rRNA extracted from Mount St. Helens pyroclastic deposits

Bands	Related bacterial sequences	% similarity	Samples present[a]	Accession no.
ms1	*Desulfovibrio aminophilus*	100	L, D	AF067964
ms2	Uncultured eubacterium	100	L, D, F	AJ232804
ms3	*Pseudomonas* sp. QSSC1-9	99	All samples	AF170731
ms4	Uncultured bacterium C3	100	L, D	AF204684
ms5	*Brevibacillus brevis*	100	All samples	M10111
ms6	*Pseudomonas* sp. G2	99	All samples	AF326356
ms7	Uncultured bacterium WD235	99	All samples	AJ292680
ms8	Uncultured *Actinobacterium QSSC8L-12*	100	All samples	AF170772
ms9	*Megasphaera elsdenii*	100	F	U95029
ms10	*Streptomyces autolyticus*	99	F	AF056716
ms11	Uncultured bacterium *G81*	100	B, F	AJ011365

[a]B: soil from sites without plants, D: soil with dead lupine plants, F: soil from the forest area, and L: soil from locations with live lupine plants.

Table 3
Phylogenetic affiliations of 16S rRNA genes belonging to the domain *Bacteria*

Phylum	Forest OTUs	Live OTUs	Dead OTUs	Bare OTUs	% of total	% similarity
Proteobacteria					29	
Alpha	16	18	12	9	*15*	92–100
Beta	1	6	1	3	*5*	91–99
Gamma	2	4	10	2	*9*	96–100
Delta	2	0	0	0	*0.9*	95–100
Acidobacteria	6	4	4	1	6	96–100
Actinobacteria	14	4	8	9	19	96–98
Division OP10	2	4	0	0	0.9	96–98
Division TM7	0	4	0	0	0.9	96–98
Verrucomicrobia	0	2	1	1	1.8	97–99
Cyanobacteria	0	4	0	0	1.8	97–100
Bacteroides	0	3	2	1	1.8	96–99
Planctomycetes	1	0	0	0	0.9	97
Unidentified	20	22	22	10	38	92–100
Total	51	77	58	42	100	

[a]Number of OTUs was defined as a group of sequences with >98% similarity.

change in soil microbial community structure. On the other hand, PLFA offers limited insight into changes in specific microbial populations. It is well documented that certain PLFAs can be used as biomarkers for specific populations (White and Ringelberg, 1998); however, the resolution of population level can change within communities. Ramsey et al. (2006) noted several factors including: (1) overlap in the PLFA biomarker composition of microorganisms; (2) determination of signature PLFAs for specific microbes requires their isolation in pure culture; and (3) PLFA patterns for individual populations can vary in response to environmental stimuli. Therefore, where population level information is needed, PCR-based methods offer avenues for hypothesis testing not available through PLFA. We first used PLFA to detect differences in the four soils, and subsequently PCR-DGGE and phylogenetic analysis based methods were used to resolve population differences in the soils.

Halvorson et al. (1991b) reported very low amounts of active soil microbial biomass carbon and cumulative respiration in pyroclastic-flow soil in 1987. During that study, overall microbial activity was correlated with soil carbon and nitrogen and was significantly higher in lupine root zones than in uncolonized soil. Samples of pyroclastic soil collected in 1990 were also used to detect patterns of soil microbial biomass carbon and carbon and nitrogen mineralization from lupine biomass-amended and non-amended soils (Halvorson and Smith, 1995). Surface soil under broadleaf lupine contained the most microbial carbon, followed by dead lupine and uncolonized soil, respectively. These authors found that the average soil microbial biomass carbon to soil carbon ratio in the 0- to 5-cm depth under lupines was about 24%, and in uncolonized soil 10%, higher than in samples collected only 3 years earlier. Halvorson et al. (2005) continued to monitor biological indicators of soil quality under lupine in 2000. Soil collected under lupines contained significantly more microbial-biomass carbon than did uncolonized soil, a value about two orders of magnitude greater than in 1987 and about a 67% increase from 1990. Development of larger or more active microbial populations under lupines was also reflected in significantly more soil dehydrogenase

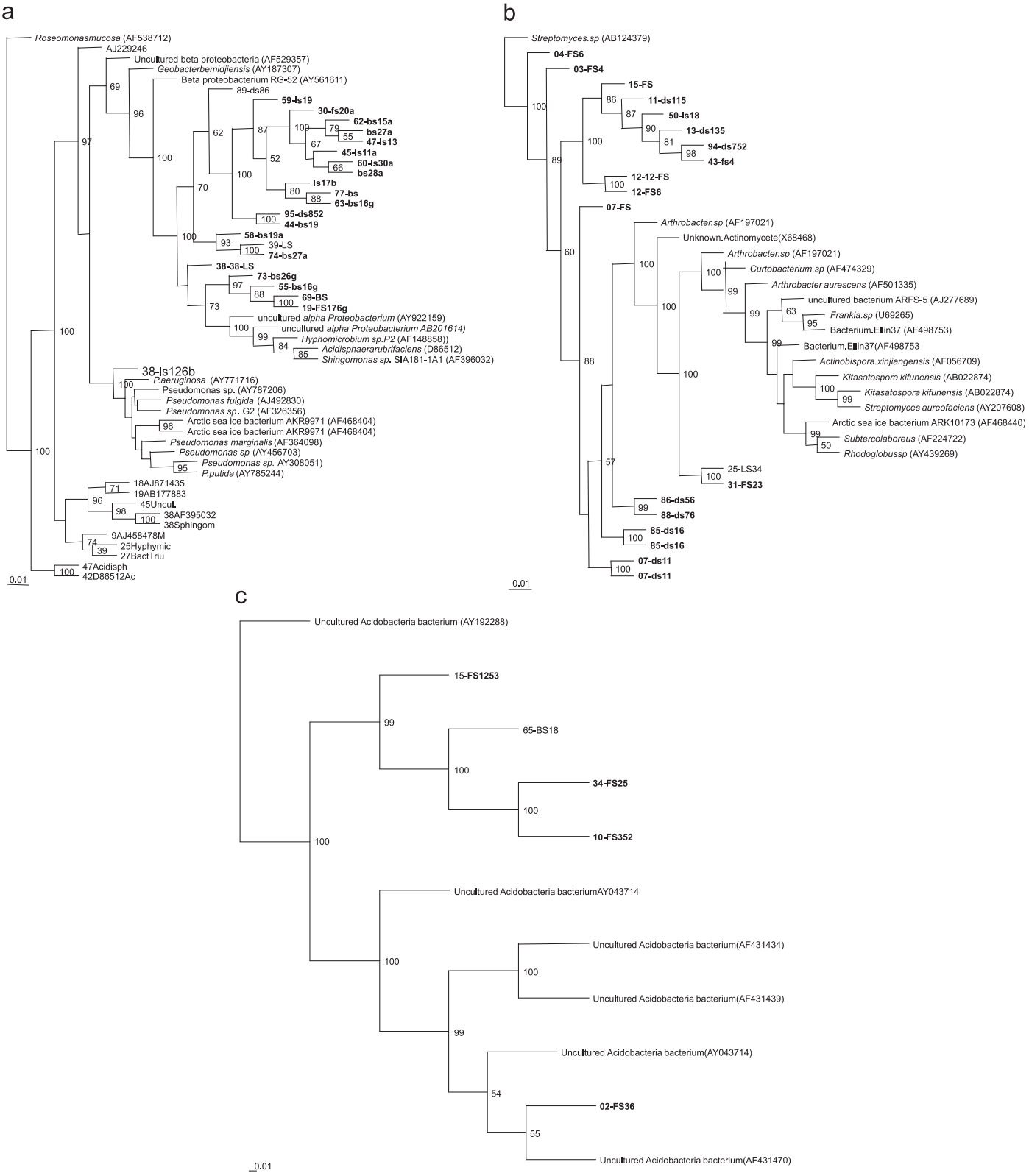

Fig. 4. Neighbor-joining analysis of 16S rRNA gene sequences from cloned 16S rRNA pyroclastic material for (a) *Proteobacteria*, (b) *Actinobacteria*, (c) *Acidobacteria*. All clones recovered from this study are marked in bold. Numbers at the nodes represent values of 1000 bootstrap replicates that support each node. Bootstrap values are not shown if less that 50%. Scale at the nodes are 0.01.

and phosphatase, important enzymes that affect biological oxidation of soil organic matter and plant nutrition, and in a higher metabolic quotient (qCO_2), the amount of basal respiration per unit of soil microbial biomass. The increase in microbial activities suggests increase in microbial composition of the lupine rhizosphere as the agent

responsible for the development of new communities. In our study, we examined total microbial populations in the four soils to determine the major bacterial composition responsible for the successional changes before1987 and up to 1997. A large proportion of the sequences from the four soils, approximately 29%, belonged to the *Proteobacteria* division with most of the sequences retrieved under live lupine plants and only 6% of the sequences were from *Acidobacterium* division, of which sequences are detected in soil worldwide (Barns et al., 1999; Dunbar et al., 1999). The low level of *Acidobacteria* in our soils may be an indication that the soils are still developing their microbial populations. Mailley and Aragno (1999) found that the rhizosphere, which is a relatively nutrient-rich niche for bacteria, has a positive selection for *Proteobacteria* and reduced percentage of *Acidobacterium* division. Therefore, the dominance of *Proteobacteria* sequences in this study may demonstrate that this division contributes most to the pioneering communities of the pyroclastic soils.

The high number of *Proteobacteria* OTUs represented in the soil with live lupine (Fig. 4a; Table 3) suggests dominance of this phylum near the rhizosphere compared to the other samples. This is indicative of the fact that many *Pseudomonas* species are *r*-strategists and have copiotrophic characteristics, and many are associated with plant roots which provide them with nutrients (Smit et al., 2001; Van Overbeek and Van Elsas, 1997). A number of OTUs exhibiting high homology to common rhizosphere species, including *Pseudomonas* species and potential plant symbiont species of the *Rhizobium* group, were obtained from soil with live lupine, but were absent in the forest soil. Our study is in agreement with Van Overbeek and Van Elsas (1997). These authors showed that the growth of *Pseudomonas* species are less favorable to nutrient-limited and relatively warm and dry conditions in bulk soils. Clone 30-ls30a obtained from soil with lupine was closely related to bacterium Ellin335 that was previously isolated from Australian pasture soil (Sait et al., 2002). These authors were the first to cultivate this strain and they concluded that the strain is globally distributed in most soils. It should be noted that the 16S rRNA gene of this strain is very similar to 16S rRNA gene sequences obtained from the rhizoplane of *Brassica napus* (Kaiser et al., 2001) and from other habitats (Benson et al., 2002). Based on the sequence identities (89–100%) between these 16S rRNA genes, these organisms may all fall into at least two unnamed genera according to the authors. They concluded that phylogenetic analysis of 16S rRNA gene sequences obtained from the novel members of the class *alpha-Proteobacteria* isolates Ellin335 and others (Ellin359 and Ellin362), indicates that they comprise a monophyletic cluster unaffiliated to all currently described orders within the *alpha-Proteobacteria*. They likely represent a novel order in this proteobacterial class, with no significant sequence identity to cultivated organisms.

The phylum *Actinobacteria* was more abundant in forest soil (14) than the other soils (Table 3, Fig. 4b). Although *Actinobacteria* appear to be dominant in the forest soil, most of the OTU representatives were associated with subdivision *Actinobacteridae* and seconded by environmental samples with no special subdivision. Although little is known about this phylogenetic group, representatives have been shown to be relatively abundant in soils of a soybean field (Ueda et al., 1995) and grassland (McCaig et al., 1999) and were recently found to be abundant in earthworm casts (Furlong et al., 2002). Phylogenetic analysis revealed that two groups of clones were produced that were quite different from 16S rRNA clone sequences obtained from GeneBank and were members of *Actinobacterium*. They, however, formed their own bootstrap-supported cluster without other GenBank member representatives of *Actinobacterium* (Fig. 4b). Although *Acidobacterium* has few cultivated members, DNA sequences from uncultivated representatives have been frequently recovered from soil (Hugenholtz et al., 1998; Barns et al., 1999) including forest environments. Other clones were closely related to an uncultured *Acidobacteria* (Fig. 4c). *Verrucomicrobium* are highly abundant in many soils, suggesting their ecological importance (Kuske et al., 1997). In this study, although only four *Verrucomicrobium* clones were obtained, two were from soil with live plants.

Lupines strongly influenced PLFA profiles and the diversity of microorganisms in Mount St. Helens pyroclastic flows by providing specific niches for microorganisms to survive and help in the decomposition of plant material and cycling of nutrients. Soils undergoing primary succession are initially very similar to their parent material and are thus strongly influenced by the type and magnitude of the initial site-formation processes (Halvorson et al., 2005). As succession proceeds, soil ecosystems are increasingly influenced by vegetation and the cumulative effects of climate.

This study is the first that has applied both biochemical and molecular methods to study the development of the microbial community composition in Mount St. Helens pyroclastic material since its eruption 17 years from the time of sampling. It shows that microbial community composition was affected by: (1) the proximity to lupine plants, and (2) possibly by different plant types. Living and dead plant materials have a direct and profound impact on whole soil lipid profiles and microbial development in early successional ecosystems.

Acknowledgments

We thank Pamela Fletcher for her assistance in obtaining samples and some of the experimental data. This research was supported in part by the 206 Manure and Byproduct Utilization Project of the USDA-ARS. Mention of trademark or proprietary products in this manuscript does not constitute a guarantee or warranty of the property by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

References

Allen, M.F., Crisafulli, C.M., Morris, S., Egerton-Warburton, L.M., MacMahon, J.A., Trappe, J.M., 2005. Mycorrhizae and Mount St. Helens: story of symbiosis. In: Dale, V.H., Swanson, F.J., Crisafulli, C.M. (Eds.), Ecological Response to the 1980 Eruptions of Mount St. Helens. Springer, New York, NY, pp. 221–231.

Amann, R.L., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143–169.

Balkwill, D.L., Leach, F.L., Wilson, T.J., McNabb, F.J., White, D.C., 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in the subsurface aquifer sediments. Microbial Ecology 16, 73–84.

Barns, S.M., Takala, S.L., Kuske, C.R., 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Applied and Environmental Microbiology 65, 1731–1737.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., Wheeler, D.L., 2002. GenBank. Nucleic Acids Research 30, 17–20.

Braatne, J.H., 1989. Comparative physiology and population ecology of *Lupinus lepidus* and *Lupinus latifolius* colonizing early successional habitats on Mount St. Helens. PhD Dissertation, University of Washington, Seattle, WA, USA.

Braatne, J.H., Bliss, L.C., 1999. Comparative physiological ecology of lupines colonizing early successional habitats on Mount St. Helens. Ecology 80, 891–907.

Braatne, J.H., Chapin, D.L., 1986. Comparative water relations of four subalpine plants at Mount St. Helens. In: Keller, S.A.C. (Ed.), Mount St. Helens: Five Years Later. Eastern Washington University Press, Cheney, Washington, USA, pp. 163–172.

Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., et al., 2003. The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Research 31, 442–443.

del Moral, R., 1988. Dynamics of herbaceous vegetation recovery on Mount St. Helens, Washington, USA after volcanic eruption. Vegetation 74, 11–27.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., Kuske, C.R., 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Applied and Environmental Microbiology 65, 1662–1669.

Federle, T.W., 1986. Microbial distribution in soil—new techniques. In: Megusar, F., Gantar, M. (Eds.), Perspectives in Microbial Ecology. Slovene Society for Microbiology, Ljubljana, pp. 493–498.

Furlong, M.A., Singleton, D.R., Coleman, D.C., Whitman, W., 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and burrows and casts of the earthworm *Lumbricus rebellus*. Applied and Environmental Microbiology 68, 1265–1279.

Halpern, C.B., Harmon, M.E., 1983. Early plant succession on the Muddy River Mudflow Mount St. Helens, Washington. American Midland Naturalist 110, 97–106.

Halvorson, J.J., Smith, J.L., 1995. Decomposition of lupine biomass by soil microorganisms in developing Mount St. Helens pyroclastic soils. Soil Biology & Biochemistry 27, 983–992.

Halvorson, J.J., Black, R.A., Smith, J.L., Franz, E.H., 1991a. Nitrogenase activity, growth, and carbon and nitrogen allocation in wintergreen and deciduous lupine seedlings. Functional Ecology 5, 554–561.

Halvorson, J.J., Smith, J.L., Franz, E.H., 1991b. Lupine influence on soil carbon nitrogen and microbial activity in developing ecosystems at Mount St. Helens. Oecologia (Berlin) 87, 162–170.

Halvorson, J.J., Smith, J.L., Kennedy, A.C., 2005. Lupine effects on soil development and function during early primary succession at Mount St. Helens. In: Dale, V.H., Swanson, F.J., Crisafulli, C.M. (Eds.), Ecological Response to the 1980 Eruptions of Mount St. Helens. Springer, New York, NY, pp. 243–254.

Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology 180, 4765–4772.

Ibekwe, A.M., Kennedy, A.C., 1998. Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. FEMS Microbiology Ecology 26, 151–163.

Ibekwe, A.M., Papiernik, S.K., Gan, J., Yates, S.R., Yang, C.-H., Crowley, D.E., 2001. Impact of fumigants on soil microbial communities. Applied and Environmental Microbiology 67, 3245–3257.

Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.-H., Crowley, D.E., 2002. Microbial diversity along a transect of agronomic zones. FEMS Microbiology Ecology 39, 186–191.

Jolliffe, I.T., 1986. Principal Component Analysis. Springer, New York, NY.

Jordan, D., Kremer, R.J., Bergfield, W.A., Kim, K.Y., Cacnio, V.N., 1995. Evaluation of microbial methods as potential indicators of soil quality in historical agricultural fields. Biology and Fertility of Soils 19, 297–302.

Jukes, T.H., Cantor, 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, NY, pp. 2113–2132.

Kaiser, O., Pühler, A., Selbitschka, W., 2001. Phylogenetic analysis of microbial diversity in the rhizoplane of oilseed rape (*Brassica napus* cv Westar) employing cultivation-dependent and cultivation-independent approaches. Microbial Ecology 42, 136–149.

Kates, M., 1986. Techniques in Lipidology: Isolation Analysis and Identification of Lipids, second ed. Elsevier Press, Amsterdam, The Netherlands.

Kop, J., Kopylov, A.M., Magrum, L., Siegel, R., Gupta, R., Woese, C.R., Noller, H.F., 1984. Probing the structure of 16S ribosomal RNA from *Bacillus brevis*. Journal of Biological Chemistry 259, 15287–15293.

Kuske, C.R., Barns, S.M., Busch, J.D., 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Applied and Environmental Microbiology 63, 3614–3621.

Leckie, S.E., 2005. Methods of microbial community profiling and their application to forest soils. Forest Ecology and Management 22, 88–106.

MacNaughton, S.J., Stephen, S.R., Venosa, A.D., Davis, G.D., Chang, Y.-J., White, D.C., 1999. Microbial population changes during bioremediation of an experimental oil spill. Applied and Environmental Microbiology 65, 3566–3574.

Mailley, L., Aragno, M., 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Applied Soil Ecology 13, 127–136.

McCaig, A.E., Glover, L.A., Prosser, J.I., 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Applied and Environmental Microbiology 65, 1721–1730.

McCune, B., Mefford, M.J., 1999. PC-ORD for Windows. Multivariate analysis of ecological data. Version 4.01. MjM Sofware, Gleneden Beach, OR.

Morris, W.F., Wood, D.M., 1989. The role of lupine in succession on Mount St. Helens: facilitation or inhibition? Ecology 70, 697–703.

Øvreås, L., Forney, L., Daae, F.L., Torsvik, T., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied and Environmental Microbiology 63, 3367–3373.

Parmenter, R.R., 2005. Patterns of decomposition and nutrient cycling across a volcanic disturbance gradient: a case study using rodent carcasses. In: Dale, V.H., Swanson, F.J., Crisafulli, C.M. (Eds.), Ecological Response to the 1980 Eruptions of Mount St. Helens. Springer, New York, NY, pp. 233–242.

Pennanen, T., 2001. Microbial communities in boreal coniferous forest humus exposed to heavy metals and changes in soil pH-a summary of the use of phospholipid fatty acids, Biolog (R) and H-3-thymidine incorporation methods in field studies. Geoderma 100, 91–126.

Petersen, S.O., Klug, M.J., 1994. Effects of sieving, storage, and incubation temperature on the phospholipid fatty acid profiles of a soil microbial community. Applied and Environmental Microbiology 60, 2421–2430.

Rajendran, N., Matsuda, O., Imamura, N., Urushigawa, Y., 1992. Variation in microbial biomass and community in the sediments of eutrophic bays as described by phospholipid ester-linked fatty acids. Applied and Environmental Microbiology 58, 562–571.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Holben, W.E., Gannon, J.E., 2006. Choice of methods for soil microbial community analysis: PLFA maximizes power compared to CLPP and PCR-based approaches. Pedobiologia 50, 275–280.

Ratledge, C., Wilkinson, S.G., 1988. Microbial Lipids, Vol. 1. Academic Press, New York, NY.

Ringelberg, D.B., Davis, J.D., Smith, G.A., Pfiffner, S.M., Nichols, P.D., Nickels, J.S., Henson, J.M., Wilson, J.T., Yates, M., Kampbell, D.H., Reed, H.W., Stocksdale, T.T., White, D.C., 1988. Validation of signature phospholipid fatty acids biomarkers for alkaline utilizing bacteria in soil and subsurface aquifer materials. FEMS Microbiology Ecology 62, 39–50.

Sait, M., Hugenholtz, P., Janssen, P.H., 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environmental Microbiology 4, 654–666.

SAS Institute, 1988. SAS user's Guide: Statistics. SAS Institute, Cary, NC.

Shannon, A.E., Weaver, W., 1963. The Mathematical Theory of Communication. University of Illinois Press, Urbana, IL.

Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S., Wernars, K., 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Applied and Environmental Microbiology 67, 2284–2291.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876–4882.

Ueda, T., Suga, Y., Matsuguchi, T., 1995. Molecular phylogenetic analysis of a soil microbial community in a soybean field. European Journal of Soil Biology 46, 415–421.

Van Overbeek, L.S., Van Elsas, J.D., 1997. Adaptation of bacteria to soil conditions: applications of molecular physiology in soil microbiology. In: Van Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology. Marcel Dekker, Inc., New York, NY, pp. 441–447.

White, D.C., 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. Social and General Microbiology Symposium 34, 37–66.

White, D., Ringelberg, D., 1998. Signature lipid biomarker analysis. In: Burlage, R., Atlas, R., Stahl, D., Geesey, G., Sayyer, G. (Eds.), Techniques in Microbial Ecology. Oxford University Press, Oxford, pp. 255–272.

Wilkinson, S.G., 1988. Gram-negative bacteria. In: Ratledge, C., Wilkinson, S.G. (Eds.), Microbial Lipids, Vol. 1. Academic Press, New York, NY, pp. 299–488.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology & Biochemistry 24, 317–323.

Zelles, L., Rackwitz, R., Bai, Q.Y., Beck, T., Beese, F., 1995. Discrimination of microbial diversity by fatty acid profiles of phospholipids and lipopolysaccharides in differently cultivated soils. Plant and Soil 170, 115–122.