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Research Article

Culture-dependent methods increase observed soil bacterial diversity from Marcellus shale temperate forest in Pennsylvania

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Soil bacteria comprise a largely untapped resource for improvements in environmental and biomedical sciences, yet only 1-10% are culturable in the laboratory (Hirsch and Valdés, 2009). This poor representation has been attributed to the stringent growth demands and low growth rate of some species (Torsvik *et al.*, 1990). Developing culture-dependent protocols that identify unique bacterial operational taxonomic units (OTUs) is an important research topic in soil bacterial ecology. Establishing new OTUs in culture will permit the study of their morphology and physiology that may advance agriculture and pharmacology. Culturability may be improved by employing different media to satisfy inherent preferences of growth substrate utilization. Therefore, soil-extract agar, R-2A agar and 1% nutrient agar were used in this study. Soil bacteria were isolated in the winter from Abernathy Field Station, a Marcellus shale temperate forest in Washington, Pennsylvania. Monitoring bacterial diversity in this ecosystem can be used to assess the early environmental consequences of anthropological factors, such as hydraulic fracturing in the Marcellus shale region. For long term monitoring, this sample collection was analyzed in conjunction with previous years' assessments. Isolates were analyzed taxonomically and phylogenetically. Unique OTUs were identified through comparative analysis of 16S rDNA. The Shannon-Weaver and Simpson's diversity indices ranked isolates on soil-extract agar highest for species richness. Rarefaction analysis suggested that sampling saturation of OTUs identified on soil-extract agar had not yet been reached. Each medium studied supported isolates of four common phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Soil-extract agar supported the greatest proportion of pigmented colonies including a cyanobacterium with intra-16S rDNA polymorphism. Each medium supported the growth of unique OTUs and genera with *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Streptomyces* found on each. This study suggests that utilizing different media can increase the culturability of soil bacteria, giving a wider representation of soil bacterial communities.

Keywords: 16S rDNA; Soil bacteria; Bacterial phylogeny

Introduction

The ecology of soil bacteria is becoming of increased interest as an early indicator of potential changes within an ecosystem. Alterations in climate change, pollution levels, and ecosystem inhabitants each impact the overall biodiversity of an ecosystem (Hannah *et al*., 2002). Responses to such changes are first apparent in the dynamic bacterial flora in the soil (Lundquist *et al.*, 1999). Specifically, changes in soil bacterial diversity can be used to assess the impact of anthropological factors on surrounding areas (Stan *et al.*, 2011). For this reason, long term ecological monitoring studies can be established to predict the effects of human behavior, such as hydraulic fracturing in the Marcellus shale region, by first understanding the responses of the native prokaryotes.

In the process of hydraulic fracturing, a fluid is injected into the ground under high pressure. This injection fractures the underlying rock formation, and a propping agent is administered to prevent the fractures from closing so that natural gas can be collected (Cooke, 1975). The injected fluid consists of chemically enhanced water containing compounds like polysaccharides and polyacrylamides and salts or organic acids to crosslink these polymers. These chemicals are digested after fracking with breakers including enzymes and radical peroxides (Harris *et al*., 1997). Unfortunately, not all compounds are removed from the rock, and some of the chemicals may leak into the surrounding ground, thereby contaminating the soil. This invasive fracturing method may also create unexpected fractures in the ground through which the natural gas can escape to the surface. All of these chemicals might have an influence on the diversity of soil bacteria present in the soil. Overall, the effects of the recent expansion of hydraulic fracturing, in the Marcellus shale region of Pennsylvania are not well understood. Therefore it is ever more important to establish and maintain baseline definitions of the region's soil bacterial populations to better depict the bacterial responses resonating from such activity (Kohler *et al*., 2011).

While some soil bacteria are easily cultured in the laboratory, there persist unidentified populations of unculturable bacteria that are thus overlooked in routine culture-dependent analyses (Janssen *et al*., 2002). It is estimated that only 1-10% of all soil bacteria are considered to be culturable (Torsvik *et al.*, 1990). This incomplete picture may be remedied by allowing bacteria to grow under different conditions, perhaps to mimic their natural niches and allow for the growth of certain populations over others. The use of methods that permit the growth of previously unculturable bacteria is necessary to understand the physical and biological properties of such bacteria beyond the sequences of their 16S rDNA (Joseph *et al*., 2003; Sait *et al.*, 2002). Furthermore, increasing bacterial culturability will more accurately reflect bacterial diversity in the soil, thus giving a better

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understanding of early prokaryotic responses to environmental changes.

After obtaining soil samples from Abernathy Field Station, a mixed-temperate forest in Washington, Pennsylvania, three different culture media were utilized with the intent of favoring different bacterial groups with inherent differences in preferred growth substrate composition. Previous work conducted by Joseph *et al*. (2003) suggests that many unculturable groups of bacteria may be isolated in pure culture with simple media. Soil-extract agar, R-2A agar, and 1% nutrient agar have been reported to select for certain bacterial populations based primarily on bacterial growth characteristics. It is crucial to consider the differing bacterial growth rates because slow growing colonies are typically overwhelmed by faster growing populations and are therefore missed in culture-dependent diversity analyses. By selecting for different growth patterns between media, one can more inclusively assess the bacterial diversity within a given soil sample.

Bacterial diversity was assessed in terms of unique operational taxonomic units (OTUs) (defined as having less than 97% sequence similarity) which were identified via comparative analysis of 16S rRNA genes amplified from crude bacterial genomic DNA extracts (Sait *et al*., 2002). This method is generally accepted for the phylogenetic classification of bacteria and has been used for the identification of novel groups in previous studies (Joseph *et al*., 2003; Sait *et al*., 2002; Janssen *et al.*, 2002). Sequences used for analysis are products of sample collection in 2011 (Papale *et al*., 2012) and 2012 (current study). This study will contribute to a better understanding of the microbial ecosystem present in Abernathy Field Station including seasonal shifts in bacterial populations and long term responses to anthropological factors in the Marcellus shale region.

Results

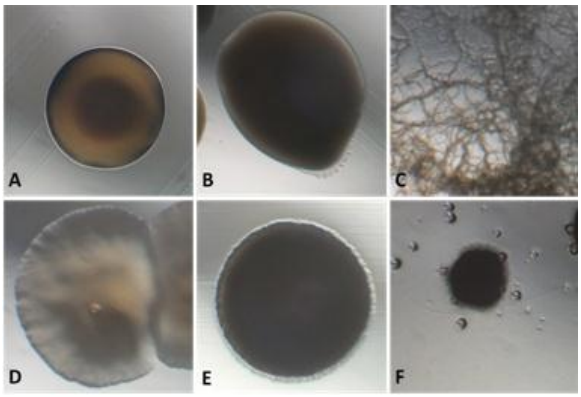

Figure 1. Bacterial colony morphological diversity. A) LSR3; Gray with a deep purple center, round with a smooth margin, convex, 1 mm diameter. B) LSR6; Off white and glossy, irregular with smooth and rhizoid margins, convex, 4 mm diameter. C) LSR7; Off white, filamentous, flat. D) LSR8; Translucent, irregular with smooth/irregular margin, flat, 1.5 mm diameter. E) LSR13; Marigold, round with filamentous margins, convex, 2.5 mm diameter. F) ASR2; Pale orange, irregular with filamentous margins, very convex, 0.5 mm diameter.

Bacterial culture gave morphologically distinct colonies

Plating diluted soil samples on 1% nutrient agar, R-2A agar, and soil-extract agar gave rise to many morphologically distinct colonies between and within media types (Figure 1). Macroscopically unique colonies were subcultured on nutrient agar to establish pure cultures for further experimentation.

While morphologically distinct colonies were isolated on each culture medium studied, different proportions of pigmented colonies were observed across media types (data not shown). Pigmentation was arbitrarily defined as colony coloration with the exceptions of clear/translucent, white, and off white, which were deemed not pigmented. Sixty-seven percent of soil-extract agar isolates were pigmented compared to 42% on R-2A agar and only 34% on 1% nutrient agar. All culture media supported the growth of waterborne bacteria without substantial differences. (Waterborne isolates were those obtained from the streambeds of Abernathy Field Station.)

PCR of 16S rDNA confirmed genomic DNA extraction

Following genomic DNA extraction and PCR amplification of 16S rDNA, PCR products were confirmed via gel electrophoresis with amplified 16S rDNA appearing as a band at 1600 base pairs (bp). Visualizing the successful amplicons provided an unexpected distinction for one isolate, ASR12 (Figure 2A). This bacterium exhibited two amplicons for 16S rDNA amplification which persisted when tested in duplicate (Figure 2B). PCR products were sequenced for those isolates that gave bands for 16S rDNA (data not shown).

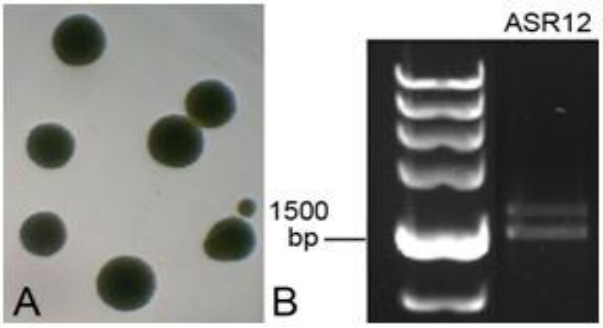

Figure 2. Unique results observed through gel electrophoresis confirmation of bacterial 16S rDNA amplification. A) Green pigmented bacterial colonies (ASR12; round, irregular margin, 0.5 mm diameter) isolated on soil-extract agar from streambed soil in Abernathy Field Station, Washington, Pennsylvania. B) 16S rDNA is indicated by a band at 1600 bp. ASR12 exhibited two amplicons for 16S rDNA amplified by PCR. Only the lower molecular weight band was successfully sequenced and characterized as genus *Chlorophyta*.

16S rDNA sequences analysis suggests increased culturability with different media

After compiling sequence data for 2011 (Papale *et al*., 2012) and 2012 isolates, bacterial diversity indices and OTU counts were determined using MOTHUR, a microbial community analysis platform (Schloss *et al*., 2009). As shown in Table 3, different indices conveyed mixed conclusions. The Shannon-Weaver and Simpson's indices showed the most soil bacterial diversity obtained on soil-extract agar, followed by R-2A agar and 1% nutrient agar. The ACE and Chao1 indices showed the highest diversity obtained from R-2A agar, followed by soil-extract agar and 1% nutrient agar.

Table 1. Bacterial diversity indices.

	NA	R-2A	SEA	Total
Sequences analyzed	38	98	46	182
OTUs	15	37	31	24
OTU to sequence ratio	0.39	0.38	0.67	0.13
Shannon-Weaver Index	2.47	3.14	3.34	2.62
Simpson's Index	0.08	0.06	0.017	0.09
ACE	20.75	93.76	60.86	31.57
Chao1 Index	17.5	72	52	38

Sequences were obtained in 2011 (Papale *et al*., 2012) and 2012 from isolates cultured on 1% nutrient agar (NA), R-2A agar, and soil-extract agar (SEA). All diversity indices were calculated from sequence data using MOTHUR (v.1.24.1) (Schloss *et al*., 2009). Shannon-Weaver diversity index=$-\Sigma[(n_i/N)\ln(n_i/N)]$ where n is the number of individuals in the ith OTU and N is the total number of individuals; Simpson's index=$[\Sigma n_i (n_i -1)]/ N(N-1)$ where n 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_1 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 by MOTHUR (Schloss *et al*., 2009).

All isolates were classified under the domain Bacteria. Bacterial isolates predominantly belonged to four common phyla for each culture medium studied (Figure 3). Classifications with greater than or equal to 80% sequence identity as determined by RDP Classifier (Wang *et al*., 2007) were considered to be accurate. ASR12, the unique bacterium isolated on soil-extract agar, belongs to the phylum Cyanobacteria and is the first of its phylum and 16S rDNA banding pattern to be cultured from soil of Abernathy Field Station. Observed phyla and their percent contributions to the whole were as follows: Actinobacteria (15%), Bacteroidetes (30%), Cyanobacteria (0.6%), Firmicutes (12%), and Proteobacteria (44%). Bacterial isolates belonged to 40 genera, 24 families, and 13 orders.

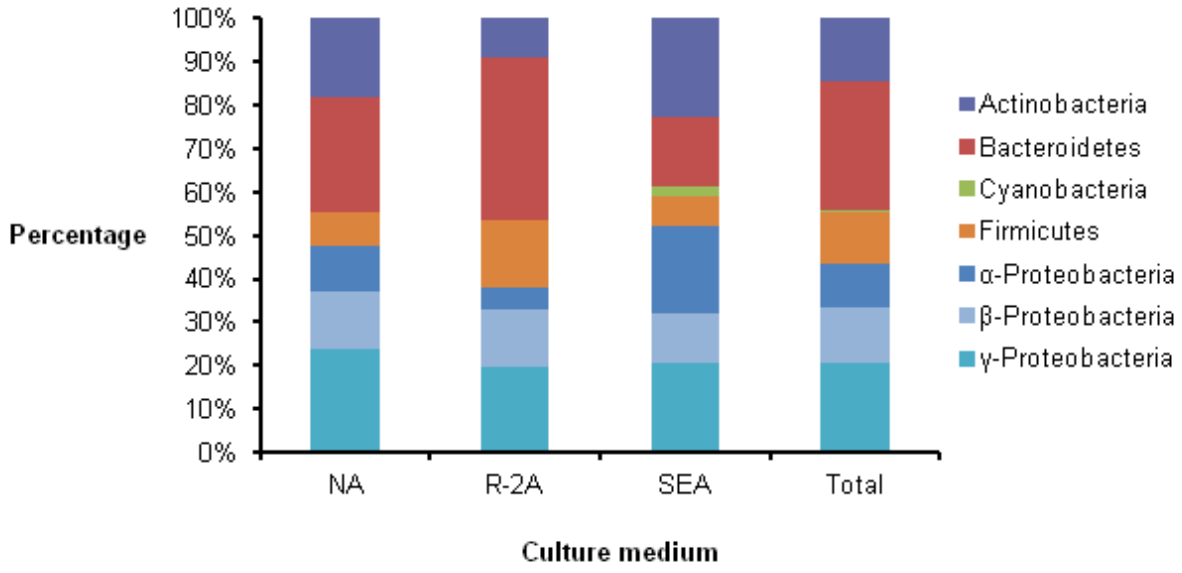

Figure 3. Taxonomic distribution of isolated soil bacteria. Depicted isolates were obtained in winter 2011 (Papale *et al.*, 2012) and 2012. Each culture medium gave isolates belonging to phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. (NA = 1% nutrient agar; SEA = soil-extract agar)

The use of three culture media increased soil bacteria culturability as supported by the presence of unique isolates obtained on each medium (Figure 4). While the OTU level overlap displayed increased similarity between colonies isolated on each medium compared to the genus level overlap, each culture medium demonstrated the ability to support unique bacterial isolates of less than 97% sequence similarity. The five common genera observed on each medium were *Bacillus, Flavobacterium, Pseudomonas, Rhizobium, and Streptomyces.*

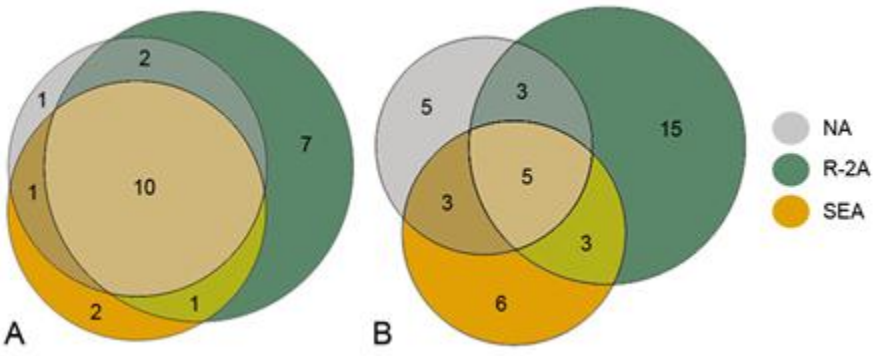

Figure 4. Venn diagrams depicting overlap of bacterial isolate classifications. Venn diagrams displaying overlap at the OTU (A) and genus (B) levels for bacteria isolated on 1% nutrient agar (NA), R-2A agar, and soil-extract agar (SEA).

Representative isolates from each OTU observed were plotted in a phylogenetic tree based on multiple sequence alignments of 16S rDNA (Figure 5). The represented phylogeny of the bacterial isolates cultured in this study conform to the evolutionary history accepted of domain Bacteria.

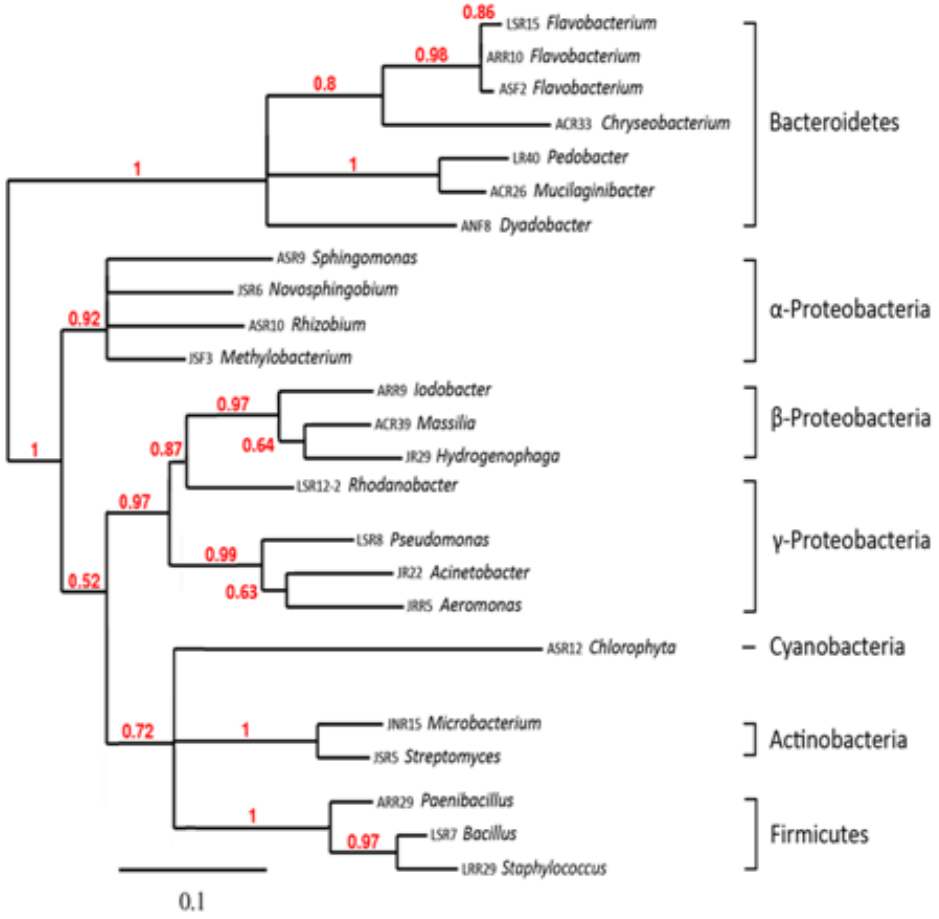

Figure 5. Phylogenetic distribution of isolated soil bacteria. Multiple alignments of 16S rDNA sequences and phylogenetic trees were constructed using the platform phylogeny.fr (Dereeper *et al.,* 2008) [settings "Advanced Mode" (MUSCLE v.3.7) for multiple alignment, GBlocks (v.0.91b) for alignment refinement, and PhylMl (v.3.0) for phylogeny using the maximum likelihood method with 500 replicates for bootstrap values]. After analysis, phylogenetic trees were assembled using the TreeDyn platform (v.1.3.1) (Chevenet *et al., 2006)*. One bacterial genus representing each OTU identified is depicted.

The use of 1% nutrient agar, R-2A agar, and soil-extract agar for bacterial isolation yielded heterogeneous bacterial colonies. Rarefaction curves, constructed with data obtained from MOTHUR (Schloss *et al.,* 2009) suggest that sampling saturation had not been reached for R-2A agar and soil-extract agar (Figure 6). Given an unsaturated sample, continued isolation could identify a greater number of OTUs.

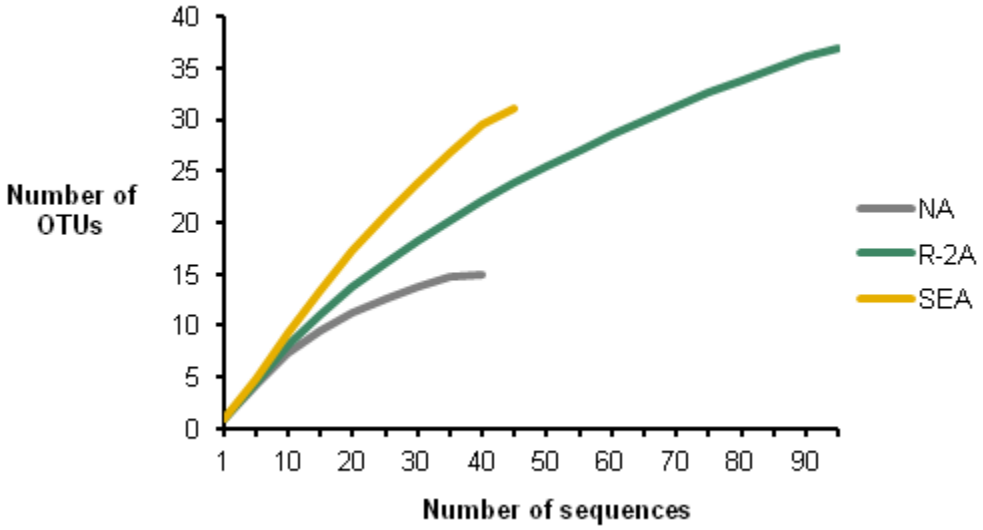

Figure 6. Rarefaction analysis estimation of observed bacterial diversity. Rarefaction curves were generated using MOTHUR (v.1.24.1) by a randomized re-sampling procedure (frequency=5) without replacement (Schloss *et al.,* 2009). The average numbers of OTUs of less than 97% similarity found per number of bacteria isolated on each culture medium are depicted. (NA: 1% nutrient agar; SEA: soil-extract agar)

Discussion

Hydraulic fracturing has raised many concerns regarding the long term effects it may cause for entire ecosystems (Rozell and Reaven, 2011). Understanding the bacterial responses to such effects is fundamental to predicting the changes that may occur within the ecosystem as a whole. Through the use of culture media with a range of nutritional components and selective properties, it was hypothesized that different bacterial populations would be obtained from the same initial soil sample. This increased culturability will more accurately represent the soil bacterial diversity of Abernathy Field Station and the local Marcellus shale region, an area that may soon reflect the impact of local fracking in its soil.

The soil-extract agar used in this study appears to be competent in obtaining morphologically distinct colonies and may favor the growth of slow-growing pigmented colonies as suggested by the relatively high proportion observed on this medium. Like soil-extract agar, R-2A agar permits the growth of bacterial colonies traditionally repressed or unnoticed in culture. R-2A agar is reported to favor the growth of pigmented bacteria (*Carter et al*., 2000) as well as waterborne species (Harding *et al*., 1989). Such slow growing colonies are typically overwhelmed by faster growing populations and are therefore missed in culture-dependent diversity analyses. One colony isolated from soil-extract agar, ASR12, illustrates the need to observe the primary bacterial cultures over an extended period of time. After two weeks of incubation at room temperature, this small green colony (round, irregular margin, 0.5 mm diameter) was observed on soil-extract agar plated with a diluted soil sample obtained from a streambed in Abernathy Field Station. As recommended by Carter *et al.* (2000), prolonged incubation periods may facilitate the identification of slow growing pigmented bacteria. Findings of the present study supported this claim. Furthermore, Hamaki *et al.* (2005) used soil-extract agar to discover potentially novel species of *Acrocarpospora* and *Streptosporangium*, among others. Soil-extract agar was noted to support a greater diversity of bacteria than nutrient agar (Taylor, 1951) and may offer growth rate advantages for certain bacterial groups that are particularly well-suited to the nutritional environment of their soil habitat.

Upon PCR amplification of 16S rDNA, ASR12 proved to have unique properties beyond its green pigment. Certain mycobacteria (Ninet *et al*., 1996), as well as human pathogens *Bartonella henselae* (Viezens and Arvand, 2008) and

Campylobacter helveticus (Linton *et al*., 1994) have been reported to yield two amplicons from a single PCR reaction for 16S rDNA, a phenomenon attributed to the existence of multiple *rrn* operons with intra-16S rDNA polymorphism (Moreno *et al*., 2002). ASR12 represents the first example of this particular banding pattern for a bacterium isolated from Abernathy Field Station. However, unlike the aforementioned examples, ASR12 was classified through the Ribosomal Database Project (Wang *et al*., 2007) as phylum Cyanobacteria, genus *Chlorophyta*. Cyanobacteria, like many bacterial groups, have been reported to exhibit multiple *rrn* operons and paralogous 16S rRNA gene copies. One genus in particular, *Lyngbya*, presented 16S rRNA gene copies with sequence divergence of a greater magnitude than the 3% difference threshold typically employed to distinguish bacterial OTUs (Engene and Gerwick, 2011). Since only the lower molecular weight 16S rDNA band was successfully sequenced for the *Chlorophyta* spp. isolated in this study, it would be of great interest to continue investigating the functional properties and sequence identity of this bacterium.

Bacterial diversity was assessed between culture media using diversity indices calculated by MOTHUR (Schloss *et al*., 2009). The Shannon-Weaver and Simpson's indices showed the most soil bacterial diversity obtained on soil-extract agar, while the ACE and Chao1 indices show the highest diversity with R-2A agar. These discrepancies arise from the different approaches taken to calculate each diversity index. The Shannon-Weaver and Simpon's indices take into account the number of unique OTUs as a function of the total number of isolates in a sample set. Since soil-extract agar gave the highest ratio of OTUs per number of sequences analyzed, it was calculated to display the greatest bacterial diversity. The ACE and Chao1 indices are based on the whole number value of OTUs observed and therefore attribute the highest level of bacterial diversity to R-2A agar. As displayed in Table 2, observed OTUs varied between culture media. When the OTUs in the overall sample were assessed by combining the sequences from the individual sample sets, the number of observed OTUs was found to be lower than the sum of the separate groups. This can be explained by the fact that when OTUs are calculated the result is entirely dependent on the "neighbors" or other sequences involved in the analysis. Increasing the number of sequences for the combined analysis increased the likelihood of sequence similarity thus decreasing the overall number of observed OTUs.

Of the bacterial phyla observed in this study, most isolates belonged to Bacteroidetes and Proteobacteria at 30% and 44%, respectively. High levels of these oligotrophic bacteria contradict the sampling outcomes of Kohler *et al*. (2011) for soil collection in Abernathy Field Station during the summer. A study conducted by Manganelli *et al*. (2009) reported greatly increased proportions of γ-Proteobacteria in aquatic environments during winter months, contributing a greater number of isolates from this group. It must be noted that bacterial populations fluctuate with seasonal changes, and nutrient levels tend to diminish in the winter months with increased soil moisture (Bardgett *et al*., 1999). An examination of bacterial genera obtained between culture media illustrates the ability of each medium to select for unique genera not seen on the other media studied. This supports the need for increased culturability in soil bacterial diversity studies.

This study has supported claims that selective media and prolonged incubation can drastically improve the bacterial diversity detected in studies of soil microbial ecology (Hamaki *et al*., 2005). This improvement is particularly important in understanding how bacterial populations change over time and in response to changes in the environment. In accordance with the observations of Carter *et al*. (2000), R-2A agar supported a greater percentage of pigmented colonies than did 1% nutrient agar while both showed proportionately fewer pigmented colonies than soil-extract agar. Utilizing each culture medium and experimenting with the selective properties of others should be aims of future bacterial diversity studies.

Experimental Procedures

Soil sample collection and media preparation

Soil-extract was prepared by autoclaving 200 g soil suspended in 400 ml tap water. The suspension was allowed to settle, and fluid was decanted and centrifuged for 10 minutes at 3,400 rpm. Supernatant was stored at -20 °C overnight. The thawed solution was filtered through a paper towel with the filtrate being the final soil extract. Soil-extract agar was prepared, with slight modifications, according to a previously established protocol (Rao, 1977). Briefly, soil-extract agar contained D-(+)-glucose (Sigma) (0.1%), potassium phosphate dibasic (Sigma) (0.02%), Bacto-Agar (Difco) (1.5%), soil-extract (26%), and tap water to 1 L. Medium was autoclaved for sterilization.

Soil samples of approximately 50 grams were collected from Abernathy Field Station, a mixed temperate forest in Washington, Pennsylvania, on February 9, 2012 from three separate sampling sites, including one land (N 40° .07.912 W 080° .10.984) and two stream bed locations. Leaf litter was cleared and soil was collected at a depth of about 5 cm into sterile 50 ml conical tubes. Soil was also collected at the land site for preparation of the soil-extract agar culture medium. Two streambed soil samples were collected in the same manner nearby in streams (N 40° .07.883 W 080° .11.010; N 40° .08.046 W 080° .11.059). Sequences used in analysis were also obtained by Papale *et al*. (2012).

Culturing of soil bacteria

Stream bed samples were centrifuged at 3,200 rpm for 2 minutes, and the supernatant was discarded. Suspensions were made for samples from each of the three collection sites by agitating 1 wet gram of soil in 100 ml sterile deionized water at 200 rpm for 15 minutes. Each suspension was serially diluted to 10^{-6}, and dilutions were mixed at 50 rpm for 10 minutes prior to plating. 200 µl of each dilution were spread onto two plates of each 1% nutrient agar (Difco), R-2A agar (Fluka), and soil-extract agar. One plate of each pair was incubated at room temperature and one at 4 °C. Plates were first examined for bacterial growth after 24 hours of incubation. Unique colonies, as determined by differences in color, shape, margin, and elevation characteristics, were subcultured on nutrient agar and incubated at room temperature. Subculturing continued as more macroscopically unique colonies were identified, up to three weeks after the initial cultures were established.

Bacterial DNA isolation

Bacterial DNA was extracted from colonies via mechanical lysis according to a previously established protocol (Sait *et al*., 2002). Briefly, bacterial colonies from pure culture were suspended in 200 µl sterile water with 0.1 mm glass beads and shaken in a mini-bead-beater (Biospec Products) for 1 minute. Samples were then boiled for 10 minutes followed by 10 minutes of incubation on ice. Samples were centrifuged at 14,000 rpm for 10 minutes. Supernatants containing genomic DNA were collected and transferred to sterile tubes. The crude lysates were used immediately in PCR or stored at -20 °C for later use (Kohler *et al*., 2011).

PCR of 16S rDNA

Genomic DNA was used to amplify 16S rDNA by PCR. A 25 µl PCR reaction was prepared with 2X PCR Master Mix (Fermentas), 0.6 µM UnivF primer [GAG TTT GAT YMT GGC TC], 0.6 µM UnivR primer [GYT ACC TTG TTA CGA CTT], and 3 µl genomic DNA extract. PCR was performed (PCR System 2700, Applied Biosystems) at 95 °C for 5 minutes followed by 32 cycles of 94 °C for 1 minute, 55 °C for 2 minutes, and 72 °C for 3 minutes. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and confirmed through agarose gel electrophoresis. The concentrations of purified samples were determined using the NanoDrop 2000c (Thermo Scientific).

In cases where multiple 16S rDNA bands were obtained after PCR, the PCR product was run on a 2% agarose gel, and the individual bands were cut out with a sterile scalpel. DNA was extracted using the QIAquick gel extraction kit (Qiagen).

Sequence analysis of 16S rDNA

Successful samples were sequenced at the Genomics Core Facility, West Virginia University. Returned sequences were trimmed at the 5'- and 3'-ends for quality and selected for further analysis based on nucleotide quality value and probability of error using Sequence Scanner Software (v.1.0 Applied Biosystems). Trimmed sequences were characterized using the Ribosomal Database Project (RDP). Classifier tool for 16S rDNA (Wang *et al*., 2007), MOTHUR, a microbial community analysis platform (Schloss *et al*., 2009), was used to analyze unique OTUs and provide indices of bacterial diversity with an OTU defined as a sequence with greater than 3% difference from its neighbors. Multiple alignments of 16S rDNA sequences were made against a known 16S rDNA gene database (Pruesse *et al*., 2007). Representative 16S rDNA sequences for unique OTUs were used to build a phylogenetic tree using the platform phylogeny.fr (Dereeper *et al*., 2008) [settings "Advanced Mode" (MUSCLE v.3.7) for multiple alignment, GBlocks (v.0.91b) for alignment refinement, and PhylMl (v.3.0) for phylogeny using the maximum likelihood method with 500 replicates for bootstrap values]. After analysis, phylogenetic trees were constructed using the TreeDyn platform (v.1.3.1).

Acknowledgments

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