Seasonal Shifts in Soil Bacterial Diversity in Southwestern Pennsylvania

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Abstract

Soil bacteria are essential components of terrestrial ecosystems. Soil bacterial composition and distribution are affected by anthropogenic factors as well as fluctuations in naturally occurring environmental factors such as the change in seasons. In this study, a total of 438 culturable heterotrophic soil bacteria were isolated in the winter, fall and summer so as to evaluate the effects of seasons on bacterial diversity in Southwestern Pennsylvania. 16S rDNA sequences of the isolated bacteria were analyzed in the microbial community analysis platform, MOTHUR. We found that the same phyla were represented in all seasons, albeit to different levels. While Bacteroidetes were most common in the winter, Firmicutes dominated during the summer. Firmicutes and Gamma-Proteobacteria were isolated throughout the year. Bacterial diversity fluctuated between seasons, being highest in the summer with 57 different bacterial species and lowest in the winter with 25 different bacterial species. Thus, this study has not only illustrated seasonal shifts in soil bacterial diversity, but has also been useful in establishing a baseline of soil bacterial diversity in this region of Southwestern Pennsylvania. Continued monitoring of soil bacterial diversity will enable long-term ecological monitoring and study of the potential environmental impacts of the Marcellus shale gas drilling.

Keywords: 16S rDNA, microbial phylogeny, community analysis

1. Introduction

Soil bacteria are vital to normal functioning of terrestrial ecosystems by playing important roles in nutrient cycling\(^1,14,29\) and detoxifying heavy metals and pollutants\(^12\). Soil bacteria also contribute to plant productivity, health and diversity\(^34,31\). Not only are they essential, soil bacteria are also abundant in terrestrial ecosystems. A gram of soil has been estimated to contain as many as \(10^{10}-10^{11}\) soil bacteria with as many as \(10^3\) unique bacteria\(^16\). Bacterial abundance and diversity in the environment emphasizes their vital role in ecosystems.

In addition to being vital to the ecosystem, soil bacteria are also excellent biological indicators of soil health and ecosystem status\(^23,32,26,36\). The ratio of oligotrophic bacteria, which can thrive in low-nutrient conditions to copiotrophic bacteria, which can thrive only in high-nutrient conditions, has been used as a measure of environmental conditions\(^33,6,18\). Indeed, the presence of copiotrophs such as members of phylum Bacteroidetes and Beta-Proteobacteria have been associated with soil rich in organic content whereas the presence of oligotrophs such as members of phylum Acidobacteria have been associated with soil low in organic content\(^10\). Thus, soil bacterial diversity and composition can be used as an ecological monitoring tool and reflect on soil composition.

The diversity and abundance of soil bacteria are affected by naturally occurring environmental variables, including soil type\(^3\), soil pH\(^26,11,19\), moisture content\(^3\) and nutrient availability\(^10\). Since many of these environmental factors naturally fluctuate, it is not surprising that soil bacterial diversity changes during different seasons\(^20,29\). Previous studies have shown that fast-growing bacteria dominate during the summer and slow-growing bacteria dominate in
the winter\textsuperscript{39}. Also, highest bacterial diversity has been observed in fall and spring\textsuperscript{39}. Monitoring such seasonal shifts in soil bacterial diversity allow for the bacterial composition of a particular ecosystem to be better characterized.

In this study, culturable, heterotrophic soil bacteria were isolated from 2009-2011 during the fall, summer and winter seasons from a field station in Southwestern Pennsylvania. The 16S rDNA gene was isolated from the cultured bacteria and sequences were analyzed in MOTHUR\textsuperscript{28}, a microbial community analysis computing platform. The 16S rDNA gene is ubiquitously found in all prokaryotes, is functionally constant and phylogenetically significant\textsuperscript{22}. From the 438 bacteria included in this study, we found that the same bacterial phyla were represented in all seasons, albeit in different levels. While a few genera were consistently found in all seasons, there were trends that indicated that bacterial diversity changed between seasons. Bacterial diversity was highest in the summer and lowest in the winter. While Bacteroidetes were common in the winter, Firmicutes were common in the summer. Both Gamma-Proteobacteria and Firmicutes were isolated throughout the year. These fluxes in bacterial groups suggest that seasonal shifts in bacterial diversity occur. The results of this study help establish a baseline of soil bacterial diversity in Southwestern Pennsylvania and are a continuation of the long-term ecological monitoring of soil health and ecosystem status for this region.

2. Materials and Methods

2.1 Soil Sampling, Culture And Isolation Of Soil Bacteria:

Soil samples were collected in summer (June 10, 2010, July 10, 2010 and July 28, 2010), fall (October 8, 2009 and October 6, 2010) and winter (February 15, 2011) from Abernathy Field Station, a 57-acre temperate mixed forest in Washington, Pennsylvania. Samples were taken from multiple locations within the field station at a depth of 1-3 cm from leaf-litter cleared land or from stream beds. All samples were transported back to the laboratory within an hour. Soil samples were suspended in sterile deionized water (1 g in 100 ml) and agitated at 250 rpm for 15 minutes at room temperature. Soil suspensions were then serially diluted in sterile deionized water to $10^{-8}$. The diluted samples were thoroughly mixed (50 rpm, 10 minutes). 200 µl of each suspension dilution or the soil itself was directly transferred onto Petri plates containing one of the following types of agar: nutrient agar, 1 % nutrient agar supplemented with 0.6 mM CaCl\textsubscript{2}, YMG agar, R2A agar, Norris’ nitrogen free medium and EMB agar. Agar plates were incubated at room temperature and observed daily for bacterial colony growth. Morphologically distinct colonies were identified and were subcultured on the same type of medium used for original isolation.

2.2 Bacterial Genomic DNA Extraction:

Genomic DNA was extracted as described previously\textsuperscript{27} with slight modifications. Briefly, bacterial colonies from pure cultures were suspended in a mixture of 200 µl of sterile water with 0.1 mm diameter glass beads in sterile microfuge tubes. The bacteria were lysed using the bead beater (Biospec Products) [mix setting, 1 min]. The lysates were boiled in a water bath for 10 minutes, cooled on ice for 10 minutes, and centrifuged at 14,000 rpm for 10 minutes. Supernatants containing the genomic DNA were transferred to sterile microfuge tubes and stored at -20 °C until further use. For those isolates that were hard to lyse using the above method, genomic DNA was extracted using the Microbial DNA isolation kit (MoBio Laboratories), using the manufacturer’s protocol.

2.3 PCR Amplification Of Bacterial 16S rDNA:

16S rDNA was amplified from genomic DNA for each bacterial isolate. A 25 µl PCR reaction was set up with 2X PCR master mix (Fermentas), 600 nM UnivF primer, (GAG TTT GAT YMT GGC TC), 600 nM UnivR primer [GYT ACC TTT TCA CTA CTT] and 3 µl of genomic DNA. PCR was performed in a thermocycler (Applied Biosystems) under the following conditions: 94°C for 5 min, followed by 32 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min with final extension performed at 72°C for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer’s instructions. Purified PCR products were analyzed by agarose gel electrophoresis and 16S rDNA concentration was estimated with reference to ZipRuler DNA Express ladder (Fermentas).
2.4 16S Rdna Sequence Analysis:

Purified PCR products were sequenced at the Genomics Core Facility, West Virginia University. Sequences were trimmed at the 5’ and 3’ ends based on nucleotide signal quality as observed using Sequence Scanner (v 1.0) [Applied Biosystems]. Sequences were identified using Basic Local Alignment Search Tool (BLAST)\(^3\) and the Ribosomal Database Project (RDP) Classifier tool, a naïve Bayesian classification method\(^{35}\) with >70% confidence interval. Sequences were aligned based on the Greenegenes database\(^8\) using MOTHUR (v.1.16.0)\(^{28}\). All statistical analyses were performed on MOTHUR\(^{28}\) which uses the furthest neighbor algorithm to assign sequences to Operational Taxonomic Units (OTUs) at distance thresholds of 0, 0.03 (unique), 0.05 and 0.1. Diversity for each respective season as well as overall total diversity among the sequences was depicted using the Shannon-Weaver index, Chao1 estimates, abundance-based coverage estimator (ACE), and Simpson’s index. Overlaps in unique species observed (SOBs) and genera identified in each season were illustrated using Venn diagrams. To assess sampling saturation, rarefaction analysis of species observed was performed.

2.5 Phylogenetic Analysis:

Phylogenetic trees were constructed using the maximum likelihood method on the platform phylogeny.fr\(^7\) using the “Advanced mode” (MUSCLE [v 3.7] for multiple alignment, GBlocks [v 0.91b] for alignment refinement, and PhyML [v. 3.0] for phylogeny using the maximum likelihood method with 500 replicates for bootstrap value with >50% bootstrapping confidence interval). After analysis, phylogenetic trees were constructed using the FigTree platform (v. 1.3.1)\(^{25}\). Trees were rooted with Desulfurobacterium (DQ413023.1) as an out-group.

3 Results

At Abernathy Field Station, a field station location in Washington, PA, we cultured and identified heterotrophic soil bacteria to establish a baseline of soil bacterial diversity in this region. The sampling was carried out in fall, summer and winter seasons (2009 – 2011) in an effort to capture potential seasonal shifts in bacterial diversity. Different culture media were used to cultivate diverse bacteria with different nutritional requirements.

Of the 438 bacteria isolated initially in the fall, summer and winter, 16S rDNA sequences were obtained from 387 isolates, but only 335 16S rDNA sequences were of quality fit for further analysis (Table 1). After the sequences were trimmed at the 5’ and 3’ ends, the length of the 16S rDNA analyzed was between 200-1000 bp. Approximately 15% of the sequences were either too short or of poor quality and were considered unclassified (Figure 1).

Table 1: Sequence data summary

<table>
<thead>
<tr>
<th></th>
<th>Fall</th>
<th>Summer</th>
<th>Winter</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria isolated</td>
<td>91</td>
<td>207</td>
<td>140</td>
<td>438</td>
</tr>
<tr>
<td>Sequences obtained</td>
<td>91</td>
<td>168</td>
<td>128</td>
<td>387</td>
</tr>
<tr>
<td>Sequences analyzed</td>
<td>80</td>
<td>141</td>
<td>114</td>
<td>335</td>
</tr>
</tbody>
</table>

Table 1. Soil bacteria were isolated during the fall, summer and winter from Abernathy Field Station, using culture dependent methods. Bacterial genomic DNA was extracted and 16S rDNA was isolated from each bacterium. The number of bacteria and sequences included in the study are represented.

To identify the bacteria, 16S rDNA sequences were analyzed using RDP Classifier\(^{34}\). Sequences with greater than 70% sequence identity in the RDP Classifier\(^{35}\) were considered to be accurate. We found that all isolates were classified under domain Bacteria. Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria were represented in the three sample sets derived from fall, winter and summer. Although the same phyla were represented in each sample set, the percentage of isolates belonging to each phylum differed widely between seasons. For example, while Firmicutes and Gamma-Proteobacteria constituted 32% and 17% of the isolates in the summer sample set respectively, they decreased by 20-fold and 8-fold, respectively, in the winter sample set. Conversely, the Bacteroidetes increased to 40%, an 8-fold change in the winter. Beta-Proteobacteria increased by two-fold from fall.
to summer, but remained relatively stable from summer to winter. Despite these fluxes, the percentage of the Alpha-Proteobacteria and the Actinobacteria remained relatively unchanged across the three sample sets. When the diversity within each phylum was considered, we found that Proteobacteria contained the highest number of taxonomic families (number of taxonomic families = 16) and bacterial genera (number of genera = 39).

Of the 73 genera identified, 49 were isolated in the summer alone. *Bacillus*, *Flavobacterium*, *Micrococcus* and *Pseudomonas* were isolated consistently in each season. Of these four genera, *Flavobacterium* and *Bacillus* were the most prevalent, representing 13% and 11% of all isolates obtained from all seasons, respectively. 29 isolates were singletons (represented only once in the sample set) and 14 were doubletons (represented by two isolates in the sample set).

**Figure 1.** Taxonomic distribution of soil bacterial isolates

16S rDNA sequences were also analyzed using MOTHUR, a microbial community analyzing platform, which assigns sequences to operational-taxonomic units (OTUs). Overall, among all three seasons, 94 OTUs were observed, indicating that 1 in every 4 bacteria isolated was taxonomically unique (Table 2). Of these 94 OTUs, 57 unique OTUs were isolated in the summer. Diversity indices were calculated for each sample set derived from each season. While the various ecological diversity indices were similar for the fall and winter sample sets, the diversity in the summer sample set was much higher (Table 2).

**Table 2: Ecological Diversity Indices**

<table>
<thead>
<tr>
<th></th>
<th>Fall</th>
<th>Summer</th>
<th>Winter</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operational Taxonomic Units (OTUs)</td>
<td>22</td>
<td>57</td>
<td>25</td>
<td>94</td>
</tr>
<tr>
<td>Shannon-Weaver index</td>
<td>2.58</td>
<td>3.54</td>
<td>2.55</td>
<td>3.91</td>
</tr>
<tr>
<td>Simpson’s index</td>
<td>0.10</td>
<td>0.04</td>
<td>0.14</td>
<td>0.032</td>
</tr>
<tr>
<td>ACE</td>
<td>36.1</td>
<td>296.3</td>
<td>38.4</td>
<td>240.3</td>
</tr>
<tr>
<td>Chao1 index</td>
<td>35.8</td>
<td>157.4</td>
<td>32.9</td>
<td>169</td>
</tr>
</tbody>
</table>

Table 2. OTUs (defined as 97% similar) and diversity indices were calculated using the 16S rDNA sequences analyzed in MOTHUR [v. 1.16.0]. Shannon-Weaver index = \( \sum[(ni / N) \ln(ni / N)] \) where n is the number of OTUs with i individuals and N is the total number of individuals. Simpson’s index = \( \sum[ni(ni - 1)] / (N(N - 1)) \) where ni is the number of OTUs with ‘i’ individuals and N is the total number of individuals. Chao1 index=\( S_{obs} + \{[n_1(n_1-1)]/[2(n_2+1)] \} \) where \( S_{obs} \) is the number of species observed, \( n_i \) is the number of OTUs with only 1 sequence and \( n_2 \) is the number of OTUs with only two sequences. ACE values were determined as described.
In order to determine whether sampling saturation had been reached, rarefaction analysis was performed on all 335 sequences. The 99%, 97% and 95% similarity curves continued to rise past the number of sequences analyzed, indicating that sampling saturation was not reached (Figure 2). However, the 90% similarity curve tapered off indicating that the likeliness of isolating an unrepresented taxonomic group is minimal (Figure 2).

To assess whether a relationship exists between bacterial diversity and the seasons, overlaps were determined at the levels of OTUs and genus (Figure 3). Interestingly, all the OTUs represented in the summer sample set were unique (Figure 3). However, the fall and winter sample sets did overlap and showed 10 common OTUs. Overall, there was a greater overlap at the genus level than at the species level (data not shown).

A phylogenetic tree was constructed using sequences (n=46) that satisfied a 90% similarity threshold. The tree was rooted with Desulfurobacterium (DQ413023.1) and showed wide heterogeneity (Figure 4). The branches were supported with bootstrap analysis (>50%). Bacteroidetes had the highest number of branching orders whereas Firmicutes had the fewest, despite the latter encompassing a larger proportion of bacterial isolates.

![Figure 2](image2.png)

**Figure 2. Estimation of bacterial diversity using rarefaction analysis**

Figure 2. Rarefaction curves were generated using MOTHUR [v.1.16.0] a randomized re-sampling procedure (freq =5) without replacement. The mean number of OTUs found per number of sequences analyzed is depicted.

![Figure 3](image3.png)

**Figure 3. Overlap in culturable bacterial species (OTUs) between fall, winter and summer seasons**

Figure 3. 16S rDNA sequences were analyzed (with 97% similarity) and Venn diagrams were plotted using MOTHUR.
4 Discussion

Only 1-10% of soil bacteria have been cultured and characterized. Thus, soil bacteria represent a major, untapped genetic resource. Moreover, bacteria have the genetic and metabolic capability to respond swiftly to changing environmental conditions, they can also serve as excellent biological indicators of ecosystem status. Thus, the assessment of soil bacterial composition and diversity can allow for not only the discovery of novel genes but also serve as a tool for long term ecological monitoring. Despite the recognition of the importance of soil bacteria, there is still paucity of knowledge regarding soil bacteria in general and in temperate ecosystems, in particular. We attempted to fill the gap in knowledge by characterizing soil bacterial diversity at Abernathy Field Station in Southwestern Pennsylvania, a mixed-temperate ecosystem. More significantly, Marcellus shale drilling is to begin at a site proximal to the study location. The potential environmental impact of the drilling practice is largely unknown. It is therefore especially important to establish a baseline of bacterial diversity prior to Marcellus shale drilling in this geographical region.

It is known that soil bacteria are affected by environmental variables such as moisture content, temperature, and nutrient availability all of which also fluctuate during seasons. In order to gain a better understanding of the soil bacterial composition, it was important to assess the potential seasonal shifts. To this end, we isolated bacteria at various times of the year corresponding to fall, winter and summer seasons. Culturable, heterotrophic soil bacteria that were isolated were identified and analyzed using their 16S rDNA, a phylogenetically significant gene ubiquitously found in all prokaryotes. Although limitations of using the 16S rDNA for phylogenetic analysis have been identified, the use of this gene still continues to be well accepted and widely used for determining bacterial phylogeny.

Figure 4. Phylogenetic classification of soil bacterial isolates
Figure 4. Multiple sequence alignments of the 16s rDNA sequences and phylogenetic trees were constructed on the platform, phylogeny.fr using the “Advanced Mode” (MUSCLE v 3.7) for multiple alignment, GBlocks (v 0.91b) for alignment refinement and PhyML (v 3.0) for phylogeny using the maximum likelihood method with 500 replicates for bootstrap values. After analysis, phylogenetic trees were constructed using the FigTree platform (v 1.3.1)\textsuperscript{25}. The tree was rooted with Desulfurobacterium (DQ413023.1). Only those isolates that exhibited a 90% 16S rDNA sequence similarity are included.

Over a course of two years from fall 2009 to winter, 2011, 438 culturable bacteria were isolated and 16S rDNA was obtained. Of these, 335 16S rDNA sequences were considered fit for further analysis (Table 1). Considering 97% similarity threshold, 94 OTUs were characterized (Table 2). Thus, roughly one in four bacteria that were isolated was found to be taxonomically unique. This elevated OTU ratio suggests high overall diversity in the sample set. Indeed, ecological diversity indices revealed high species richness and also corroborated the high OTU finding. The calculated diversity indices are comparable to other studies in which bacteria were sampled from soil with high nutritional status\textsuperscript{9}.

One of the goals of this study was to also assess whether soil bacterial diversity shifts with seasons. When soil bacteria isolated from individual seasons were compared, we found that the summer season was most diverse (Table 1). Across various seasons, one of the most remarkable changes in bacterial composition was evident in the phylum Bacteroidetes, which increased by 8-fold from summer to winter and constituted 45% of all isolates obtained in the winter (Figure 1). Bacteroidetes members have been shown to prevail in soils rich in organic content\textsuperscript{29}. The increase in the number of Bacteroidetes species in the winter suggests that soil organic content is higher during winter. This is not entirely surprising considering that for every 10 °C decrease in temperature, the organic content in the soil increases by two to three-fold\textsuperscript{2}. The rich organic content may have favored the growth of copiotrophs such as Bacteroidetes. Furthermore, the oligotrophic bacteria such as Alpha-Proteobacteria and Actinobacteria remained relatively unchanged across the three seasons analyzed (Figure 1). Indeed, these oligotrophs have been shown to survive across all seasons regardless of different environmental conditions\textsuperscript{4}. We also observed that the members of Gamma-Proteobacteria and Firmicutes constituted a smaller proportion of the isolates obtained during winter. This trend was previously observed that the number of fast-growing bacteria such as Gamma-Proteobacteria and Firmicutes decrease remarkably in the winter and that Firmicutes such as Bacillus are more numerous during the warmer months\textsuperscript{39}. Despite these seasonal shifts in the phylum level, four bacterial genera, Bacillus, Pseudomonas, Micrococcus and Flavobacterium were consistently isolated in all sample sets.

A phylogenetic tree was constructed (Figure 4) using the 16S rDNA sequences at the 90% similarity threshold. The tree showed wide heterogeneity. Rarefaction analysis (Figure 2) was also performed and showed that sampling saturation was not met, indicating that more unique OTUs still remain to be characterized in this ecosystem. It must be noted that despite great efforts, not all soil bacteria are culturable\textsuperscript{32}. The results presented here are representative of only the culturable bacterial species. Efforts are underway to also pursue culture-independent methods of determining soil bacterial diversity. Despite this shortcoming, this study has added to our understanding of soil bacterial diversity in a mixed-temperate ecosystem. Also, it has revealed trends that are consistent with seasonal shifts taking place in soil bacterial composition and diversity.

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6 References

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