To the Graduate Council:

I am submitting herewith a thesis written by Katherine Elizabeth Sides entitled "Agricultural Soil Bacteria; A Study of Collection, Cultivation, and Lysogeny." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental and Soil Sciences.

Michael Essington, Major Professor

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(Original Signatures are on file with official student records.)

Agricultural Soil Bacteria;
A Study of Collection, Cultivation,
and Lysogeny

A Thesis Presented for the
Master of Science Degree
University of Tennessee, Knoxville

Katherine Elizabeth Sides
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Dedication

This thesis is dedicated to my mother, Margueritte Jeannette Guido, and to my partner, David Curtis Sides, who have encouraged me through many adventures, including this one.

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I would like to acknowledge the people in my life who have provided encouragement and inspiration. Dr. Dhritiman Ghosh and Dr. Krisnakali Roy have been terrific sources of inspiration throughout this research, helping to maintain the awe and wonder of the science, and illuminating the beauty of this unseeable microbial world that we study. My dear globe-trotting friend, Michelle Dodd, has been an endless source of motivation and perspective. Susan “Marathon” Fiscor helped me find and hold onto my determination, as well as provided many fun adventures. Emmi Felker Quinn, Carolyn Reilly Sheehan, Themis Mahalia Stone, and Tiffany Kingston Morrison also provided encouragement, advice, and regular well-timed distractions.

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Abstract

The aim of this research project was to test new collection and cultivation techniques that may increase the range of cultivable diversity of soil bacteria. Fortified BioSep beads were employed *in situ* to capture soil bacteria, and the success of the beads was analyzed using Phylochip microarray analysis. In the cultivation phase, three different media substrates and increased incubation period were evaluated for the ability to select novel or rare bacteria. Over 700 agricultural soil bacterial isolates were classified, including a rare *Gemmatimonadetes sp.*, a rare *Verrucomicrobia sp.*, several *Acidobacteria sp.*, and many novel isolates. Land management, media, and incubation period each resulted in lineage specific preferences. The yeast fortified BioSep bead cultivation collection was significantly different from the bulk soil or acyl homoserine lactone (AHL) fortified bead cultivation collections, and there were lineage specific differences in all three collection types.

Phylochip analysis showed a significant difference between bulk soil and all BioSep bead (water, yeast, or AHL fortified) communities based on microarray analysis of 16S rDNA. The yeast fortified BioSep bead community was richer in operational taxonomic units (OTU) than all others. The number of phyla determined by the Phylochip analysis was much higher than that seen in the overall cultivation collection.

Prophage induction assays of 21 isolates were performed, using mitomycin C (mitC) and a mixture of six AHLs, to examine soil lysogenic phage-host interactions. The fraction induced by mitC was 29%, and 10% were induced by AHL. There was no correlation between induction and land management or host growth rate.

This research showed that increases in cultivable diversity can be attained by the use of BioSep beads in the collection process, varying media substrates, and by extending incubation of inoculate cultures. Phylochip analysis, however, revealed that even with altered cultivation methods, there is still a wealth of soil bacterial diversity that remains to be cultivated from this site. We also found that AHLs impact the interactions between soil bacterial hosts and prophage.

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Introduction

Biogeochemical processes and soil bacteria

Soil bacteria dominate, or participate in, a number of earth's biogeochemical cycles, including the carbon, nitrogen, oxygen, phosphorus, and sulfur cycles (Giri et al., 2006; Wu et al., 2007). Some of the specific processes of soil bacteria include carbon fixation, decomposition of organic matter, respiration, nitrogen fixation, nitrogen mineralization, nitrification, denitrification, phosphorus mineralization, and sulfur oxidation and reduction (Giri et al., 2006). These processes can directly or indirectly affect the concentrations of atmospheric gases, as well as the availability of nutrients to plants and animals (Giri et al., 2006).

Such large scale effects by these tiny cells are possible because of their fantastic abundance. One gram of soil contains, on average, a billion bacterial cells (Schloss and Handelsman, 2006), though not all of these may be viable. The majority of bacteria in soils are found in the top 1 meter of soil (Whitman et al., 1998), and according to the Food and Agricultural Organization (FAO) of the United Nations (2000), there are approximately 31,823,000 km^2 of non-constrained agricultural soil on earth. Presuming one gram of soil is approximately equal to 15 cubic centimeters, then there are 2.12 X 10^{24} total bacterial cells in arable soil on earth. Whitman et al. (1998), however, determined there are approximately 2.6 X 10^{29} total soil bacterial cells on earth by including the estimated bacterial abundance of desert and other non-arable soils, and adding a calculated abundance from depths of 2 to 8 meters.

Bacteria have been evolving diverse metabolic processes for around 3.8 billion years (DeLong and Pace, 2001), much longer than the several hundred million years of evolution seen by plants and animals (Giri et al., 2006). This evolution has resulted in complex, enzyme-driven metabolism that allows bacteria to survive in nearly every conceivable environment on earth (Schlegel and Jannasch, 2006). Large and complicated molecules, such as cellulose, are degraded by extracellular enzymes that bacteria exude directly into the environment (Guggenberger, 2005). Other life forms,

such as plants, are dependent upon such degradation processes from soil bacteria to gain access to nutrients such as nitrogen (Smith and Goodman, 1999).

The diverse degradative abilities of soil bacteria have been utilized in an array of bioremediation projects, from oil spills to acid mine drainage to sewage waste (Crawford, 2006). Other enzymes, such as cellulases, xylanases, and proteinases, are utilized in a variety of industries, including textile, ethanol, paper, and dairy production (Quax, 2006; Doyle and Meng, 2006). Furthermore, antibiotics, anti-tumor medications, bio-herbicides, and bio-pesticides are successfully exploited in human enterprise (Demain, 1999; Handelsman and Stabb, 1996; Dayan et al., 2009).

What we don't know

For the past 80+ years scientists have become increasingly aware that our efforts to inventory soil bacterial diversity have been dismally inadequate (Staley and Konopka, 1985; Sait et al., 2002; Schloss and Handelsman, 2004). "The Great Plate Count ‚Anomaly" was first used by Staley and Konopka (1985) to express the frustration of scientists who realized that most of the microbes from various natural environments were not cultivable in laboratories (Nichols, 2007; Amann et al., 1995). This was determined by comparing microscopic direct counts to the number of colonies that arose on plates inoculated from the same samples (Staley and Konopka, 1985). Ideally, each cell in a sample should form an individual colony during cultivation. By current estimates, we are only able to cultivate 1 in 100 cells, or around 1% (Amann et al., 1995; Davis et al., 2005). Furthermore, the bacteria that are most commonly cultivated in laboratories are not necessarily the most commonly occurring bacteria in soils (Pace, 1999; DeLong and Pace, 2001).

There are currently 52 recognized phyla of prokaryotes, half of which had no cultivated representatives as of 2003 (Rappe and Giovannoni, 2003). Curtis et al. (2002) estimated that there are 10^6 species of soil bacteria on earth, assuming a total abundance of 10^{29}, and Dykhuizen (1998) estimated there are 10^9 global bacterial species in 1998. Schloss and Handelsman (2004), however, determined that the number of bacterial species in the world is far lower.

Increasing cultivation efficiency

Researchers continue to try new techniques in efforts to increase cultivable diversity, mainly because sequence based knowledge of an organism often leaves many questions unanswered. Success is seen even through the simple expansion of existing methods, such as media choice, incubation period, sample dilution rate, pH, and O_2 exposure (Janssen et al., 2002; Joseph et al., 2003; Sait et al., 2002; Overmann, 2006). Cultivation based studies can be paired with molecular surveys of 16S rRNA genes to determine the rate of success in this endeavor. Additional areas of possible enhancement include increasing dislodgement of cells that are adsorbed to soil particles during the initial extraction process (Dhand, 2009), and selective trapping of soil bacteria through specialized collection methods (Peacock et al., 2004; Chang et al., 2005; Geyer et al., 2005; Biggerstaff et al., 2007; Ghosh et al., 2009).

BioSep beads – an alternative sampling method

BioSep beads (Microbial Insights, Inc, Rockford, TN, USA) are 2-3 mm oval spheres formed from a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (PAC). The interior pore space mimics that of soil and provides a matrix for microbial growth (see Chapter 1, Fig. 3). These beads were initially created to deliver specific microbes into wastewater treatment facilities, however their utility as a microbial trapping device became apparent. The use of BioSep beads in sampling of soil bacteria can aid sampling of targeted taxa or functional guilds via *in situ* enrichment. The beads can be fortified prior to deployment with almost any absorbable substrate, which allows manipulation similar to selective enrichment (Peacock et al., 2004; Chang et al., 2005; Geyer et al., 2005; Biggerstaff et al., 2007; Ghosh et al., 2009). The beads can also be deployed for varying lengths of time or at multiple depths.

Bacteriophage

The discovery of bacteria killing viruses called bacteriophage (or phage) was made between the years 1915 and 1917 (Duckworth, 1976). Phage are parasites that hijack bacterial systems to replicate and produce new phage. Most phage found so far -

Myoviridae, Podoviridae and Siphoviridae - have a relatively small genome packed into an isometric protein capsid and a tail of varying length (Fig. 1) (Marsh and Wellington, 1994; Ackermann, 2007). The impacts of phage on agricultural soil bacterial communities may be great due to their affects on host abundance, diversity, and activity (Marsh and Wellington, 1994; Weinbauer and Rassoulzadegan, 2004).

Phage exhibit two main life cycles; lytic and lysogenic - though there are also variations such as pseudolysogeny (Weinbauer, 2004). In the lytic cycle, phage adsorb to host outer membranes, inject their genetic material into the host, hijack the hosts replication and synthesis systems to create more phage genomes and structures, assemble into new phages, then lyse the host cell to release the newly constructed phages. In the lysogenic cycle, phage do not immediately replicate after injecting their genome into the host. Rather, the phage genome integrates with the host genome for a period of time, and is replicated into each host daughter cell genome. Host stress or

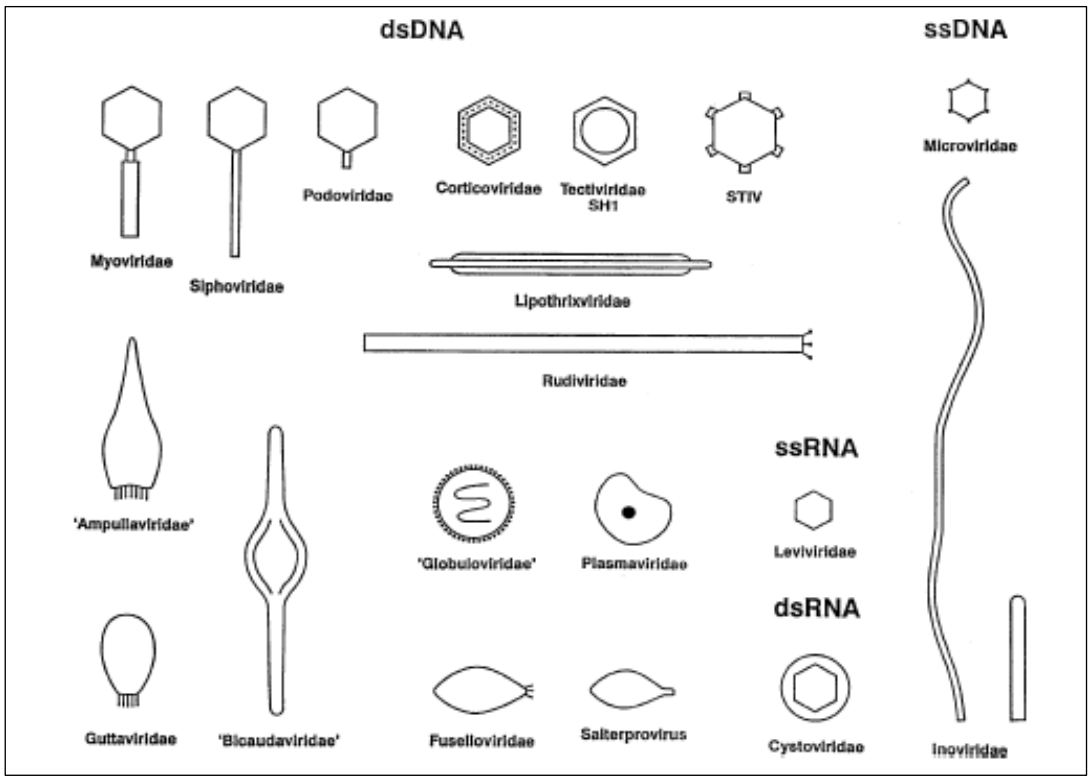

FIG. 1. Phage morphologies. Reprinted from H.W. Ackerman (2007) “5500 Phages Examined in the Electron Microscope”.

other environmental cues, however, can induce the phage genome to excise from the host genome and resume the lytic cycle, replicating and lysing the host (Weinbauer, 2004; Ghosh et al., 2009). Evidence of lysogeny, in the form of partial or entire prophage genomes, has been found in most bacterial genomes (Canchaya et al., 2003)

Lytic and induced lysogenic phage can have immediate effects on host populations by killing a portion of the community, which releases carbon and other nutrients into the food web and may shift the microbial community structure (Weinbauer and Rassoulzadegan, 2004). Lysogenic phage, however, can also transfer genetic material between hosts, resulting in lateral gene transfer (LGT), and sometimes confer pathogenicity through phage conversion (Weinbauer and Rassoulzadegan, 2004). It has also been speculated that lysogeny may be more advantageous than the lytic phage life cycle in the soil environment, where host nutrient sources are sporadic and conditions can be harsh (Stewart and Levin, 1984; Marsh and Wellington, 1994; Ghosh et al., 2009). During inhospitable times, bacteria populations may dwindle, or cells may exist in a state of torpor, or as spores, for long periods (Ghosh et al., 2009).

Phage conversion

The lateral transfer of genes by prophage in prokaryotes makes the task of classification more difficult, however it can increase host fitness and survival capabilities (Chiura, 1997; Chibani-Chennoufi et al., 2004). For example, Chiura (1997) reported phage mediated generalized gene transfer between a mutant *Escherichia coli* strain and 5 marine bacteria from other families that recovered the *E. coli* strain. Host fitness can also be increased by infection with lysogenic phage carrying antibiotic resistance, virulence, or toxin encoding genes (Wagner and Waldor, 2002), and lysogenic infection can grant the host immunity to further infection (Marsh and Wellington, 1994).

Research Objectives

Our increasing dependence on soil corresponds to our need for a better understanding of soil processes. The focus of this thesis research, therefore, was to increase knowledge of the bacteria and phage that are present in agricultural soils.

Objective 1: To evaluate a diverse agricultural bacterial collection by; 1) cultivating communities from bulk soil and fortified BioSep beads, 2) sampling from a variety of land management regimes, 3) utilizing a range of substrates during the cultivation process, and 4) extending the incubation period of inoculate plates beyond that used in the majority of cultivation studies.

Objective 2: To assess the efficiency of the BioSep bead microbial trapping technique by; 1) incubating BioSep beads in situ, fortified with substrates or purified water, and 2) comparing 16S rDNA extracted from BioSep beads and bulk soil utilizing the Phylochip G2 microarray.

Objective 3: To examine lysogeny and prophage induction within the bacterial collection by; 1) using two inducing agents to evaluate prophage induction under different host conditions, 2) assessing the prevalence of prophage within *Variovorax* sp., and 3) determining whether relationships exist between lysogeny and host colony formation rate or land management.

Chapter 1

Cultivation of Diverse Agricultural Soil Bacteria Utilizing BioSep Beads

Abstract

Traditional methods for the cultivation of soil bacteria have failed to result in the cultivation of half of the known soil bacterial phyla. Cultivable diversity, however, has been improved by the use of a variety of substrates, and by extending the incubation time. We utilized a new collection method, BioSep beads fortified with either acyl homoserine lactones (AHL) or yeast extract, along with multiple substrates and extended incubation time to establish a collection of bacteria for future study. Soil and BioSep Bead cultivable communities were established from two agricultural land management regimes and a late successional forest. The diversity of cultivated bacteria from the yeast beads differed significantly from bulk soil or AHL bead cultivable diversity, and lineage specific preferences occurred with both AHL and yeast bead operational taxonomic units (OTUs). Land management and media type, as well as incubation time, also resulted in taxa specific preferences between cultivable OTUs. Rare isolates were obtained from Gemmatimonadetes (1), Verrucomicrobia (1), and Acidobacteria (10), and 6% of the overall cultivable community are potentially novel at the genus or higher level.

Introduction

For many years, researchers have been devising new techniques to increase the fraction of soil microbes that can be cultivated in the laboratory. Current estimates from microscopic direct counting versus colony formation range from 1 to 10% (Staley and Konopka, 1985; Amann et al., 1995; Nichols, 2007). Cultivation independent techniques have shown that microbial diversity is captured at an equally dismal rate (Schloss and Handelsman, 2004), with reported cultivated representatives of only half of the 52 bacterial phyla as of 2003 (Rappe and Giovannoni, 2003). Surveys based on sampling of small sub-unit ribosomal RNA genes, for example, show the occurrence of acidobacteria in many soils is as high as 20% (Janssen, 2006), however as of 2003 there were only 37 representative isolates (Joseph et al., 2003). Though the causes for

the incongruence are not completely understood, laboratory cultivation conditions may fail to adequately represent *in situ* conditions, resulting in an inability to support growth for the majority of soil bacteria.

Several studies have documented successes in the past few years by altering a variety of traditional cultivation methods, including media richness, substrate, incubation period, pH, and O_2/CO_2 concentration. Mitsui et al.,, in 1997, showed that dilute nutrient broth (DNB) media selected for different bacteria from soil than regular nutrient rich concentrations. Janssen et al. (2002) were able to cultivate soil bacteria from Acidobacteria and Verrucomicrobia, as well as novel species from Actinobacteria and Proteobacteria, utilizing nutrient broth media at $1/100^{th}$ normal concentration. Likewise, Joseph et al. (2003) successfully cultivated three Gemmatimonadetes isolates from soil, as well as representatives from Acidobacteria and Verrucomicrobia, by utilizing a variety of substrates at low concentrations in VL55 based media (Sait et al., 2002). Similar success has been achieved with a combination of low nutrient media and increased incubation periods of up to 90 days (Davis et al., 2005). Additionally, Acidobacteria subdivision-1 cultivation was significantly increased by Eichorst et al. (2007) through a combination of lower pH and increased CO_2.

Another possible method to increase cultivable diversity is the use of density-dependent gene regulation, or quorum sensing (QS), molecules. These are exuded by some bacteria and can result in swarming, increased degradation, virulence, or other group activities (Fuqua et al., 2001; Manefield and Whiteley, 2007). In the cultivation of bacterioplankton, the addition of cyclic adenosine mono-phosphate (cAMP) to synthetic freshwater media was found to significantly alter the community (Bruns et al., 2003). CyclicAMP is a regulator of many genes, including metabolic and stationary phase genes, and can be taken up by cells directly from the environment (Bruns et al., 2003). Acylated homoserine lactones (AHLs) are another type of QS molecules, produced by some proteobacteria, that impact gene expression at certain concentrations and are generally able to move across cell membranes (Manefield and Whiteley, 2007). Owing to the ecological importance of AHLs is the fact that some non-AHL synthesizing bacteria, such as *E. coli*, have AHL receptors. Furthermore, non-proteobacteria such as

some *Bacillus* and Actinobacteria species, have the ability to degrade AHLs (Manefield and Whiteley, 2007).

In this study, the objective is to isolate a broad range of diverse bacteria from soil and to examine the interactions of hosts and their temperate phage in agricultural soils. Many rarely cultivated bacteria have not been examined for temperate phage, so one goal of the project was to isolate particularly rare or novel soil bacteria. By examining the successes of recent studies, we developed an experimental design to extract and cultivate bacteria from soils of three different land management regimes, ranging from forest to conventional agriculture, at the Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site in Hickory Corners, Michigan. The design included the use of three substrates at low concentrations and a range of low to high complexity, media pH values to simulate that of the soils sampled, and the placement of fortified BioSep beads in the field for one month to attract bacteria. One set of BioSep beads was fortified with a mixture of six AHLs, the other with yeast extract. Bacteria were also cultivated directly from soils, and incubation periods were extended to 76+ days.

We hypothesized that each of these experiment variables would select for the cultivation of particular soil bacteria: 1) the use of BioSep beads fortified with AHLs and yeast would result in changes to cultivation diversity compared to the soil cultivable community , 2) the communities from each of the three land management regimes would differ overall, 3) the three media types would select for different isolates, and 4) cultivable community diversity would change over the extended incubation period.

Methods and Materials

Study Site

The study was conducted at the NSF-sponsored, Long Term Ecological Research (LTER) site located on the Kellogg Biological Station (KBS), Hickory Corners, MI. The average precipitation at KBS is 890 mm/y, and the mean annual temperature is 9.7℃. The soils are predominantly mesic Typic Hapludalfs, with fine-loamy to coarse-loamy texture of the Kalamazoo and Oshtemo series. Additional information regarding the

experimental design of the site and plot maps are available at http://lter.kbs.msu.edu/.

The main experimental site at KBS is a complete randomized block design consisting of seven different 1 ha cropping systems replicated in one of six blocks and an eighth never-tilled control site. The cropping systems include four annual crop rotations, two perennial and two successional systems in native vegetation. KBS also has three unmanaged sites that include old growth native deciduous forest, 40-60 year old conifer plantations, and a set of old-field sites 40+ years post-agricultural abandonment. All main treatments are replicated six times.

For this investigation soils were collected from treatments T1 (corn-soybean-wheat rotation, intensive tillage, high chemical input), T4 (zero input organic corn/soybean/wheat + clover cover crop), and one forest site (SF2) (Fig. 2). Cultivation of bacteria was also performed with BioSep beads fortified with various substrates that had been buried in plots from these treatments for four weeks (see description below).

FIG. 2. Aerial view of the Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site in Hickory Corners, MI. Solid white rectangles are three replicates of conventional agriculture plots (T1), dashed white rectangles are three replicates of organic agriculture plots (T4), and black square (left) is one successional forest replicate (SF2).

Soil sampling

Soil samples were taken in August, 2007. Three soil cores (2.5 cm dia x 10 cm depth) were collected from each of five established sampling stations (15 cores) in each plot and homogenized to form three replicate composite samples for treatments T1 and T4. Soils were collected and homogenized in a similar fashion from five stations across one SF plot. Only one forested site was utilized due to the large differences in vegetation present in the three SF replicates.

BioSep beads

BioSep beads (Microbial Insights, Inc, Rockford, TN, USA) fortified with various carbon sources were also utilized to recruit a substrate-responsive sub-set of the heterotrophic microbial community in the soils from the same treatments described above. The beads are 2-3 mm oval spheres formed from a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (PAC). The interior pore space mimics that of soil and provides a matrix for microbial growth (Fig. 3). The highly

FIG. 3. Magnified bisected BioSep® bead showing porous interior. These small (2-3 mm) beads, made from powdered activated carbon (75%) and Nomex® composite, were fortified with either yeast or acyl homoserine lactones (AHL) and incubated belowground for 30 days to collect soil bacteria. Image from Microbial Insights (http://www.microbe.com/how-bio-traps-work.html).

adsorptive beads (Ghosh et al., 2009) were amended with either yeast extract (YE) (0.025 g) or an acyl-homoserine lactone (AHL) mixture (0.0083 µM) containing six compounds (N-ß-ketocaproyl, N-butyryl, N-tetradecanoyl, N-octanoyl, N-heptanoly, and N-hexanoyl) by soaking the beads in AHL or YE solutions. The AHLs utilized in this study represent a range of acylated hydrocarbon chains (4 to 14 carbons). AHLs have been identified as quorum sensing compounds in gram-negative bacteria (Leadbetter and Greenberg, 2000; Gonzalez and Keshavan, 2006; Manefield and Whiteley, 2007).

These compounds were selected for *in situ* enrichment based on previous reports that they serve as carbon and energy sources for some bacteria (Leadbetter and Greenberg, 2000; Flagan et al., 2003; Uroz et al., 2005; Manefield and Whiteley, 2007) and that they may signal some uncultivable bacteria to grow under laboratory conditions (Barer and Hardwood, 1999; Guan et al., 2000; Bruns et al., 2002). Prior to burial in soil, 100 beads were soaked in 100 ml of YE (0.3 g/L) or AHL (1 µM/L) overnight. The fortified beads were then deployed at one station in two replicate plots of treatments T1 and T4, and at two stations within the SF2 plot. For ease of recovery from field sites, the beads were placed inside a nylon mesh bags that allowed for free movement of soil solution and contact with adjacent soil. The netted beads were then buried between 6 and 10 cm depth for one month.

Extraction, plating, enumeration, and isolation of bacteria

Beads were first rinsed with sterile deionized water to remove surface soil, and cut into smaller fragments with a flame sterilized scalpel. Bacteria were extracted from BioSep beads using a 5mM sodium pyrophosphate solution (0.01 g beads/ml) and 20 minutes of horizontal shaking. Bead extracts were serially diluted (10^{-4} to 10^{-7}) in 1% sodium phosphate solution. Soils were sieved to 2 mm, extracted with autoclaved and deionized H_2O (0.01 g soil/ml H_2O), and placed on a horizontal shaker for 20 minutes. Single strength VL55 base media (Sait et al, 2002) was used to serially dilute soils for plate inoculation (10^{-4} to 10^{-8}). Diluted bead and soil extracts were spread onto VL55 basal medium (1.5% agar) (pH 5.5) containing either pentose sugars (fructose,

arabinose and xylose; media I), = peptone (media II), or a mixture of chitin, cellulose and N-acetyl glucosamine (media III). Three replicate plates for each media type, soil or bead combination, and dilution were prepared.

Colonies were counted and marked as they appeared on the plates after 6, 18, 36, and 76 days of incubation to establish an "ecocollection" as previously described (Hattori et al., 1997). Approximately 10% of the colonies appearing during these four time intervals were randomly picked and streaked onto new plates of the same media type for isolation. These were then transferred two additional times for further isolation. All incubations were done at room temperature (22-25˚C) in the dark.

DNA extraction and isolate archiving

Isolates were subsequently grown in 25 ml of either media I, II, or III. Upon visible turbidity, a 10 ml aliquot of culture was centrifuged at 10,000 g for 20 min and the pellet was utilized for DNA extraction with the MO BIO UltraClean™ Microbial DNA Isolation Kit following the manufacturer's protocol except that the centrifugation steps were extended to either 1 or 3 min for the intermediate and final steps, respectively. Additionally, the columns were air evaporated for 10 min prior to elution. The remaining 15 ml of liquid culture was centrifuged at 10,000 g and the supernatant removed. The pellet was suspended in approximately 5 ml of with the appropriate media type containing 20% glycerol for storage in cryovials at -80℃.

PCR and purification

Polymerase chain reactions were conducted for over 800 isolates using the following mix: 25 µl Taq polymerase, 1 µl 8F primer (AGAGTTTGATCATGGCTCAG), 1 µl 536r primer (CGTATTACCGCGGCTGCTGG), 2 µl template DNA, and 21 µl purified H_2O. Both primers were at 10pm/µl concentration. PCR conditions were: 5 min 45 s at 95℃, 45 s at 58℃, 45 s at 72℃, times 30 cycles, with a final 7 min at 72℃. PCR purification was accomplished using either the Promega Wizard SV 96 Clean-Up System, or the Promega Wizard SV Gel and PCR Clean-Up System. Additional column

incubation at 37℃ for 15 minutes was utilized to evaporate ethanol prior to elution with nuclease-free water.

Cloning

Sequencing of 1465 bp of the 16S rRNA gene was conducted for rare and novel isolates by cloning PCR products from 27f (AGAGTTTGATCCTGGCTCAG) and 1492r d (TACGGYTA- CCTTGTTACGACTT) primers. The same PCR conditions were utilized as above except the extension time was increased to 1 min 30 s. The PCR products were purified with the Promega Wizard SV Clean-up System and cloned using Promega's pGEM-T Vector System. White colonies were picked and grown overnight in LB liquid media with 50 µg/ml ampicillin and submitted for sequencing using the M13 f/r primers.

Sequencing and classification

The DNA concentrations for each sample were measured on a Hoefer DynaQuant DNA Fluorometer. Sanger sequencing was performed on the Applied Biosystems ABI 3730 capillary electrophoresis instrument using either 8f or M13 primer. Partial 16S rRNA gene sequences were classified using the Ribosomal Database Project version 10 (Wang et al., 2007). Assignment of Operational Taxonomic Units (OTUs) was accomplished by aligning sequences through an Infernal secondary-structure based aligner (Nawrocki et al., 2009), and using the RDP Complete Linkage Clustering method.

Analyses and statistics

Colony formation time groups were analyzed with SAS one-way and three-way ANOVA, using Tukey-Kramer for multiple and class level comparisons. Each variable (collection method, land management type, media type) was modeled against cumulative colony formation units (CFU) from the 10^{-5} dilution plates.

Dendrogram trees were generated using RDP (Wang et al., 2007), converted to Newick or Nexus format using ClustalX (Larkin et al., 2007; Thompson et al., 1997), and

imaged using Dendroscope software (Huson et al., 2007). UniFrac was utilized for significance testing (Lozupone et al., 2005; Lozupone et al., 2006; Lozupone et al., 2007); differences between communities were evaluated with the paired P-test (parsimony based phylogenetic test) (Martin, 2002), and lineage specific analyses utilized a G-test, similar to a chi-squared test for fit (Sokal and Rohlf, 1995). Clustering environments was accomplished with UniFrac hierarchical cluster analyses, which is based on distance matrix data applied to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Martin, 2002).

Results

Growth of isolates

Throughout the duration of the incubation period, cumulative CFU by collection type showed that the plates with the most colonies came from AHL fortified beads, while yeast bead plates had the second highest, and soil plates had the fewest (Fig. 4). These counts were taken from the 10^{-5} dilution plates. Cumulative CFU was not affected by either land management or media type. Slopes of the CFU curves from all variables were continuing to rise at the end of the incubation period, suggesting that new colonies would have formed had the incubation continued (Fig. 4a-c).

In contrast to plate CFU data, the total number of isolates was 834 from soil, 373 from AHL beads, and 316 from yeast beads. The large difference between bead and soil isolates is due to colony overgrowth on the 10^{-4} dilution plates from both AHL and yeast extract, which removed these plates from the isolate pool. Many plates were lost to fungus, contamination, or mixed culture, and several isolates would not grow in liquid media (required for the DNA extraction process), so the final number of isolates evaluated further was lower than the original collection (751 for partial 16S rRNA gene sequencing).

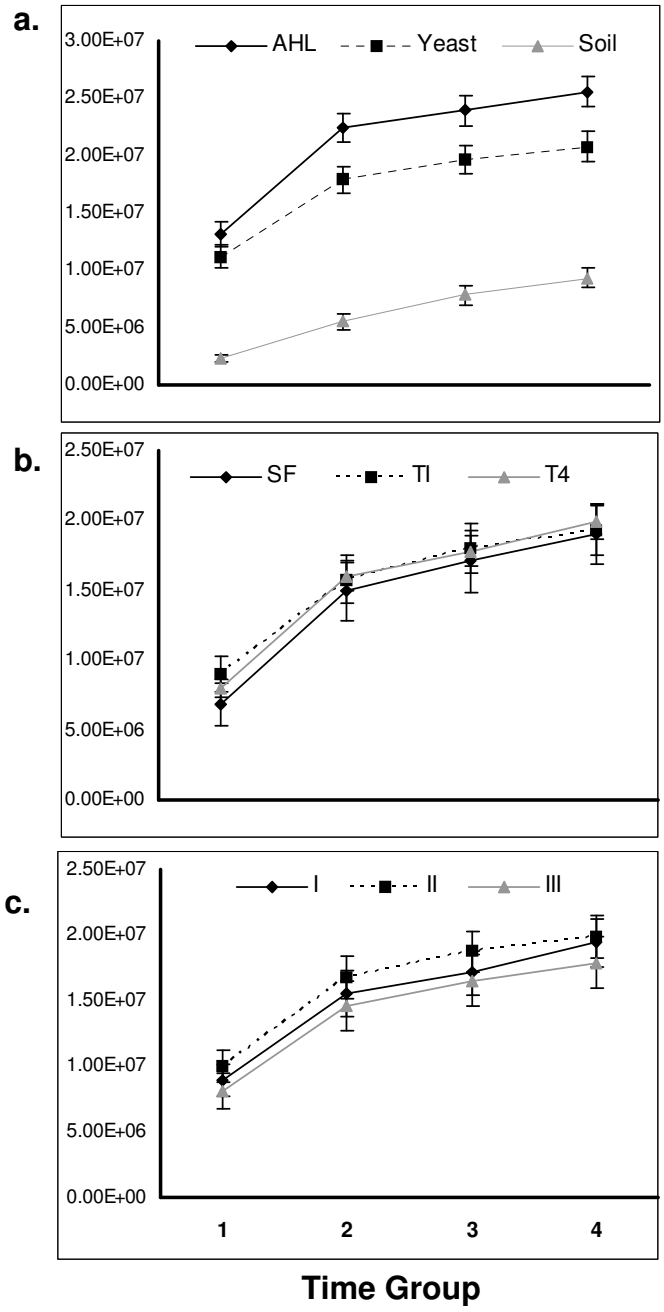

FIG. 4a-c. Cumulative colony forming units (CFU) from initial inoculate plates plotted by time group (TG). TG 1 = days 1 to 5, TG 2 = days 6 to 14, TG 3 = days 15 to 36, TG 4 = days 36 to 75. Graph A is CFU by collection method, acyl homoserine lactone fortified BioSep beads (AHL), yeast fortified BioSep beads (Yeast), or bulk soil (Soil); B is CFU by land management, successional forest (SF), conventional agriculture (T1) or organic agriculture (T4); and C is CFU by media substrate type, media I = five carbon sugars (arabinose, fructose, xylose), media II = peptone, media III = complex (chitin, cellulose and n-acetyl glucosamine). Error bars are standard error.

Diversity

Partial 16S rRNA gene sequences of 751 isolates were classified and aligned through the RDP (version 10) website. The sequences belong to 7 phyla, 9 classes, 14 orders, and 42 families at 97% or greater confidence threshold. The use of fortified BioSep beads resulted in the addition of 7 families that were not represented in the soil isolates, as shown in the classifications of 662 isolates in Table 1. Table 2 gives the class distribution and percentage of all 751 sequences, as shown in the tree in Fig. 5.

Distribution of Operational Taxonomic Units

Most of the aligned sequences (662) were clustered into 157 OTUs using a 3% sequence similarity cutoff. The use of AHL and yeast beads in the collection process contributed 33 OTUs that were not present in the soil isolate community. Soil isolates contributed the highest number of unique OTUs overall (Fig. 6), however this is likely partially due to the uneven distribution of isolates from the different collection methods as stated previously (292, 195 and 175 from Soil, yeast bead, and AHL bead). Land management unique OTUs were distributed at a fairly even rate across the three types (Fig. 7), which agrees with the total number of isolates present per land management type (235, 222, and 205 for T1, T4 and SF). The greatest OTU overlap occurred between the T1 and T4 communities.

The use of multiple substrates effectively selected for unique OTUs, and the number of unique OTUs per substrate was similar to the total number of isolates per substrate (222, 274, and 164 for media I, II and III). The greatest overlap of OTUs was between media types I and II (Fig. 8). Extending the incubation period beyond 14 days resulted in adding 39 OTUs to the isolate collection, 28 from group 3 and 11 from group 4 (Fig. 9). The greatest community overlap occurred between groups 2 and 3 (Fig. 9). The total isolates per time group was 201, 208, 182, and 81 from group 1, 2, 3, and 4.

TABLE 1. Family classification of 662 bacterial isolates from the Kellogg Biological Station (KBS) developed culture collection. Highlighted genera were exclusive to yeast or Acyl homoserine lactone fortified BioSep bead sampled communities. Dashes = no isolates. Soil column represents bacteria isolated from bulk soil samples.

Class	Order	Family	Soil	Yeast Beads	AHL Beads
Acidobacteria	Acidobacteria_Gp1	Acidobacteria_Gp1	9	-	1
Actinobacteria	Actinomycetales	Actinosynnemataceae	-	1	-
		Dermabacteraceae	1	-	-
		Dermacoccaceae	-	1	-
		Geodermatophilaceae	-	-	1
		Intrasporangiaceae	5	2	1
		Microbacteriaceae	8	1	1
		Micrococcaceae	3	15	6
		Mycobacteriaceae	11	-	-
		Nocardiaceae	2	31	38
		Nocardioidaceae	8	13	2
		Propionibacteriaceae	1	1	-
		Pseudonocardiaceae	1	2	-
		Streptomycetaceae	2	1	2
		Streptosporangiaceae	1	-	-
		Thermomonosporaceae	-	1	-
Sphingobacteria	Sphingobacteriales	Chitinophagaceae	16	1	11
		Cytophagaceae	3	-	-
		Sphingobacteriaceae	9	1	6
Bacilli	Bacillales	Paenibacillaceae	3	-	-
		Bacillaceae	2	-	-
Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	1	-	-
Alphaproteobacteria	Caulobacterales	Caulobacteraceae	14	1	-
	Pseudomonadales	Moraxellaceae	-	2	-
	Rhizobiales	Beijerinckiaceae	-	2	-
		Bradyrhizobiaceae	47	23	9
		Hyphomicrobiaceae	1	4	2
		Methylobacteriaceae	12	3	-
		Phyllobacteriaceae	18	29	17
		Rhizobiaceae	2	2	-
		Xanthobacteraceae	4	25	2
	Rhodospirillales	Acetobacteraceae	1	-	-
		Rhodospirillaceae	4	-	-
	Sphingomonadales	Sphingomonadaceae	40	1	11
Betaproteobacteria	Burkholderiales	Burkholderiaceae	32	8	19
		Comamonadaceae	7	19	37
		Incertae_sedis	-	-	1
		Oxalobacteraceae	10	-	3
Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	1	4	-
	Pseudomonadales	Pseudomonadaceae	7	1	2
	Xanthomonadales	Xanthomonadaceae	5	-	3
Verrucomicrobia	Subdivision3	Incertae_sedis	1	-	-

TABLE 2. Class distribution of 751 soil bacterial isolates from the Kellogg Biological Station Long Term Ecological Research Site.

Class	Color	# of isolates	Percent of total
Alphaproteobacteria	Blue	331	44.1
Verrucomicrobia	Orange	1	0.1
Gemmatimonadetes	Light Green	1	0.1
Shingobacteria	Pink	56	7.5
Bacilli	Gold	4	0.5
Acidobacteria	Brown	10	1.3
Actinobacteria	Green	185	24.6
Betaproteobacteria	Purple	137	18.2
Gammaproteobacteria	Cyan	26	3.5
Total		751	

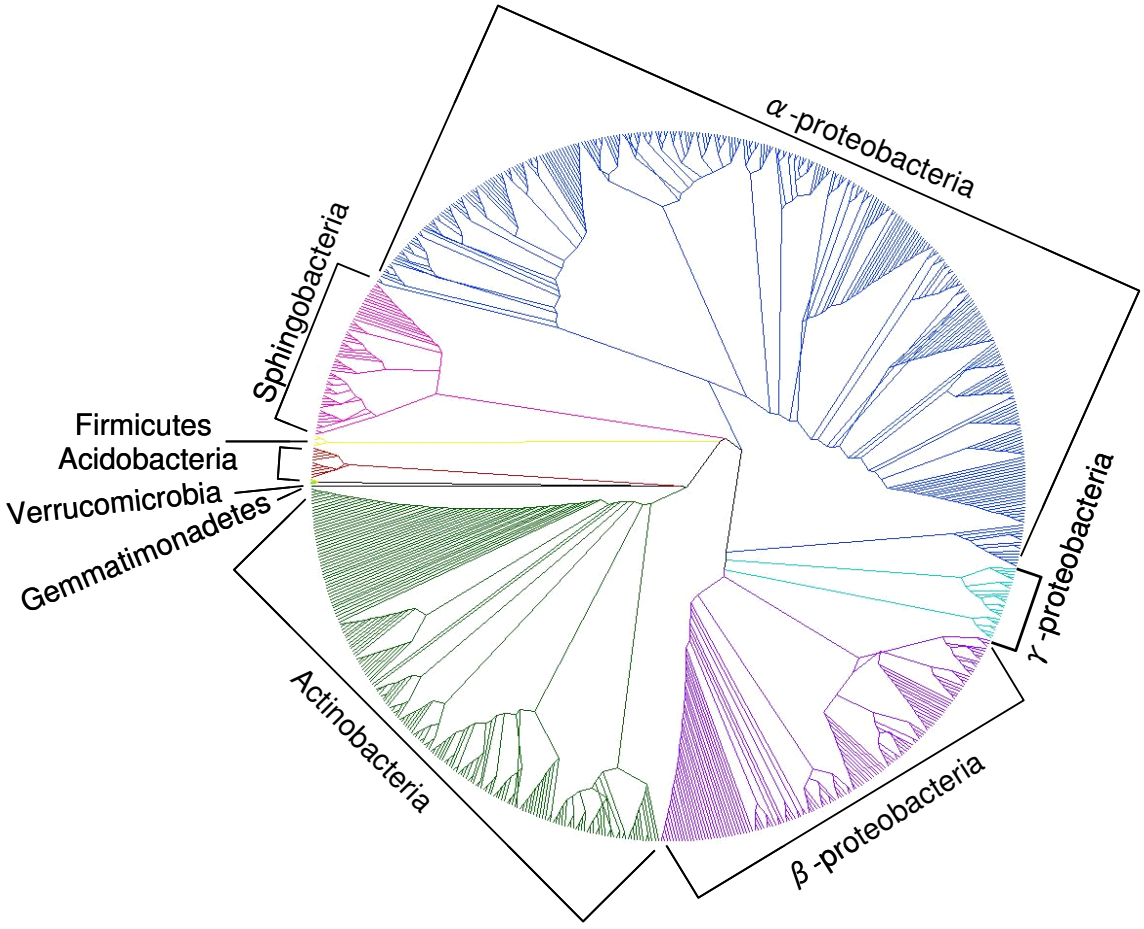

FIG. 5. Dendrogram based on partial 16S rRNA gene sequences of 751 isolates from the bacterial collection cultivated from yeast and acyl homoserine lactone (AHL) fortified BioSep beads, and from bulk soil.

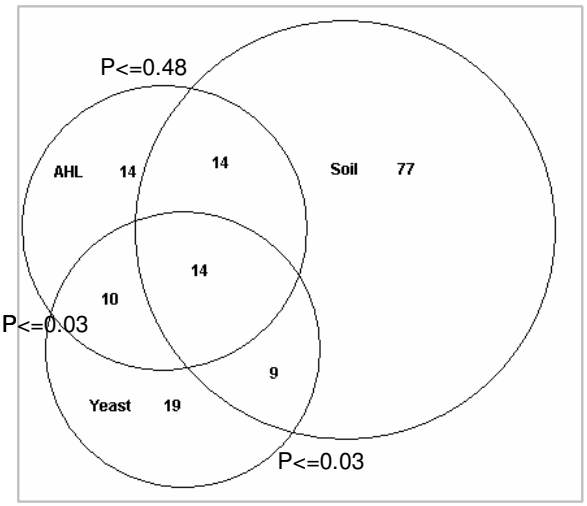

FIG. 6. Distribution of operational taxonomic units from the three bacterial isolate collection methods. AHL = acyl homoserine lactone fortified BioSep beads, Yeast = yeast fortified BioSep beads, Soil = bulk soil. P = P value from UniFrac parsimony based phylogenetic test of community similarity for adjacent cultivable communities.

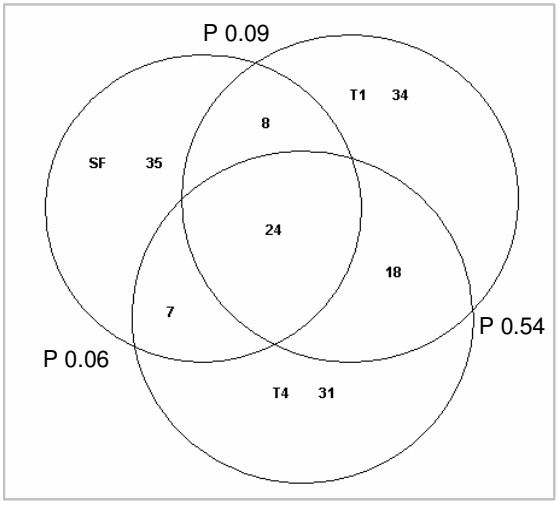

FIG. 7. Operational taxonomic unit distribution by land managements; Successional Forest (SF), conventional agriculture (T1), and organic agriculture (T4). P = P value from UniFrac parsimony based phylogenetic test of community similarity for adjacent cultivable communities.

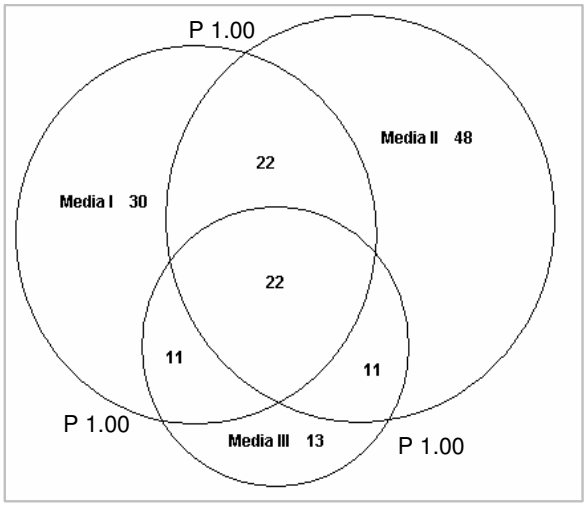

FIG. 8. Operational taxonomic unit distribution by media substrate type. Media I = five carbon sugars (arabinose, xylose and fructose), media II = peptone, media III = complex (chitin, cellulose, and n-acetyl glucosamine). P = P value from UniFrac parsimony based phylogenetic test of community similarity for adjacent cultivable communities.

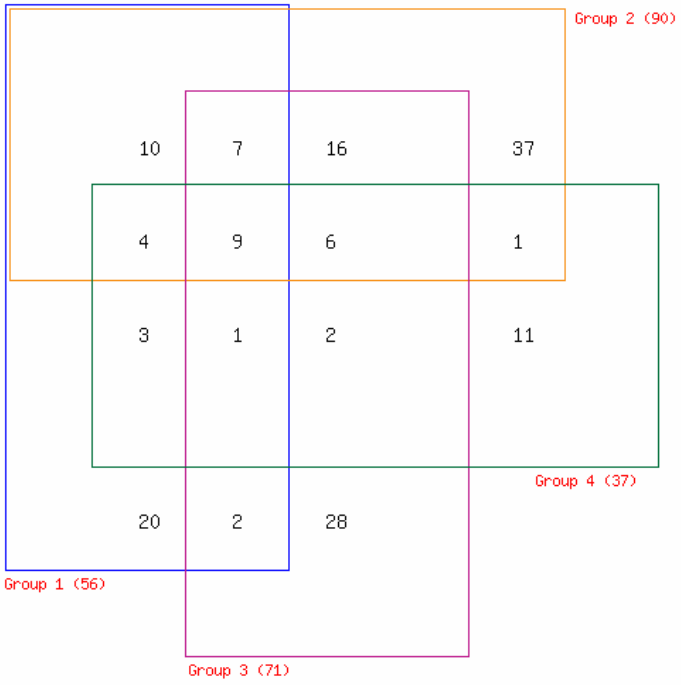

FIG. 9. Operational Taxonomic Unit distribution based on initial colony forming unit (CFU) appearance on inoculate plates within four time groups. Group 1 = CFU initial appearance between days 1 and 5, group 2 = between days 6 and 14, group 3 = between days 15 and 36, and group 4 between days 36 and 75. None of the cultivated time group communities were significantly different from other time groups.

Community and lineage specific analyses

UniFrac parsimony based phylogenetic testing (P test) of the soil bacterial collections cultivated from the three collection methods showed a significant difference between the yeast bead and AHL bead cultivated collections (Probability (P)<=0.03), and between the yeast bead and bulk soil derived collections (P<=0.03). Lineage specific analyses revealed that the Alphaproteobacteria genus *Labrys* was cultivated more frequently from yeast beads than from soil or AHL beads (Table 2, see also Tables 4 and 5). *Rhodococcus* (Actinobacteria) isolates were obtained almost exclusively from AHL and yeast beads, and *Variovorax* isolates (Betaproteobacteria) occurred more often from AHL beads (Table 2).

There were no overall significant differences in cultivable community structure between land management systems or media types, however the phylogentic test P values for land management suggested that this variable may have affected diversity of the collection when comparisons were made (i.e. 0.09 and 0.06 for T1-SF and T4-SF paired communities). Significant differences of specific lineages are shown in Table 3. Land management preference was noted in the *Rhodococcus* and *Variovorax* genera, and media preference was noted in *Chitinophaga* (Sphingobacteria) and *Sphingomonas* (Alphaproteobacteria) (Table 3).

In the analysis for time groups, the phylogenetic test values between time group 1 and each other time group was 0.06, suggesting that the time group 1 cultivated collection differed from the others. There were no significant differences between the remaining time groups. In the lineage specific analyses (Fig. 10), Betaproteobacteria colonies formed mainly in the first time group (group 1), Actinobacteria colonies formed evenly in all time groups with the exception *Arthrobacter* and *Rhodococcus* sp. that predominately formed in time group 1, and Sphingobacteria colonies occurred somewhat evenly throughout the incubation period. Alphaproteobacteria colonies formed in 3 groups; those that formed in the second time group (group 2), those that were slow to form (groups 3 and 4), and those that formed evenly across all time groups.

TABLE 3. UniFrac G test based lineage specific analyses for several genera in the bacterial isolate collection based on experiment variables of collection method, land management, and media substrate. Collection methods were: acyl homoserine lactone (AHL) fortified BioSep beads, bulk soil, and yeast (YST) fortified BioSep beads. Land managements were: successional forest (SF), conventional agriculture (T1), and certified organic agriculture (T4). Media substrates were: Sugars – arabinose, xylose, and fructose (I), peptone (II), and complex – chitin, cellulose, and n-acetyl glucosamine (III).

	Variable	Observed	Predicted	P value	Dominate genus of lineage
Collection method	AHL	2	8.77		Labrys
	SOIL	6	14.97	0.0000369	
	YST	25	9.25		
	AHL	38	18.88		Rhodococcus
	SOIL	1	32.22	0.0000000	
	YST	32	19.9		
	AHL	32	15.16		Variovorax
	SOIL	7	25.86	0.0000177	
	YST	18	15.98		
Land Mgmt	SF	41	22.57		Rhodococcus
	T1	9	25.14	0.0004150	
	T4	21	23.29		
	SF	4	18.12		Variovorax
	T1	36	20.18	0.0007830	
	T4	17	18.7		
Media substrate	I	0	5.75		Chitinophaga
	II	4	7	0.0019200	
	III	13	4.25		
	I	5	15.55		Sphingomonas
	II	35	18.95	0.0030900	
	III	6	11.5		

UniFrac hierarchical cluster analyses, which is based on distance matrix data applied to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Martin, 2002), was conducted using the four experiment variables (Fig. 11 a-d). The clusters revealed that; yeast and AHL communities were more similar to each other than to the bulk soil community; the two agricultural land management communities (T1 and T4) were more similar to each other than to the successional forest (SF) community; media I and II communities were clustered together; and time groups 2, 3 and 4 were clustered apart from group 1, with groups 2 and 3 the most similar (Fig. 11 a-d).

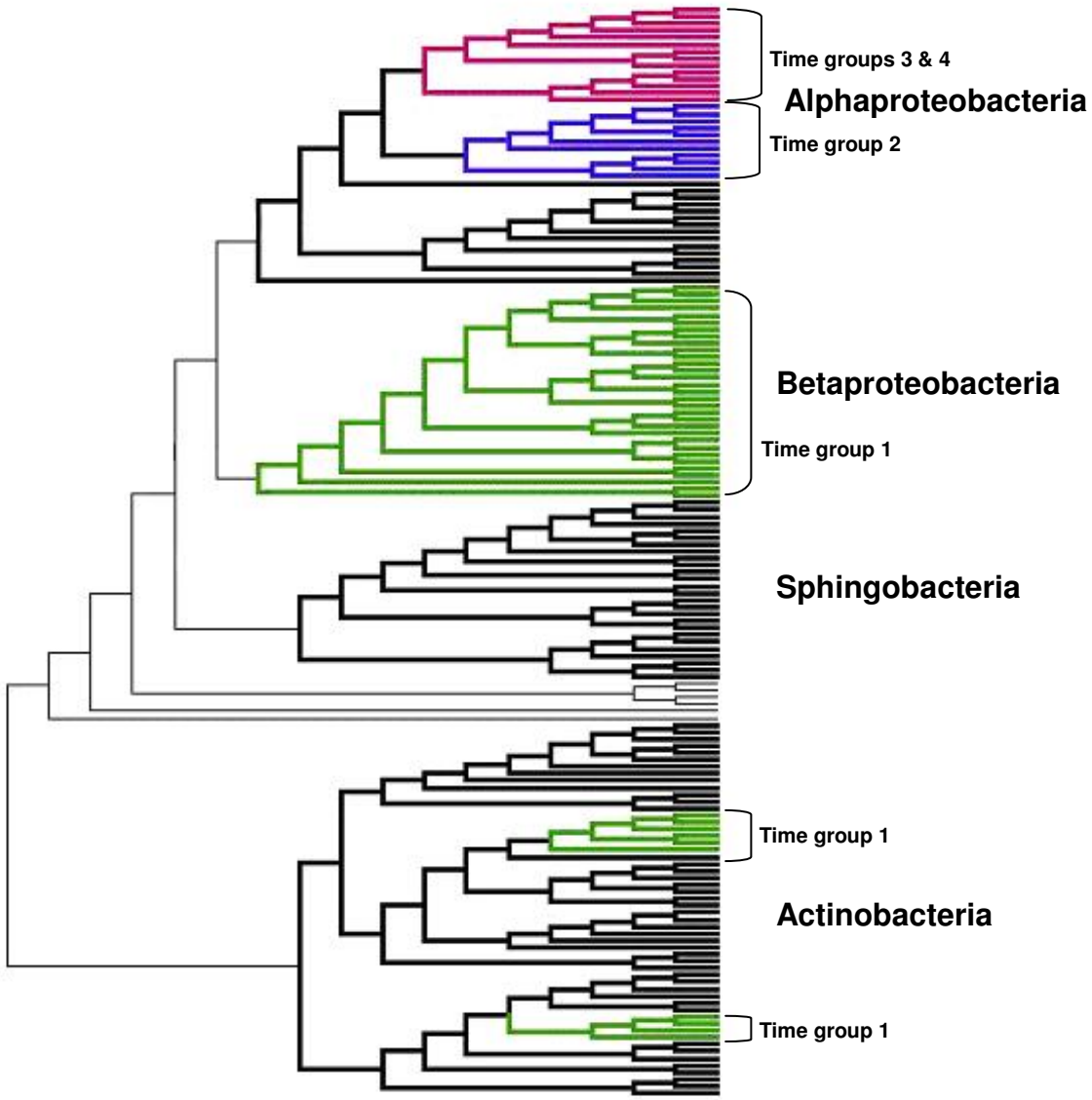

FIG. 10. UniFrac G test based lineage specific analysis of operational taxonomic units (OTU) within the four time groups, determined by initial colony forming unit (CFU) appearance on inoculate plate and displayed in hierarchical cluster. Group 1 = CFU initial appearance between days 1 and 5, group 2 = between days 6 and 14, group 3 = between days 15 and 36, and group 4 between days 36 and 75. Colonies arose evenly throughout all 4 time groups unless noted with dominant time group.

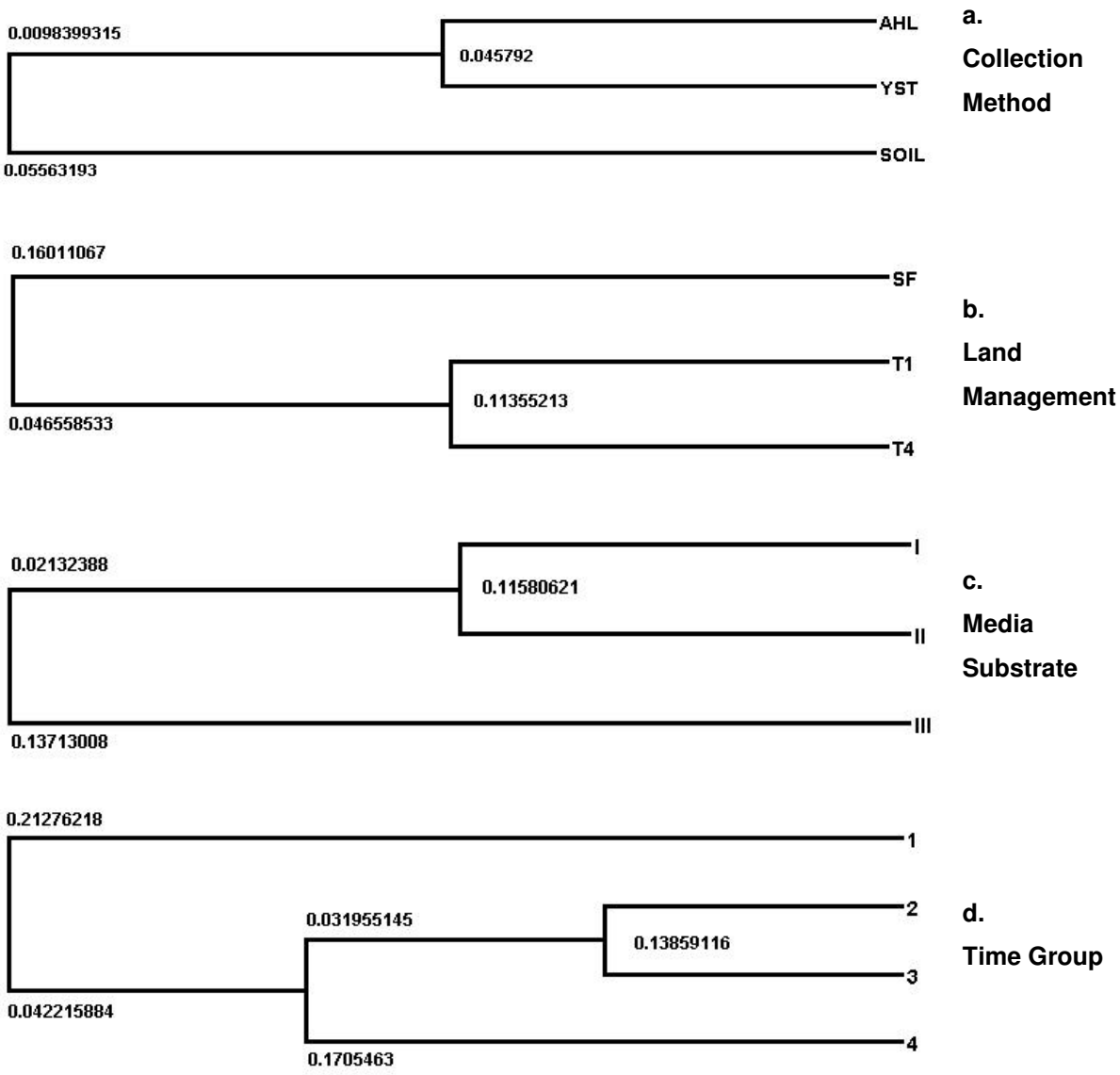

FIG. 11a-d. UniFrac hierarchical cluster analyses of the soil cultivable communities by the four experimental variables. a. Collection methods were: acyl homoserine lactone (AHL) fortified BioSep beads, yeast (YST) fortified BioSep beads, and bulk soil. b. Land managements were: successional forest (SF), conventional agriculture (T1), and certified organic agriculture (T4). c. Media substrates were: Sugars – arabinose, xylose, and fructose (I), peptone (II), and complex – chitin, cellulose, and n-acetyl glucosamine (III). d. Time groups were assigned by initial colony forming unit (CFU) appearance on inoculate plates within four time periods. Group 1 = CFU initial appearance between days 1 and 5, group 2 = between days 6 and 14, group 3 = between days 15 and 36, and group 4 between days 36 and 75. Edge weights represent fraction of similarity of the environments, with a distance of 1 being mutually exclusive, and 0 being identical.

Rarely cultivated isolates

Soil samples were responsible for most of the rare and novel organisms in the collection, however the greater abundance of soil isolates overall may account for this result. Table 4 shows the distribution of 21 genera represented by 83 isolates in the collection that occur in low frequencies in both the GenBank and RDP databases, including Acidobacteria GP1, Verrucomicrobia Subdivision 3, and *Gemmatimonas*. An examination of the collection method showed that five of these less frequent genera occurred from yeast and AHL bead extracts only, 10 were exclusively from soil, and 6 were present from a combination of bead and soil isolates (Table 4).

As a group, the 83 isolates from the rarely cultivated genera occurred almost evenly across the land management types (28, 31 and 24 from SF, T1 and T4, respectively). Media II, however, was preferred by this group of isolates over

TABLE 4. Distribution of infrequently cultivated genera by collection method; acyl homoserine lactone (AHL) fortified BioSep beads, yeast (YST) fortified BioSep beads, and bulk soil. Infrequency was determined by relatively low (<=20) isolate representation of genera as queried in GenBank or Ribosomal Database Project databases.

Class	Order	Family	Genus	Soil	Yeast Bead	AHL Bead
Acidobacteria	Gp1	(Acidobacteria GP1)	Acidobacteria Gp1	9	-	1
Actinobacteria	Actinomycetales	Propionibacteriaceae	Friedmanniella	1	1	-
		Nocardioidaceae	Marmoricola	3	6	-
		Microbacteriaceae	Okibacterium	1	-	-
		Intrasporangiaceae	Phycicoccus	3	1	-
			Tetrasphaera	1	1	-
Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Altererythrobacter	3	-	-
	Rhizobiales	Xanthobacteraceae	Labrys	6	25	2
		Beijerinckiaceae	Methylocella	-	1	-
Betaproteobacteria	Burkholderiales	Incertae sedis	Aquincola	-	-	1
		Comamonadaceae	Ramlibacter	2	-	-
			Pseudorhodoferax	-	-	1
Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rudaea	1	-	-
Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	1	-	-
Sphingobacteria	Sphingobacteriales	Cytophagaceae	Dyadobacter	1	-	-
	Sphingobacteriales	Chitinophagaceae	Segetibacter	1	-	-
		Cytophagaceae	Spirosoma	1	-	-
		Chitinophagaceae	Terrimonas	1	-	-
			Sediminibacterium	-	1	5
			Flavisolibacter	-	-	1
Verrucomicrobia	Subdivision 3	(Verrucomicrobia Subd3)	Incertae sedis	1	-	-

media I or III, and media I resulted in the fewest rarely cultivated isolates (15, 41 and 27 from I, II and III, respectively). The rarely cultivated isolates also showed a preference for the middle time groups 2 and 3 (12, 33, 27, and 11 from time groups 1, 2, 3 and 4, respectively). Three of these genera, *Labrys*, *Marmoricola*, and the *Acidobacteria*, were examined individually by land management, media type, and time groups in Table 5. The *Gemmatimonas* isolate came from bulk soil extracted from a T4 plot, was isolated on media II, and was from time group 3. The Verrucomicrobia isolate came from soil extracted from the SF site, was isolated on media I, and was also from time group 3.

The RDP classification showed that 145 isolates in the collection may be novel at the genus level using a 10% similarity cutoff. GenBank, however, was able to match many of these to within 10%. A conservative estimate is that 44 isolates, or 6%, are novel at the genus or higher classification level. This will have to be confirmed through additional analyses.

TABLE 5. Distribution of soil bacterial isolates from three rare genera by experiment variables. Land managements were: successional forest (SF), conventional agriculture (T1), and certified organic agriculture (T4). Media substrates were: Sugars – arabinose, xylose, and fructose (I), peptone (II), and complex – chitin, cellulose, and n-acetyl glucosamine (III). Time groups were assigned by initial colony forming unit (CFU) appearance on inoculate plates within four time periods. Group 1 = CFU initial appearance between days 1 and 5, group 2 = between days 6 and 14, group 3 = between days 15 and 36, and group 4 between days 36 and 75.

	Land Management		
Genus	**SF**	**T1**	**T4**
Acidobacteria Gp1	7	-	3
Labrys	15	9	9
Marmoricola	-	9	-
	Media Type		
Genus	**I (Sugars)**	**II (Peptone)**	**III (Polymers)**
Acidobacteria Gp1	4	3	3
Labrys	3	12	18
Marmoricola	1	8	-
	Time Group			
Genus	**1**	**2**	**3**	**4**
Acidobacteria Gp1	-	1	8	1
Labrys	10	14	7	2
Marmoricola	-	4	-	5

Discussion

Initial Growth

Many previous cultivation studies have used incubation periods of two weeks or less, however, the present results indicate that many soil bacteria require longer lag periods on agar based media. This isolate collection provides further evidence of the need for longer incubation. As noted by the cumulative colony formation (Fig. 4), even after 80 days the growth curves had positive slopes. Additionally, community comparisons and cluster analysis suggests that the cultivated collection from time group 1 was different from the other groups (Fig. 11d). The use of a higher volume of agar based media might be required, however, as plates tended to dry out even though wrapped in parafilm.

Plates inoculated with soil extract had lower CFUs overall, which is surprising since the average dry weight of soil was higher than the average dry weight of beads per extract solution (0.835 and 0.288 mg). There are several possible explanations for this, including higher extraction efficiency from BioSep beads. Bacteria can adsorb to soil surfaces due to soil charge (Dhand et al., 2009), or be difficult to extract from within aggregates. Some researchers have used sonication in the extraction process, which could be beneficial despite the obvious damage that can be caused to whole cells (Janssen et al., 2002; Joseph et al., 2003).

Another possibility is that beads may have selected for faster growing opportunistic species. This is supported by the sequence data which showed AHL and yeast bead isolates arose quickly and were dominated by *Arthrobacter* and *Rhodococcus* (Actinobacteria), as well as *Variovorax* (Proteobacteria). *Burkolderia* species were also present in both bead communities, but were more numerous in the bulk soil community. These four genera contributed 42% and 33% of the total AHL and yeast bead isolates, yet only 14% of the bulk soil isolates.

Diversity of the collection

Janssen (2006) published an analysis of 32 soil sequence libraries from around the globe which found that the most commonly reported prokaryotes from cultivation independent observations were; *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*. Roesch et al. (2007) found similar results in their pyrosequencing study. In the present study, the cultivated community contained isolates from all of the above except Chloroflexi and Planctomycetes. This may have been due to the lower pH of the media used (5.5) in this study, or could have resulted from only isolating 10% of the colonies on high colony density plates.

Only one Gemmatimonadetes (KBS708) and one Verrucomicrobia (KBS606) isolate were present in the cultivation collection, despite the high number of isolates sequenced (751). Five cultured Gemmatimonadetes isolates are listed in GenBank and RDP, and the maximum identity of KBS708 is less than 89% to the GenBank listed isolates. The highest similarity score obtained using the aligned KBS708 sequence in RDP Sequence Match is 0.917 to *Gemmatimonas aurantiaca* T-27, (AB072834), as demonstrated in Fig. 12. This suggests that KBS708 is novel as well as rare.

The Verrucomicrobia isolate, KBS606, closely matches bacterium Ellin516 (AY960779) from Subdivision 3, as determined by RDP Sequence Match (Score=0.992) and GenBank (Maximum identity 99%). A previous cultivation-independent study at KBS found that Verrucomicrobia 16S rRNA was recovered from soil at approximately 1.9%, with a correlation to time of year, May having the highest Verrucomicrobia RNA (Buckley and Schmidt 2001). Their results also suggest that soil moisture may positively impact the abundance of Verrucomicrobia RNA in soils. Sampling for this experiment occurred between August and September 2007, and the KBS weather data for that time period shows that there were rainfall events before and during the sampling period.

Diversity by experiment variables

Collection method was the only experiment variable that significantly affected the cultivable community as a whole. This could be attributable to the known limitations of

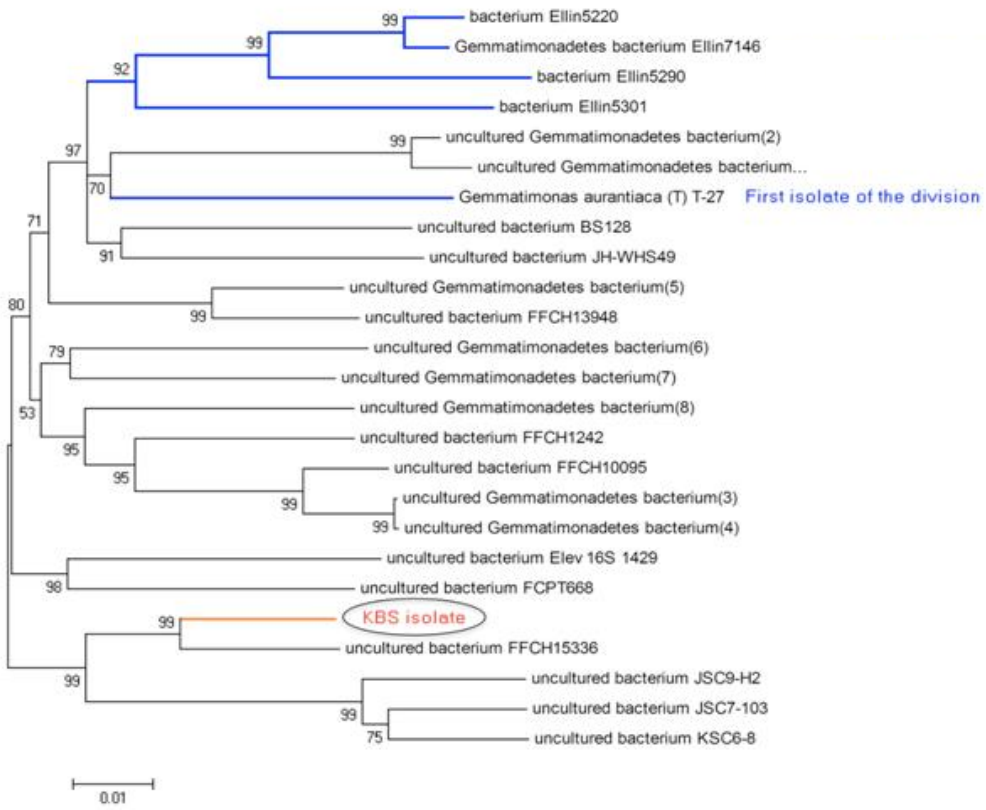

FIG. 12. Gemmatimonadetes tree showing nearest cultivated relatives to KBS708 (circled) from the cultivation collection (Ghosh et al., unplublished).

soil bacteria cultivation, or could have been the result of fungal contamination of some plates that occurred during the incubation period. Additionally, isolates found to contain mixed colonies were discarded, though some bacteria can only grow in consortia. This may have resulted in the elimination of an important fraction of the community diversity. Lineage specific differences, however, were observed for all four variables, and each change to the traditional cultivation methods resulted in the addition of OTUs to the collection. This is evidence of the need for more innovative collection and cultivation techniques, as well as extended incubation. There is further evidence that additional changes, such as increased [CO_2] and the use of dissecting microscopes to select micro-colonies from plates, may expand future cultivation collections from the KBS LTER site (Stevenson et al., 2004).

AHL and yeast fortified BioSep beads

Both *Rhodococcus* and *Variovorax* responded positively to AHL fortified BioSep beads, verifying previous studies that showed these two bacteria can utilize AHLs as substrate (Leadbetter and Greenberg, 2000; Roche et al., 2004; Uroz et al., 2005). *Arthrobacter* have been shown to degrade AHLs as well, possibly in consortia with *Variovorax paradoxus* (Flagan et al., 2003), however the only *Arthrobacter* present in this collection came from soil and yeast bead extracts.

There were several genera present in the collection that have been shown to contain AHL producing species (Manefield and Whiteley, 2007), and the responses of these genera to AHL fortified beads were mixed. *Ralstonia* appear to have been attracted to the AHL beads and *Mesorhizobium* and *Pseudomonas* did not seem affected in either direction, however *Burkholderia*, *Rhizobium* and *Bradyrhizobium* seem to have been deterred by the presence of AHL. *Agrobacterium* and *Erwinia* isolates did not occur in high enough quantity to determine a response to AHL.

The full significance of AHLs in the environment remains to be determined, however this study showed that AHLs can be utilized as selective media in the collection process. This may be particularly useful in future studies of AHL degrading microbes. Based on this collection, one might further explore the possible AHL degrading abilities of *Agromyces* (Actinobacteria), *Pedobacter* (Bacteroidetes), and *Sphingomonas* and *Pelomonas* (Proteobacteria).

Yeast fortified BioSep beads were also successful in selecting for specific soil bacteria, as *Labrys* (Alphaproteobacteria) and *Rhodococcus* (Actinobacteria) were more commonly isolated from yeast bead extract plates than from bulk soil cultivation. *Labrys* do not occur at a high intensity in either the GenBank or RDP databases. Previous publications and Bergey's Manual of Systematic Bacteriology (2005) confirm that some *Labrys* species have intensified growth with yeast extract (Miller et al., 2005). The opposite was true, however, for Sphingomonodales and Betaproteobacteria in the collection, which had significantly more isolates from bulk soil and AHL bead plates and very few from yeast plates.

Novelty in the collection

There are 44 potentially novel isolates in the collection by conservative estimates. These are isolates that had below 90% confidence threshold to the nearest cultivated isolate in the RDP 10 database, and were generally 97% or less identical to the nearest cultivated neighbor when submitted to a BLAST nucleotide search. These potentially novel isolates appear to be unevenly distributed across experiment variables when compared to the distribution of all 662 sequenced isolates. For instance, 28 novel isolates came from media II plates, though only 18 were predicted as determined by comparison to the ratio of all isolates by media substrate. Distribution of all 44 novel isolates by the four experiment variables is shown in Table 6. Most of the novel isolates from AHL bead plates were from the Bacteroidetes class (6 out of 10), while the yeast bead novel bacteria were mainly Actinobacteria (5 out of 9), and the bulk soil novel isolates were dominated by Alphaproteobacteria (15 out of 25).

One of the novel isolates observed in the collection was BMOPS420, an Alphaproteobacterium from AHL bead extract, T4 land management plot, plated on media I, and appearing in the third time group. The highest similarity score in RDP is 0.924 to bacterium Ellin314 (AF498696), an unclassified Rhodospirillaceae. This isolate was later found to be a lysogen through induction assay, and a micrograph of BMOPS420 and its phage is displayed in Fig. 13.

Conclusion

It is clear from this study that our ability to cultivate soil bacteria in the laboratory, including novel or rare taxa, can be easily enhanced by simple adjustments to current methods. The addition of fortified BioSep beads increased the number of OTUs present in the total bacterial collection by 21%. The use of additional fortifying compounds is predicted to result in further cultivable diversity. Increases in OTU richness in the overall community were also gained by utilizing multiple substrates during cultivation, and by increasing the amount of time inoculated plates are incubated. Efforts in increasing the diversity of cultivation collections is a laborious process, but still warranted in the search

for industrial and medically important compounds, as well as for the host of research interests that require cultivated isolates.

TABLE 6. Predicted and observed distribution of 44 novel isolates by experiment variables. Predictions were determined from the fraction of contribution of isolates from each variable. Media substrates were: Sugars – arabinose, xylose, and fructose (I), peptone (II), and complex – chitin, cellulose, and n-acetyl glucosamine (III).Land managements were: successional forest (SF), conventional agriculture (T1), and certified organic agriculture (T4). Collection methods were: acyl homoserine lactone (AHL) fortified BioSep beads, yeast (YST) fortified BioSep beads, and bulk soil. Time groups were assigned by initial colony forming unit (CFU) appearance on inoculate plates within four time periods. Group 1 = CFU initial appearance between days 1 and 5, group 2 = between days 6 and 14, group 3 = between days 15 and 36, and group 4 between days 36 and 75.

	Variable	Observed	Predicted
Media Type	I	7	15
	II	28	18
	III	9	11
Land Mgmt	SF	13	14
	T1	10	16
	T4	21	15
Collection Method	AHL bead	10	12
	SOIL	25	19
	YST bead	9	13
Time Group	1	2	13
	2	16	14
	3	16	11
	4	10	5

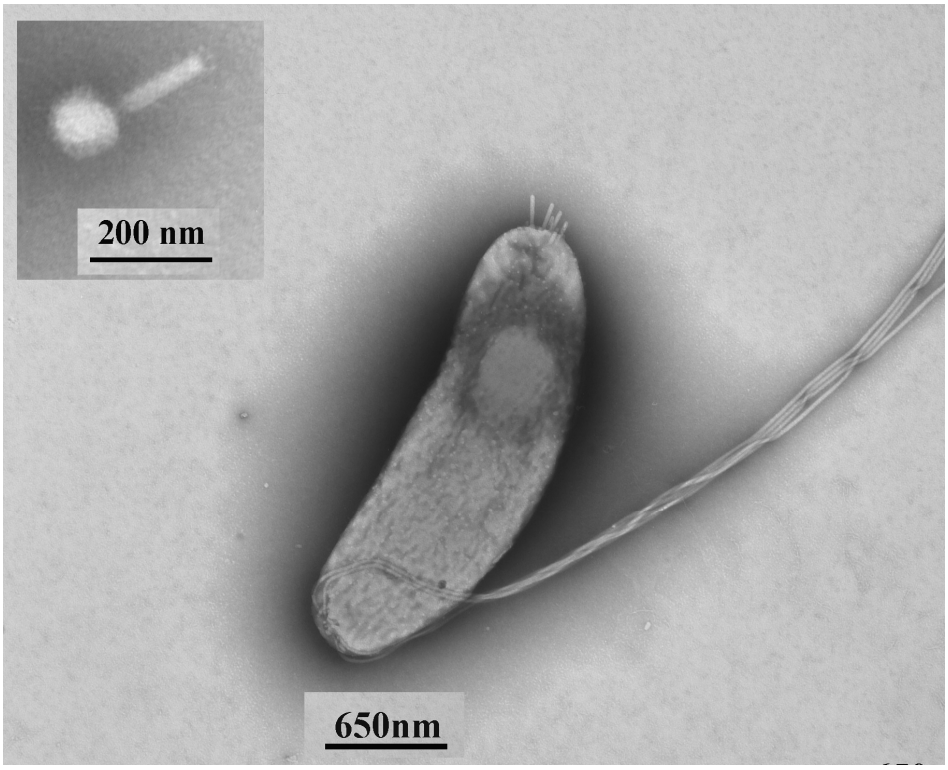

FIG. 13. Transmission electron micrograph of a novel Alphaproteobacterium isolate from the cultivation collection, BMOPS420, and its temperate phage (insert). The isolate was cultivated from a BioSep bead fortified with acyl homoserine lactones and incubated below the soil surface for 30 days. The phage was induced with mitomycin C.

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Chapter 2

Microarray Analysis of Small Sub-unit Ribosomal RNA Genes from Forest Soil Bacteria and Archaea Extracted from Fortified Bio-Sep Beads.

Abstract

Microarray analysis of the 16S rRNA gene was utilized to determine the ability of porous BioSep beads to capture soil bacterial and archaeal diversity. The BioSep beads were fortified with one of three solutions; 1) purified water, 2) yeast extract, or 3) a mixture of six acyl homoserine lactones (AHL). The beads were incubated below the soil surface for four weeks in a successional forest plot at the Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site. Extracted DNA from beads as well as bulk soil was analyzed with the G2 Phylochip from Affymetrix. Extracts from the four experimental variables were analyzed on three Phylochips per variable for analytical replication, and a positive fraction of 0.90 from each replicated chip was utilized as the cutoff to determine the presence of an Operational Taxonomic Unit (OTU). A total of 172 OTU were present; 90 from bulk soil, 93 from water fortified BioSep beads, 97 from yeast fortified BioSep beads, and 67 from AHL fortified BioSep beads. Bulk Soil bacterial operational taxonomic unit (OTU) composition was significantly different from BioSep bead OTU compositions, however there were no differences in OTU composition between the three types of fortified beads. Archaeal DNA was detected only from the bulk soil samples.

The BioSep beads were colonized by four phyla, one proteobacteria class, and 28 families that were not present from bulk soil extract, and nearly doubled the OTUs present in the overall analysis. It is clear that the use of BioSep beads can increase soil bacteria measurable diversity. Even though AHL BioSep bead extract showed the lowest number of OTU, there were none the less 34 OTU present from AHL beads that were not present from bulk soil sampling. The possibilities for BioSep bead fortification are vast, and increased cultivable diversity is predicted through the combined use of fortified beads and expanded cultivation techniques.

Introduction

The determination of soil microbial diversity has proven to be a difficult task for many reasons. Cultivation dependent techniques are inherently inefficient and skew measurable diversity toward those microbes best adapted to the limited conditions offered by traditional cultivation methods (Schneegurt et al., 2003; Sait et al., 2002). Cultivation independent techniques have expanded the known soil bacterial phyla, however these techniques introduce their own biases, as extraction of DNA (and cells) from soil is limited by the efficiency of the extraction method used (Feinstein et al., 2009; Schneegurt et al., 2003), by adsorption of nucleic acids to soil colloids and clay particles (Frostegard et al., 1999), and by the inclusion of extracellular non-viable DNA in the interrogation (Frostegard et al., 1999). Additionally, humic acids can interfere with DNA extraction and amplification processes (Sagova-Mareckova et al., 2008; Schneegurt et al., 2003).

There are currently 52 recognized bacterial phyla, at least 32 of which occur in soils (Janssen, 2006). The global inventory of 32 libraries of 16S rRNA by Janssen (2006) found that the top nine reported bacterial phyla from soil libraries were Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes, listed in approximate order of abundance. Roesch et al. (2007) had slightly different results from their pyrosequencing study. They showed that Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Firmicutes, Gemmatimonadetes, Nitrospira, Verrucomicrobia, and TM7 were the nine most abundant phyla from four western hemisphere sites.

Sides et al. (2010) (Chapter 1) conducted a cultivation dependent study from the Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site. They found that, with 662 isolates, cultivated phyla consisted primarily of Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, and Firmicutes, with one isolate each from Verrucomicrobia and Gemmatimonadetes. The KBS cultivation based study utilized BioSep beads (2 to 3 mm carbon spheres with high interior porosity), fortified with acyl homoserine lactones (AHLs) or yeast extract and placed below ground for 4 weeks, to

potentially trap less commonly cultivated soil bacteria and therefore increase cultivable diversity. BioSep beads have been successfully fortified and utilized in microbial metabolism studies, as well as placed in situ to trap and examine active degrader microbes in bioremediation studies (Ghosh et al., 2009; Biggerstaff et al., 2007; Peacock et al., 2004; Geyer et al., 2005; Chang et al., 2005). However, their efficiency as soil bacterial diversity collectors has not been well-established. Elucidation of soil bacterial diversity through the combined use of BioSep beads with specific fortifying compounds also remains to be sufficiently evaluated, although positive results were shown utilizing atrazine fortification by Ghosh et al. (2009).

Over the past 30 years, AHLs have been studied in the context of quorum sensing (QS) and gene regulation in marine, soil, and infectious bacteria (Vasil, 2003; Wang and Leadbetter, 2005; Gantner et al., 2006; Fuqua et al., 2001). These AHLs are released by select proteobacteria and potentially build up in the environment under high population densities (Gonzalez and Keshavan, 2006; Leadbetter and Greenberg, 2000; Fuqua et al., 2001). The AHLs have been shown to regulate group behaviors such as swarming, biofilm development, and pathogenesis (Fuqua et al., 2001; Manefield and Whiteley, 2007; Gonzalez and Keshavan, 2006). Bacteria from non-AHL producing phyla, such as certain Bacillus and Actinobacteria species, and even the Proteobacteria *Escherichia Coli* and *Variovorax paradoxus*, can degrade AHLs or have AHL type receptor genes, suggesting broad ecological relevance for these molecules (Flagan et al., 2003; Manefield and Whiteley, 2007; Leadbetter and Greenberg, 2000; Uroz et al., 2005; Roche et al., 2004). The use of AHLs in cultivation studies has led to mixed results, though generally microbial response has been unremarkable (Bruns et al., 2002; Bruns et al., 2003; Stevenson et al., 2004).

Sides et al. (2010) (Chapter 1), found that fortifying BioSep beads with AHLs for field collection of soil bacteria led to various genera-specific responses. For instance, *Variovorax* and *Rhodococcus* isolates were significantly more numerous than predicted among the bacteria cultivated from AHL beads compared to the bulk soil or yeast bead collections, while the number of *Bradyrhizobium* isolates was significantly lower than predicted in the AHL collection. Along with increases in isolates of certain genera, use

of AHL fortified BioSep beads resulted in the addition of two families and 14 Operational Taxonomic Units (OTUs) that were not present in the bacterial collections derived from bulk soil or yeast beads. In total, the cultivated bacteria represent only a portion of the total microbial diversity inhabiting the beads and soil samples.

In the following study we used Phylochip analysis to compare BioSep bead and bulk soil communities. The G2 Phylochip has 16S rRNA gene probes for over 8,000 OTUs, and is a quick and effective way to compare communities that also provides information about which organisms are present, without reliance upon cultivation. We utilized three BioSep bead fortifying solutions; 1) purified water, 2) a mixture of 6 acyl homoserine lactones (AHL), and 3) yeast extract. Bacterial and archaeal DNA was extracted from beads and bulk soil from a forested site at Kellogg Biological Station and hybridized on three replicate Phylochips per collection type. We predicted that; 1) beads fortified with purified water would contain a bacterial and archaeal community similar to that derived from bulk soil, and 2) beads fortified with AHLs or yeast extract would contain altered communities (relative to bulk soil and water bead based communities and to each other) due to, for example, selective enrichment, competitive exclusion, or substrate preference.

Materials and Methods

Study site

Bulk soil and BioSep bead collection of soil microbes occurred at the Kellogg Biological Station Long Term Ecological Research (KBS LTER) site in Hickory Corners, MI from August to September 2009. A successional forest plot was utilized, SF2, which was abandoned from agricultural use over 40 years ago and now consists mainly of deciduous forest and typical regional understory grasses and shrubs. Additional information about the experimental design of the site is available at http://lter.kbs.msu.edu/.

Collection methods

BioSep beads (Microbial Insights, Inc, Rockford, TN, USA) are 2-3 mm oval spheres formed from a composite of 25% aramid polymer (Nomex) and 75% powdered activated carbon (PAC). The interior pore space mimics that of soil and provides a matrix for microbial growth (Fig. 3, Chapter 1). Prior to burying the beads in the soil, the beads were fortified overnight with one of three solutions; water (control), yeast extract (0.3 g/L), or a 1 mM acyl homoserine lactone mixture (N-ß-ketocaproxy, N-butyryl, N-tetradecanoyl, N-octanoyl, N-heptanoly, and N-hexanoyl). Beads were then bundled into nets for deployment and buried 6 to 10 cm below ground. Three complete sets of fortified beads were buried within a 4 m area, with each set protected from rodent predation by a wire mesh. Care was taken to be certain that the wire mesh and netting material did not interfere with bead-soil contact. Beads were retrieved one month later, at which time bulk soil samples were collected with a 2.5 cm probe directly adjacent to bead placement sites. Three cores of soil were taken at each site, to an average depth of 8 cm, for a total of 9 samples. Soil samples were homogenized and sieved through a 2 mm sieve prior to DNA extraction.

DNA extraction protocol

The MO BIO PowerSoil® DNA Isolation Kit was used to extract DNA from beads and bulk soil. Beads were first gently rinsed to remove soil from exterior, then sliced into several pieces with a sterile scalpel. Three sliced beads were placed into each PowerSoil bead tube, with two tubes extracted per site replicate for a total of 6 extract tubes per fortifying compound, plus 6 extract tubes for the combined bulk soil sample (with 0.25g soil added to each tube). All six sets of template DNA from each fortified treatment were combined after extraction into four main samples labeled W, A, Y, and S (water beads, AHL beads, yeast beads, and bulk soil).

Sample preparation for Phylochip

Template DNA from W, A, Y, and S were used to conduct multiple polymerase chain reactions (PCR) per sample with 27f (AGAGTTTGATCCTGGCTCAG) and 1492r

d (TACGGYTACCTTGTTACGAC-TT) bacterial primers. Separate PCR's were conducted to amplify Archaeal DNA using primers 4Fa (TCCGGTTGATCCTGCCRG) and 1492r d, with 4 reactions per W, A, Y, and S. The PCR products were combined by sample type separately for bacteria and archaea, and purified using Microcon YM-100 (Millipore) columns. The DNA concentrations for each combined sample were measured on a Hoefer Dyna Quant DNA Fluorometer. Each sample type was then analyzed for bacteria and archaea together on three replicate Phylochips using the method described in Ghosh et al. (2009) and DeSantis et al. (2007).

Data analysis

Results of the Phylochip analysis, based on OTU communities, were analyzed using the Fast UniFrac phylogenetic (P) test, hierarchical cluster analysis, and principle components analysis (PCA) (Martin, 2002; Hamady et al., 2010). The P test is a parsimony based comparison of paired communities which returns a P value giving the probability of reaching the same community similarity after repeated permutations. Hierarchical cluster analysis and PCA are determined from a distance matrix for each pair of sampling environments. The distance matrix is created from the UniFrac metric, with is a branch length based sequence comparison of the communities. For analysis purposes, presence was affirmed for an OTU when all three replicate chips of a sample type showed a positive fraction (pf) at the OTU level of 0.90 or greater. A total of 172 OTUs were detected in the overall community based on the PF criteria.

Results

OTUs and Phyla

A total of 17 phyla distributed among 84 families and 174 OTUs were confirmed present from the total Phylochip analysis, utilizing a 0.90 pf cutoff across the replicates (Table 7, Fig. 14). Archaea OTUs (e.g., Table 7/Fig. 14) were present only from the bulk soil community and therefore community analyses were conducted with and without Archaeal OTUs. Differences in the number of OTUs from Actinobacteria,

Crenarchaeota, Firmicutes, and Alpha- and Beta-proteobacteria account for much of the variation between bead and bulk soil communities (Table 7). Notably, almost half of the OTUs present (83 out of 174) came exclusively from bead collection methods, and the use of BioSep beads in the collection process resulted in the addition of four phyla and one Proteobacteria class that were not present in the bulk soil community; Natroanaerobium, NC10, Nitrospira, Spirochaetes, and Epsilon-proteobacteria (Fig. 14).

UniFrac community analysis

Analysis with Fast UniFrac showed that the bulk soil community was significantly different from the water, AHL, and yeast bead communities (P = 0.002 for each pairing), with or without archaea OTUs included (Table 8). There were no significant differences between yeast, AHL and water bead communities. A principal components analysis plot of the 4 sample collection methods on 3 axes shows the separation of the bulk soil community from all of the bead communities, with 57.27% of the variation explained by PC1 (Fig. 15). A hierarchical cluster analysis based on sampling environments and using individual chip data at 0.90 pf further supports separation of the bulk soil community from the bead communities, and shows slightly ambiguous clustering between yeast, AHL, and water bead community replicates (Fig. 16).

Overlap between communities

The bulk soil community was comprised of 90 OTUs, 49 of which were unique to that community, while 38 OTUs were shared with the water bead community (Fig. 17), 39 with the yeast bead community, and 33 with the AHL bead community. All four communities shared 32 OTUs, 15 of which were Alphaproteobacteria mainly from the Bradyrhizobiaceae family. The three bead communities shared 52 out of 125 total bead community OTUs (Fig. 18). The additional 20 shared OTUs for the bead communities were mostly Actinobacteria (Corynebacteriaceae and Micrococcaceae) and Betaproteobacteria (Comamonadaceae).

TABLE 7. Distribution of Operational Taxonomic Units (OTU) detected by collection method. Soil = DNA extracted from bulk soil, Water beads = DNA extracted from purified water fortified BioSep beads, Yeast beads = DNA extracted from yeast fortified beads, and AHL beads = DNA extracted from acyl homoserine lactone fortified BioSep beads. All BioSep beads were incubated below ground for 30 days prior to extraction.

	Collection Method			
Phylum or class	Soil	Water beads	Yeast beads	AHL beads
Acidobacteria	1	2	2	2
Actinobacteria	12	24	25	26
Bacteroidetes	2	1	1	-
Chloroflexi	2	1	-	-
Crenarchaeota	12	-	-	-
Cyanobacteria	2	1	2	1
Deinococcus-Thermus	1	-	-	-
Firmicutes	12	1	3	-
Natronoanaerobium	-	-	1	-
NC10	-	2	2	-
Nitrospira	-	1	1	1
OP10	1	-	1	-
Planctomycetes	3	-	-	-
Proteobacteria				
alpha	23	27	32	18
beta	6	20	14	12
delta	1	3	-	-
epsilon	-	1	-	1
gamma	6	6	8	3
unclassified	1	1	1	1
Spirochaetes	-	-	1	-
TM7	1	1	2	1
Unclassified	2	1	1	-
Verrucomicrobia	2	-	-	1
Total OTUs by Collection Method	90	93	97	67

FIG. 14. Presence (shaded) or absence (white) of families within phyla or proteobacterial class, as determined by Phylochip 16S rRNA gene microarray analysis, for each sample collection method**;** bulk soil, water fortified BioSep beads, yeast fortified BioSep beads, and acyl homoserine lactone (AHL) fortified BioSep beads. Three Phylochip replicate analyses are combined per column, with presence confirmed by a positive fraction value of >=0.90 for at least one operational taxonomic unit (OTU) per family on all replicates.

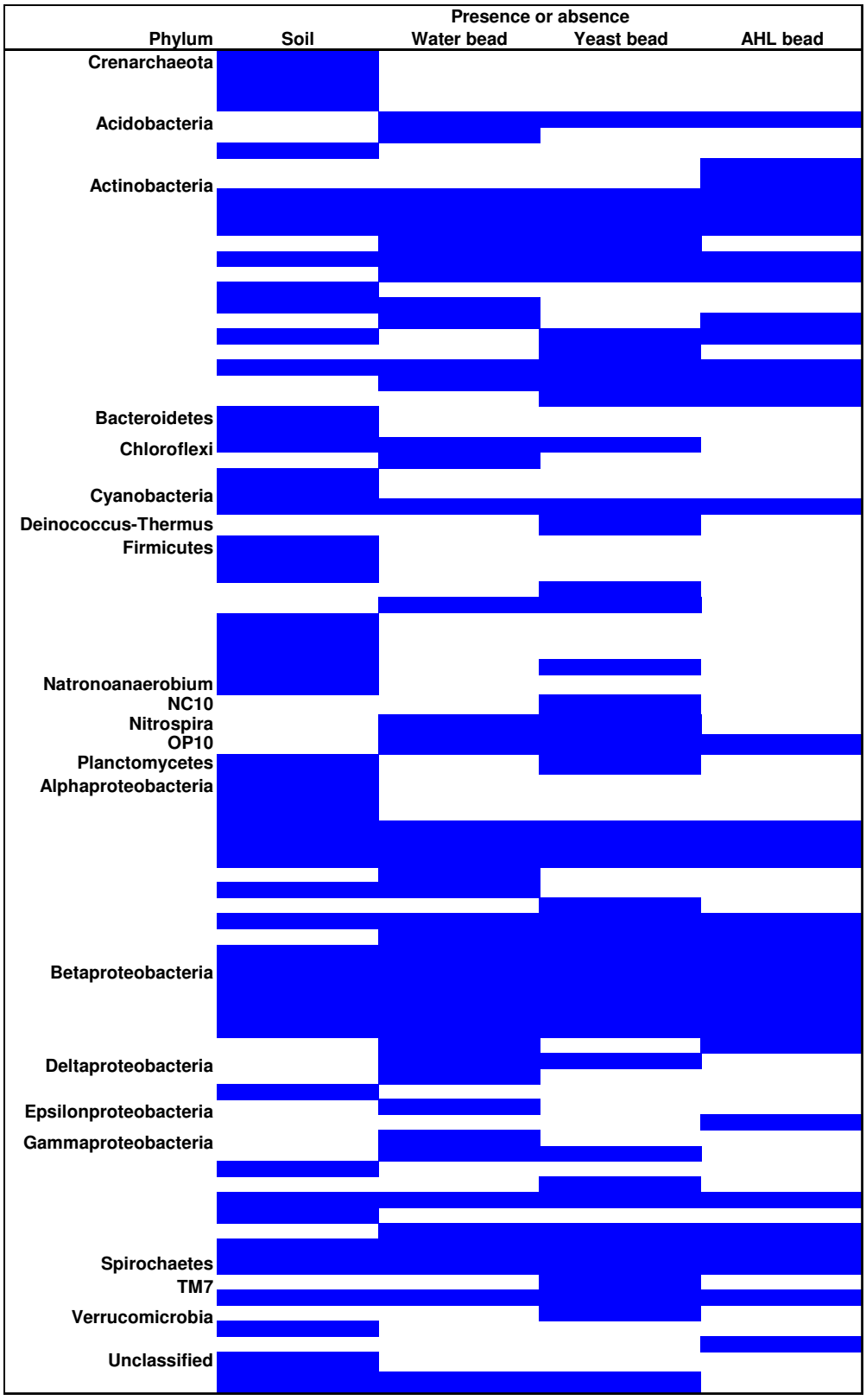
Presence or absence
Phylum
Soil
Water bead
Yeast bead
AHL bead
Crenarchaeota
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Firmicutes
Natronoanaerobium
NC10
Nitrospira
OP10
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Epsilonproteobacteria
Gammaproteobacteria
Spirochaetes
TM7
Verrucomicrobia
Unclassified

TABLE 8a-b. UniFrac paired phylogenetic test P values for the four Phylochip communities based on Operational Taxonomic Unit (OTU) distribution, with Bonferroni correction for multiple pairings. **a.** including Archaea OTUs, **b.** excluding Archaea OTUs. Values with ** are significantly different at alpha < 0.05.

a.	Bulk soil	H_2O beads	Yeast beads	AHL beads
Bulk soil	-	0.002**	0.002**	0.002**
H2O beads	0.002**	-	0.156	0.384
Yeast beads	0.002**	0.156	-	1.000
AHL beads	0.002**	0.384	1.000	-

b.	Bulk Soil	H2O beads	Yeast beads	AHL beads
Bulk soil	-	0.002**	0.002**	0.002**
H_2O beads	0.002**	-	0.096	0.324
Yeast beads	0.002**	0.096	-	1.000
AHL beads	0.002**	0.324	1.000	-

FIG. 15. UniFrac scaled three dimensional principal components analysis (PCA) plot showing the first three axes and relative distances between communities from the four environments. Soil = the community derived from bulk soil, AHLbeads = the community derived from acyl homoserine lactone (AHL) fortified BioSep beads, Waterbeads = the community derived from purified water fortified BioSep beads, and Yeastbeads = the community derived from yeast fortified BioSep beads. Plot is graphed by the UniFrac metric and distance matrix, determined by comparing the unique branch length measurements for each pair of environments.

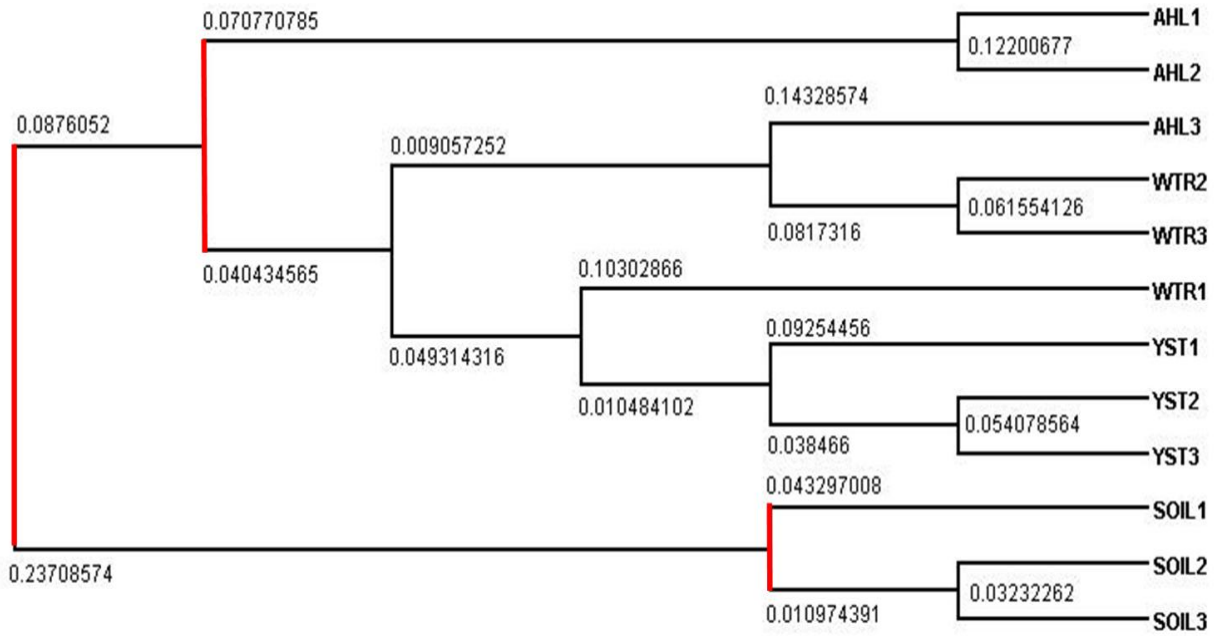

FIG. 16. UniFrac hierarchical cluster analyses of the Phylochip replicates of the four experiment environments (Martin, 2002). Soil1, 2 and 3 are the bulk soil environments, AHL1, 2 and 3 are the acyl homoserine lactone (AHL) fortified BioSep beads environments, WTR1, 2 and 3 are the purified water fortified BioSep bead environments, and YST1, 2 and 3 are the yeast fortified BioSep bead environments. Edge weights represent fraction of similarity between environments, with 0 being identical and 1 being mutually exclusive. Red branch points denote additional UniFrac Jackknife cluster analyses that showed highly repeatable branching (>99.9%).

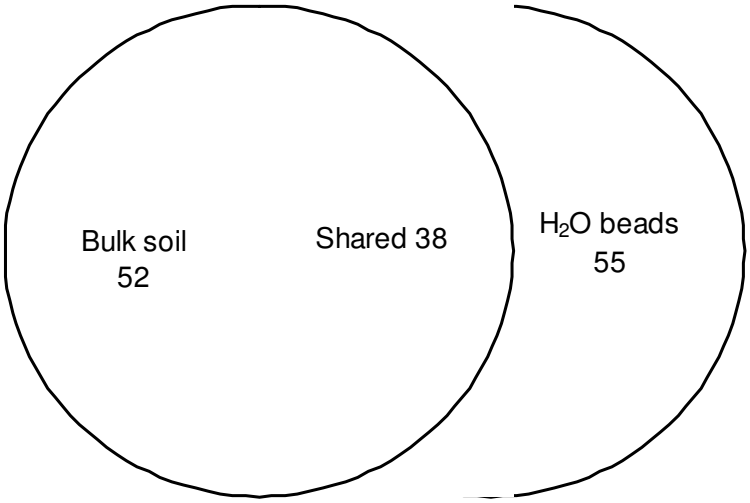

FIG. 17. Comparison of unique and shared Operational Taxonomic Units (OTU) between the bulk soil and water bead derived communities. Areas are approximate.

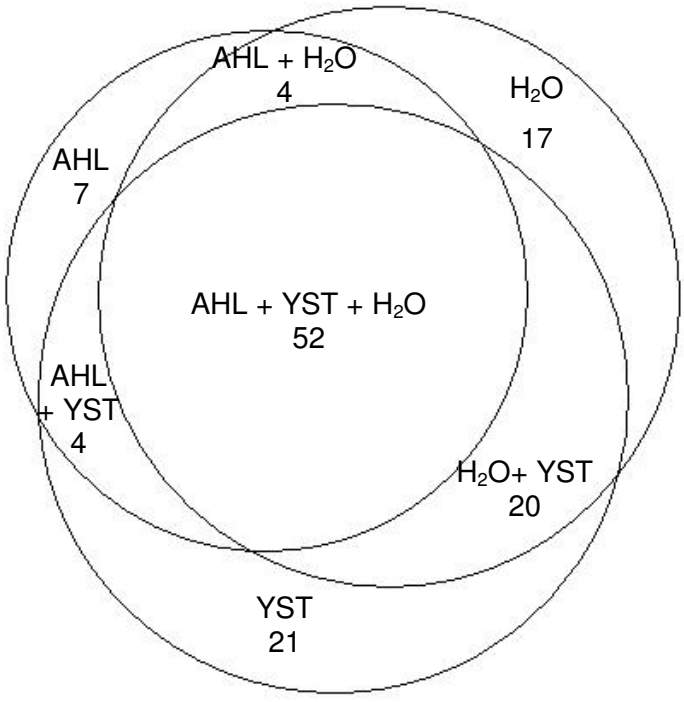

FIG. 18. Comparison of Operational Taxonomic Unit (OTU) distribution from water (H_2O), acyl homoserine lactone (AHL), and yeast (YST) BioSep bead derived communities. Areas are approximate.

Comparison of cultivated community to microarray community

Bulk soil and BioSep beads (yeast and AHL fortified) from the KBS successional forest plot were utilized in a previous cultivation study that resulted in the isolation of over 700 soil bacterial isolates, many of which were classified through partial 16S rRNA gene sequencing (Chapter 1). A comparison of the bead + bulk soil cultivated collection to bead + soil Phylochip communities (excluding archaea) showed that 6 phyla and 17 families were detected from both two experiments (Fig. 19, Table 9).

Discussion

Performance of Phylochips

Hybridization of BioSep bead and bulk soil DNA extracts onto the G2 Phylochip showed representation by 17 bacterial phyla, compared to 25 bacterial phyla represented in the pyrosequencing study by Roesch et al. (2007). However, we sampled at only one site and Roesch et al. (2007), sampled four sites across the western hemisphere. Interestingly, two of the phyla represented in this study, Natronoanaerobium and NC10, were not present in the pyrosequencing study.

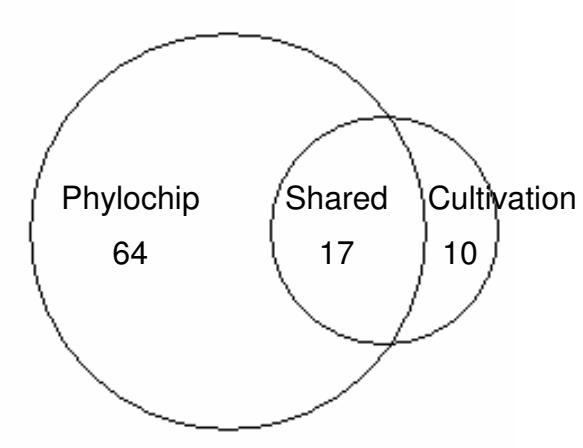

FIG. 19. Comparison of the number of soil bacterial families from a successional forest plot as determined by both Phylochip 16S rDNA microarray analysis and partial 16S rRNA gene sequencing of cultivated isolates.

TABLE 9. Comparison of soil bacterial families determined by Phylochip 16S rDNA microarray analysis and partial 16S rRNA gene sequencing of cultivated isolates from a successional forest plot. Highlighted families were detected in both studies.

Phylum (Class)	Phylochip (Order) Family	Cultivation Family
(Acidobacteria)	Acidobacteriaceae	Acidobacteriaceae
Acidobacteria-5	(Unc) Unclassified	
Acidobacteria-6	(Unc) Unclassified	
Solibacteres	(Unc) Unclassified	
(Actinobacteria)	Acidimicrobiaceae	
	Cellulomonadaceae	
	Corynebacteriaceae	
	Dermabacteraceae	
	Kineosporiaceae	
	Micrococcaceae	Micrococcaceae
	Micromonosporaceae	
	Mycobacteriaceae	Mycobacteriaceae
		Nocardiaceae
		Nocardioidaceae
	Promicromonosporaceae	
	Pseudonocardiaceae	Pseudonocardiaceae
	Streptomycetaceae	Streptomycetaceae
	(Actinomycetales) Unclassified	
	Coriobacteriaceae	
	(Unc) Unclassified	
BD2-10 group	(Unc) Unclassified	
Bacteroidetes	Crenotrichaceae	Crenotrichaceae
	Flexibacteraceae	
		Sphingobacteriaceae
Anaerolineae	(Unc) Unclassified	
Dehalococcoidetes	(Unc) Unclassified	
(Chloroflexi) unc	(Unc) Unclassified	
(Chloroflexi) Anaerolineae	(Unc) Unclassified	
(Chloroflexi) Dehalococcoidetes	(Unc) Unclassified	
Cyanobacteria	Chloroplasts	
	(Unc) Unclassified	
(Deinococcus-Thermus)	(Unc) Unclassified	
Firmicutes	Alicyclobacillaceae	
	Bacillaceae	
		Paenibacillaceae
	Thermoactinomycetaceae	
Clostridia	Clostridiaceae	
	Lachnospiraceae	
	Peptococc/Acidaminococc	
	(Clostridiales) Unclassified	
	(Unc) Unclassified	
Mollicutes	Erysipelotrichaceae	
(Natronoanaerobium)	(Unc) Unclassified	
NC10-1	(Unc) Unclassified	
Nitrospira	Nitrospiraceae	
(OP10)	(Unc) Unclassified	

(Phylum) Class	Phylochip (Order) Family	Cultivation Family
Planctomycetacia	Anammoxales	
Alphaproteobacteria	Acetobacteraceae	Acetobacteraceae
	(Azospirillales) Unclassified	
	Beijerinck/Rhodoplan/Methylocyst	Beijerinckiaceae
	(Bradyrhizobiales) Bradyrhizobiaceae	
		Hyphomicrobiaceae
	Methylobacteriaceae	Methylobacteriaceae
	Caulobacteraceae	Caulobacteraceae
	(Rhizobiales) Bradyrhizobiaceae	Bradyrhizobiaceae
	Phyllobacteriaceae	Phyllobacteriaceae
		Rhizobiaceae
	(Rhizobiales) Unclassified	
	(Rhodobacterales) Unclassified	
	Sphingomonadaceae	Sphingomonadaceae
	(unc) Unclassified	
Betaproteobacteria	Burkholderiaceae	Burkholderiaceae
	Comamonadaceae	Comamonadaceae
		Incertae sedis
	Oxalobacteraceae	Oxalobacteraceae
	Ralstoniaceae	
	Nitrosomonadaceae	
	Rhodocyclaceae	
Deltaproteobacteria	Desulfohalobiaceae	
	Desulfovibrionaceae	
	Syntrophobacteraceae	
Epsilonproteobacteria	Campylobacteraceae	
Gammaproteobacteria	Unclassified	
	Alteromonadaceae	
	Unclassified	
	Coxiellaceae	
	Unclassified	
	Thiotrichaceae	
	(Unc) Unclassified	
		Moraxellaceae
		Pseudomonadaceae
	Xanthomonadaceae	Xanthomonadaceae
(Proteobacteria)	(Unc) Unclassified	
Spirochaetes	Spirochaetaceae	
TM7-3	(Unc) Unclassified	
(TM7)	(Unc) Unclassified	
(Verrucomicrobia)	(Unc) Unclassified	
		Subdivision 3
	Verrucomicrobiaceae	
	Xiphinematobacteraceae	
(Unclassified)	Unclassified	
	81 families	**27 families**

Eight of the top nine phyla from the Janssen (2006) global survey were present in this study. The missing phylum was Gemmatimonadetes, although we cultivated a Gemmatimonadetes isolate from the KBS site in 2007 (Sides et al., 2010) (Chapter 1), and although there are 15 sets of Gemmatimondetes OTU probes on the G2 Phylochip. One reason Gemmatimonadetes may not have been captured on the Phylochips is probe specificity. The cultured Gemmatimonadetes from our previous study was only 89% similar to its nearest relative in GenBank, and this could suggest that Gemmatimonadetes from the KBS site have a more divergent 16S rRNA gene that fails to hybridize to the G2 Phylochip probes. Also, the Gemmatimonadetes cultivated from the KBS site in 2007 (Sides et al., 2010) (Chapter 1) was from an organic agriculture plot and not the forested site utilized in this study.

Bulk soil and water bead comparison

Bulk soil and water bead communities were more diverse than hypothesized (Fig. 16). Each had over 50 OTUs that were not present in the other community, while only 38 OTUs were shared. The UniFrac Phylogenetic test showed that the communities were significantly different, contradicting the hypothesis that the water bead community would mirror the bulk soil community. The most obvious differences were observed in the archaea, Firmicutes, Actinobacteria, and Gammaproteobacteria phyla (Fig. 13).

AHL and yeast bead comparison

Water beads and yeast beads showed 93 and 97 OTUs in each community, however, AHL beads showed only 67 OTUs, and the bulk soil community contained 90 OTUs. The three extractable BioSep bead communities were not significantly different from each other according to the Fast UniFrac phylogentic test. This disproves the hypothesis that use of AHL and yeast fortifying compounds would result in differences in diversity. Use of beads, however, resulted in the observation of 28 families that were not observed from the bulk soil extraction, showing that BioSep beads can help expand our knowledge of soil bacterial diversity**.**

Detection of families from Phylochip versus cultivation

The Phylochip based examination of the KBS forested site showed much greater diversity than the cultivation experiment with 81 versus 27 families (Table 5, Chapter 1). However, several of the previously cultivated organisms were not detected in this study despite having probes on the G2 chip. Most notable is the Nocardiaceae family, which contains the Rhodococcus genus, which comprised almost 10% of the 751 cultivated bacteria (Chapter 1). Additional families that were not detected by the Phylochip were; nocardioideacea, sphingobacteraceae, paenibacillaceae, moraxellaceae, pseudomonodaceae, Verrucomicrobia subdivision 3, hyphomicribiaceae, and rhizobiaceae, though all of these families are represented on the G2 Phylochip.

Conclusion

The Phylochip microarray analysis showed that BioSep beads can increase the detectable diversity of soil bacteria. In this study, almost half of the OTU richness detected came from the bead samples. Even the beads fortified with sterile deionized water achieved OTUs not detected in the bulk soil samples. While the communities detected from the various BioSep bead fortifying compounds did not significantly differ from each other, this may change with further variation of fortifying compounds. The number of Phylochip detected bacterial families was far greater than those detected from cultivation methods, showing that we are still far behind in capturing soil bacterial diversity through cultivation.

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Chapter 3

The Prevalence of Mitomycin C-Inducible Temperate Phage in Agricultural Soil Bacteria.

Abstract

Very few studies have addressed phage-host interactions in soil, even though it has been suggested that lysogeny is more advantageous in soil than in other environments. The harsh conditions in soil, i.e. fluctuating nutrient and moisture availability, have resulted in the evolution of bacteria that can grow slower and exist in long torporous states. Prophage induction is generally studied using toxins or UV light as the inducing agents. However, there may be additional inducing agents or induction mechanisms in the soil environment that have not yet been detected. In this study, a set of soil isolates with varied colony formation rates on solid agar were tested for the occurrence of lysogeny. Induction assays were performed using mitomycin C and acyl homoserine lactones (AHLs). Filtrates from induced and non-induced cultures were stained with nucleic acid dye and virus particles were counted under epifluorescent microscopy. Twenty nine percent of the isolates tested were mitomycin C (mitC) inducible, and 10% were AHL inducible. Host colony formation rate and land management did not correlate to induction response, however, only 21 inductions assays were completed in this experiment. The results of this study showed that lysogeny is prevalent in the isolates tested, and that AHLs in the soil environment may result in prophage induction.

Introduction

Importance of lysogeny

Lysogeny is a bacteriophage life cycle that is indicated by the integration of the phage genome into the host genome. Eventually, the phage is induced and resumes the lytic cycle: excising from the hosts genome, generating new phage using the hosts cellular mechanisms, and lysing the host cell to release the new phage. Lysogeny in the environment has been predominately studied in marine systems (Wommack and Colwell, 2000; Weinbauer, 2004). It is reported to occur in 28 to 71% of marine bacterial isolates (Furhman, 1999; Paul, 2008), and between 0.8 and 11.4 % in whole marine

bacterial communities (Weinbauer and Suttle, 1999), and lysogeny can vary by season, nutrient level, or host population (Jiang and Paul, 1994; Cochran and Pau,l 1998; Williamson et al., 2002; Paul, 2008). It has been theorized that lysogeny may be a more advantageous life cycle for phage in soil due to inhospitable conditions, low host population density, and less detrimental effects on host population than the lytic cycle (Stewart and Levin, 1984; Pantastico-Caldas et al., 1992; Marsh and Wellington, 1994). Additionally, lysogeny can provide immunity of hosts from further infection (Marsh and Wellington, 1994).

Prophage can be induced into the lytic cycle, resulting in lysis of host cells and subsequently affecting food webs through the mortality of a portion of the population, and through the release of immobilized carbon, nitrogen, and other nutrients (Weinbauer, 2004; Weinbauer and Rassoulazagen, 2004). These effects, however, depend upon the overall rate of lysogeny and the occurrence of environmental inducing factors. Prophage can also mediate the transfer of biogeochemically important genes, sometimes across species (Chiura, 1997; Weinbauer, 2004; Weinbauer and Rassoulazagen, 2004; Lindell et al., 2004), and enhance pathogenesis and other important functions through phage conversion (Waldor and Mekalanos, 1996; Williamson et al., 2002).

Recent studies have found that lysogeny may be prevalent among soil bacteria (Williamson et al., 2005; Ghosh et al., 2008). Induction of prophage occurred in 2 to 62.5% of soil prokaryotic communities that were collected using BioSep beads (Ghosh et al., 2008). A cultivation dependent study found that 30% of soil isolates contained prophage that were inducible by mitC exposure (Williamson et al., 2008), and a cultivation independent study found that 4 to 20 % of bacteria from three Antarctic soils, and 22 to 68 % of bacteria from four Delaware agricultural soils, were lysogens (Williamson et al., 2007).

Furthermore, genomic analyses have found that well over half of bacterial genomes contain prophage genetic elements, suggesting that lysogeny is indeed quite common (Canchaya et al., 2003; Paul, 2008). This could have important implications in the soil environment, similar to those found in marine environments, due to the release

of nutrients into the environment and changes to community structure through cell lysis, gene transfer, and phage mediated host conversion (Waldor and Mekalanos, 1996; Weinbauer, 2004; Weinbauer and Rassoulazagen, 2004; Paul, 2008). Specifically, some soil bacteria provide protection to plants against insect or fungal pathogens through the release of antibiotics or other small molecules (Schwinghamer, 1970; Handelsman and Stabb, 1996; El-Tarabilya and Sivasithamparam, 2006). Further, lysogens in soil bacterial communities can have a competitive advantage over non-lysogens (Marsh and Wellington, 1994; Chibani-Chennoufi et al., 2004). Soil bacteria release extracellular enzymes that break down residues and mineralize nutrients into plant-useable forms, perform biological nitrogen fixation (BNF), and exude substances that contribute to soil aggregation (Guggenberger, 2005; Chotte, 2005; Deubel and Merbach, 2005; Quax, 2006). All of these functions are potentially affected by lysogeny. Lysogeny affects human endeavors as well, causing reduced production of culture based dairy products (Lunde et al., 2005; Doyle and Meng, 2006), and possibly slowing bioremediation, biocontrol, or bioreactor processes (Gabig-Ciminska et al., 2004).

Environmental factors, such as nutrient availability and host population density, are thought to determine whether phage will enter the lysogenic cycle or proceed directly to the lytic phase (Weinbauer and Suttle, 1999; Bongiorni et al., 2005), while host stress response to toxins or UV light is generally found to induce prophage into the lytic cycle (Schwinghamer, 1970; Cochran and Paul, 1998; Ghosh et al., 2009). However, more is known about the latter than the former, and properties of the host may also determine the most beneficial life cycle when a potentially lysogenic phage initially encounters a host (Weinbauer, 2004). Soils contain a mixture of bacteria that can grow under various nutrient conditions, and some are better suited for lower or sporadically available nutrition. One strategy for survival in harsh conditions is to grow slower, which is a host strategy that correlates with an increased occurrence of lysogeny (Paul, 2008).

The majority of induction studies of lysogenic phage have followed the tradition of using negative stress responses by hosts as the means to induce (i.e., toxin exposure). Induction has been shown to occur when the host DNA repair mechanism is enacted

(Little, 2005; Ghosh et al., 2009), as repair mechanisms result in the removal of prophage genes when discovered so it is advantageous for the prophage to enter the lytic cycle before detection. However, induction into the lytic phase might also be favorable under positive conditions, such as if hosts are plentiful in the environment. To this end, host receptors for quorum sensing compounds in the environment might cue prophage to transform to the more optical lytic phase. This has recently been shown for the first time in bacterial community samples and in *E. coli* (Ghosh et al., 2009), however it has not been explored in isolated soil bacteria.

We previously cultivated a large collection of isolates from bulk soil and AHL and yeast fortified BioSep beads from the Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site (Chapter 1). A subset of the isolates, mostly *Variovorax sp.* classified using partial 16S rRNA gene sequences, was chosen for prophage induction assays.

Variovorax sp. were found regularly throughout the cultivation collection, and *Variovorax paradoxus* was shown by Leadbetter and Greenburg (2000) to degrade and subsist exclusively on acyl homoserine lactone (AHL) molecules. Along with the ability to metabolize AHLs, *V. paradoxus* can degrade pesticides (Sorensen et al., 2009), and *Variovorax sp.* Pal2 can cleave the C-P bond in phosphonoalanine (Kulakova et al., 2009). *V. paradoxus* can also receive, via conjugation mediated horizontal gene transfer, a large catabolic plasmid donated by *Alcaligenes eutrophus* JMP134 that provides mercury resistance and other catabolic abilities (Neilson et al., 1994). *Variovorax sp.*, therefore, are potentially useful bacteria for agriculture and bioremediation.

Inoculated plates were incubated for 76+ days, and colonies isolated throughout the incubation were sorted into four time group categories. We predicted that: 1) lysogeny would be more prevalent among slower growing isolates, 2) lysogeny would occur in *Variovorax* sp., and 3) AHLs would cause induction in some isolates.

Materials and Methods

Host information

Bacterial hosts used to induce for lysogenic phage were cultivated from soils collected from the Kellogg Biological Long Term Ecological Research (KBS LTER) site in Hickory Corners, MI, as described in Chapter 1. Hosts were classified through the Ribosomal Database Project classifier software (Cole et al., 2007; Cole et al., 2009) using partial 16S r RNA gene sequences. Briefly, host DNA was extracted with the UltraClean™ Microbial DNA Isolation Kit, and PCR reactions were performed using 8f and 536r primers (AGAGTTTGATCATGGCTC-AG and CGTATTACCGCGGCTGCTGG). PCR products were purified with the Promega Wizard SV 96 Clean-Up System, and sequencing was conducted using Applied Biosystem's ABI 3730 capillary electrophoresis instrument with the 8f primer. Most of the hosts chosen were from the Variovorax genus, as this host was abundant throughout the 800+ isolate collection, and was present in all four relative growth rates from all three land management regimes.

Phage Induction assays

Phage inductions were conducted on log-phase liquid cultures, as determined by visual assessment of turbidity. Nine 250 ml flasks, each containing 25 ml of single strength VL55 media (Sait et al., 2002), were inoculated with 250 µl of stationary phase culture. Once light turbidity formed, mitomycin C (mitC) was added to 3 of the flasks (25 µl), and three flasks were transferred into new flasks containing evaporated acyl homoserine lactone (AHL) solution (1 µm/L, N-ß-ketocaproxy, N-butyryl, N-tetradecanoyl, N-octanoyl, N-heptanoly, and N-hexanoyl). The remaining three flasks were untreated controls. All nine flasks were covered in foil and incubated for 12 to 15 hours with shaking (95 rpm).

Following incubation, the cultures were transferred to nine centrifuge tubes and spun at 4150 g for 30 minutes at 4℃. Supernatants containing phage were filtered through 0.22 µm syringe filters to remove bacterial cells, and 900 µl of the filtrate was

treated with RNase free DNase for 45 minutes to remove unwanted bacterial DNA. DNase treatment was stopped with 0.5M EDTA, then 100 µl of each treated phage filtrate was added to 1700 µl of filtered water and passed through a stack of three pre-wetted filters using a Millipore vacuum manifold (XX2702550). The circular 25 mm filters, from bottom to top, were 1) Millipore glass fiber prefilter (AP4002500), 2) Millipore 0.22 µm Durapore Membrane Filter (GVWP02500), and 3) Whatman Anodisc 0.02 µm filter (6809-6002).

After all solution passed through the filters, Sybr Gold Dye was applied for 20 minutes to stain virus particles on the Anodisc filters. Filters were then washed by passing 2 ml filtered water through, and the Anodisc filters were placed on slides and viewed with Epifluorescent microscopy. IPLab software was used to photograph and count a minimum of 10 fields per slide, and each treatment had three slides (Fig. 20).

Transmission Electron Microscopy (TEM)

Several of the inducible phage were imaged, after Uranyl acetate staining, with a Hitachi H-800 TEM. Phage were first concentrated using one of two methods; 1) ultracentrifugation (10,000 g for 45 min), or 2) Amicon filtration (YM100).

Statistical analyses

Viral particle counts were converted to virus like particles per ml of culture, and paired one-tailed t-tests were performed on log-transformed data to compare the means of three (or more) treatment slides (mitC or AHL) against the means of 3 (or more) control slides. Induction was considered significant at alpha < 0.05.

Logistic regression was performed using SAS software to analyze whether land management or relative growth rate of the host was linked to the occurrence of inducible prophage. Values of 1 or 2 were assigned based on whether the t-test was significant. Models using land treatment and growth rate as separate parameters, as well as interactions between the two parameters, were tested. Time groups were also evaluated based on the following combinations; Fast = combined groups 1 and 2 (0 to

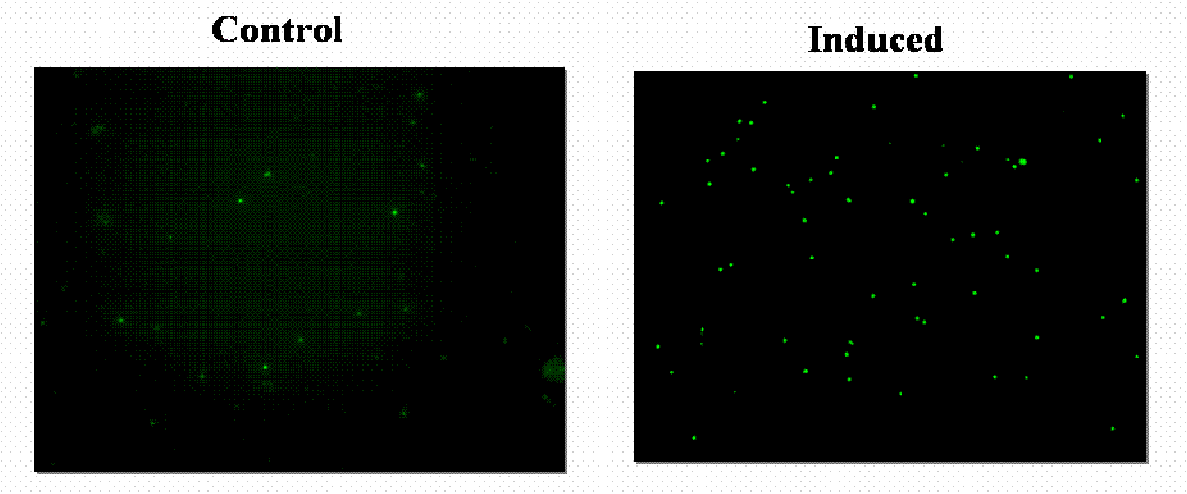

FIG. 20. Example of control and inducible phage slide images using Sybr Gold nucleic acid stain and epifluorescence microscopy.

14 days to host colony formation), and Slow = combined groups 3 and 4 (15 to 76+ days to host colony formation).

Results

Induction Assays

Of the 21 isolates tested, 6 were induced by mitC , two were induced by AHL (Fig. 21a-b), and 5 induced spontaneously, making the total occurrence of lysogens 62% (29% induced by mitC, Table 10). One of the AHL isolates was only induced in only two out of three trials (BMOPS60). The second BMOPS60 induction assay trial also exhibited spontaneous induction in the control cultures, though the AHL induction contained significantly higher in Virus Like Particles (VLP) than controls (Fig. 21a-b). Another isolate (BMOPS530) was mitC induced in the initial trial and spontaneously induced in the second trial. None of the isolates were inducible by both mitC and AHL.

Results of logistic regressions

Logistic regressions showed no significant correlations between induction response and time group (or land management type). There was a relatively high incidence of Variovorax lysogens across the growth rates by all induction types, as 10

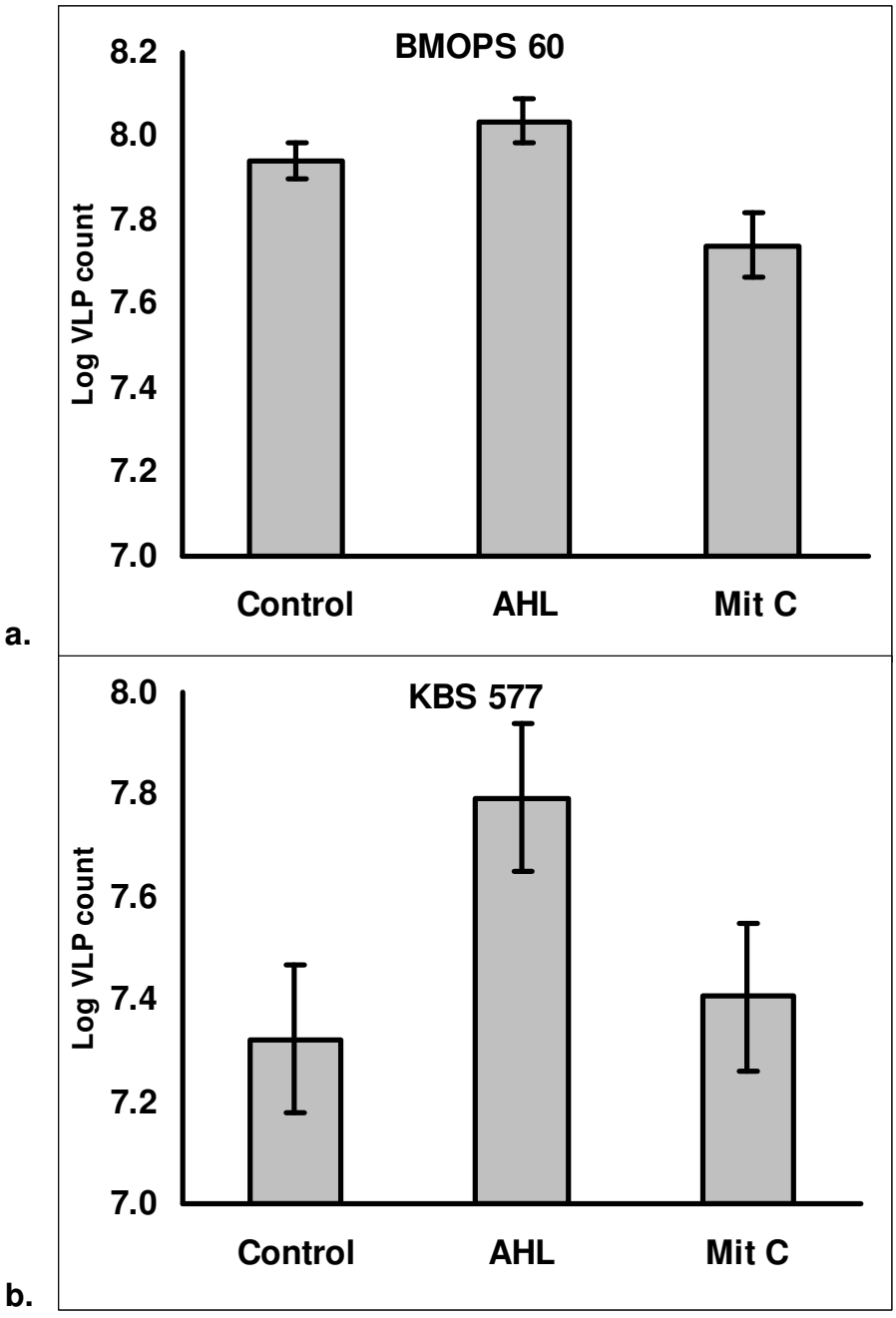

FIG. 21a-b. Log transformed virus like particle (VLP) counts from prophage inductions using acyl homoserine lactone (AHL) and mitomycin C (mitC). Hosts were *Variovorax* sp. soil isolates. Bars are standard error. P values for AHL versus control; a. BMOPS60 = 0.032184, b. KBS577 = 0.032065.

TABLE 10. T-test results of phage inductions for 21 soil bacterial isolates using mitomycin C (mitC) and acyl homoserine lactone (AHL) as inducing agents. “Yes” for mitC and AHL inductions = P value < 0.05. Time group is initial host colony formation rate on inoculate plates within four time periods; 1 = days 1 through 7, 2 = days 8 through 14, 3 = days 15 through 36, and 4 = days 37 through 76. Land management; SF2 = late successional forest, T1 = conventional agriculture, and T4 = certified organic agriculture.

Isolate ID	Genus	Time Group	Land Management	Spontaneous Induction	MitC Induced	AHL Induced
BMOPS15	Cupriavidis	1	SF2	yes	no	no
BMOPS21	Cupriavidis	1	SF2	no	no	no
BMOPS335	Cupriavidis	2	SF2	no	no	no
BMOPS48	Leptothrix	1	T1	no	no	no
KBS171	Massilia	2	T4	no	no	no
BMOPS2	Ralstonia	1	T4	no	no	no
BMOPS1	Variovorax	1	T4	no	yes	no
BMOPS60	Variovorax	1	T4	no	no	yes[1]
BMOPS410	Variovorax	2	SF2	no	no	no
BMOPS445	Variovorax	3	T1	no	no	no
BMOPS471	Variovorax	3	T1	no	yes	no
BMOPS627	Variovorax	4	T1	yes	no	no
KBS577	Variovorax	3	T4	no	no	yes
BMOPS653	Variovorax	4	T1	yes	no	no
BMOPS667	Variovorax	4	T1	no	yes	no
BMOPS38	Variovorax	1	T4	yes	no	no
BMOPS39	Variovorax	1	T1	no	yes	no
BMOPS78	Variovorax	1	SF2	yes	no	no
BMOPS530	Variovorax	3	T1	no	-[2]	no
KBS606	Verrucomicrobia	3	SF2	no	yes	-[3]
BMOPS420	Unc Rhizobiales[4]	3	T4	no	yes	no

1 AHL induced in two out of three trials.

2 Mit C induced in one of two trials.

3 KBS606 AHL induction response could not be determined due to variation.

4 BMOPS420 is novel beyond class level.

out of 13 isolates were induced either spontaneously, with mitC, or with AHL (Table 11).

Phage morphology

The presence of viruses in the supernatant of induced hosts was verified by transmission electron microscopy (TEM). The viruses had capsids with or without tails of varying lengths (Fig. 22-25), though some seemingly tail-less phage may have had their tails broken off during the staining process (Fig. 24). Based on morphology, the phages were categorized as *Myoviridae*, *Syphoviridae*, or *Podoviridae*, which are the most commonly reported types of phage (Ackermann, 2007)(Fig. 1).

Discussion

Lysogeny in KBS soils

It was not surprising to find lysogeny occurring in soil isolates at 29%, since previous research showed soil isolates had a mitC inducible fraction of 30% (Williamson et al., 2008). However, the isolates tested in Williamson et al. (2008) (primarily *Bacillus* sp. and *Enterobacter* sp.) were not related to the isolates in this study (mainly from the Burkholderiales order). The most notable finding was that AHL can cause induction of prophage, as this is the first report of such activity in a bacterial isolate, and supports the quorum sensing induction response mechanism presented by Ghosh et al. (2009).

	Relative Growth Rate	
Induction type	Fast	Slow
Spontaneous	2	2
MitC	2	2
AHL	1	1

TABLE 11. Results of Variovorax sp. prophage inductions by host initial colony formation rates. Fast = colonies formed within 14 days, Slow = colonies formed between 15 to 76 days.

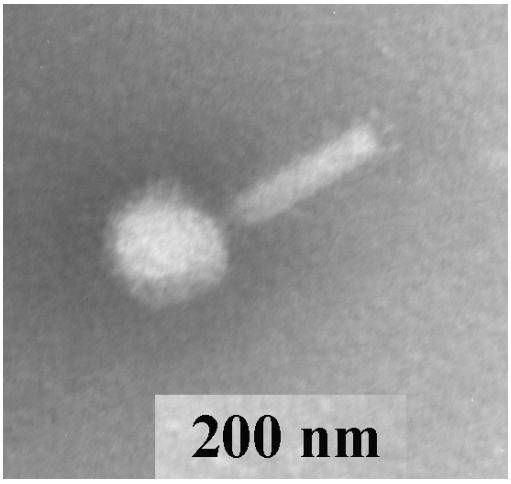

FIG. 22. *Myoviridae* prophage induced from the unclassified Rhizobiales host (BMOPS420) with mitomycin C.

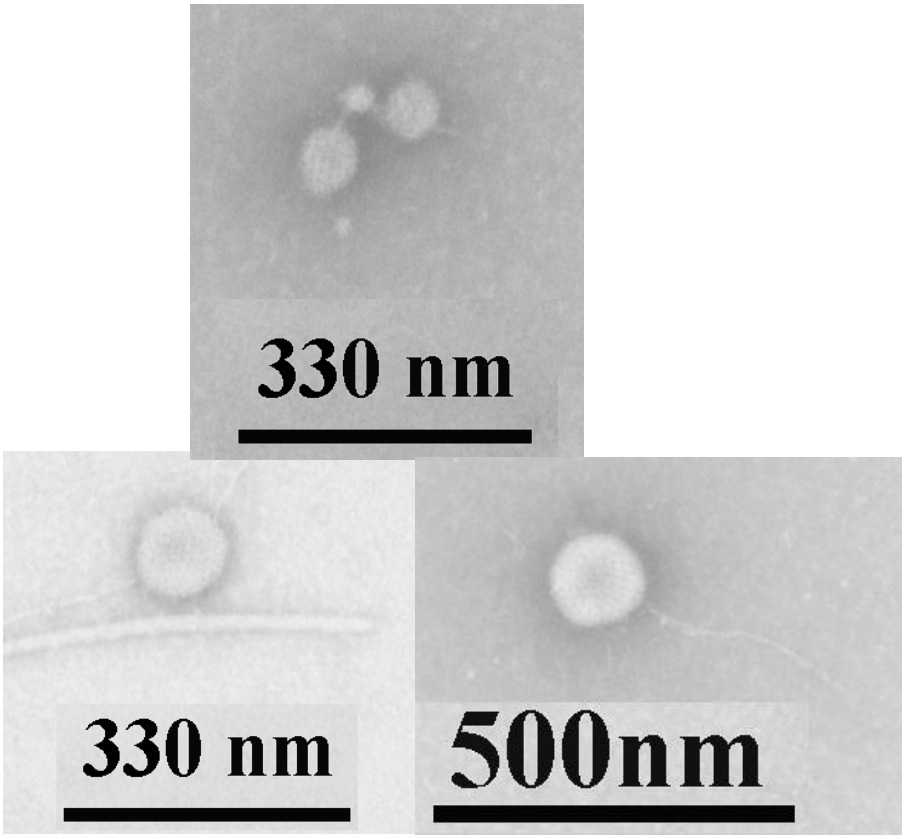

FIG. 23. *Siphoviridae* prophage (isolate BMOPS60), induced with acyl homoserine lactones and mitomycin C from a *Variovorax* sp. host.

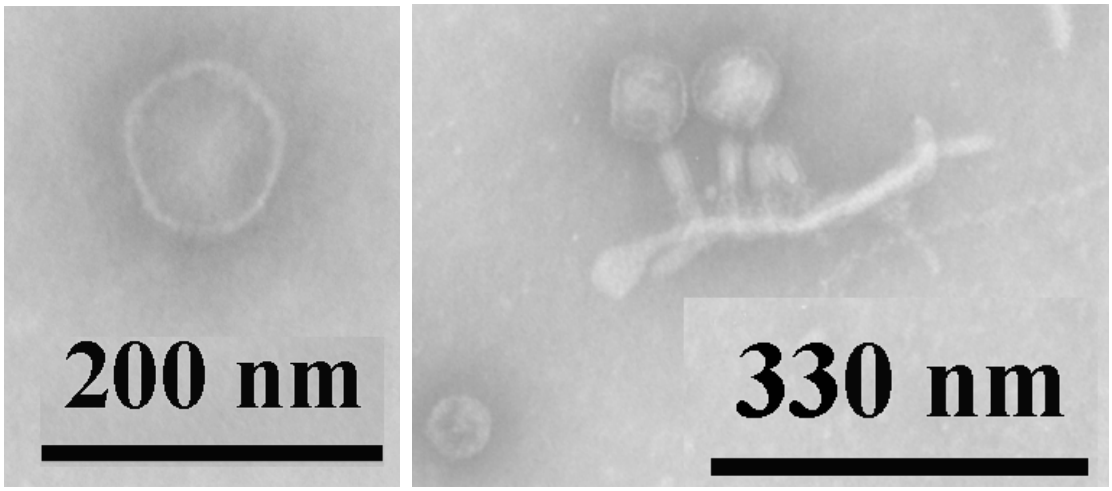

FIG. 24. *Podoviridae* or *myoviridae* phage, shown with and without tail, induced from *Variovorax* sp. host with mitomycin C (isolate BMOPS471).

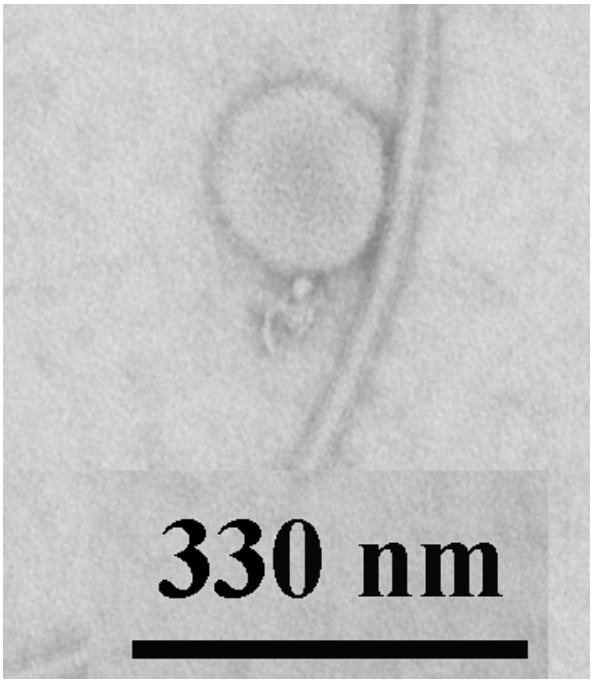

FIG. 25. *Podoviridae* phage induced from *Variovorax* sp. host with mitomycin C (BMOPS1).

However, the procedure used to interrogate the isolates in this research will require modification to achieve consistent results.

An alternative method to measure induction, which uses Quantitative Competitive (QC) PCR to quantify changes in available host attB or other suitable genes (prophage binding sites within the host chromosome) (Lunde et al., 2000), may be of use to verify induction since imaging in this study was often difficult due to flocculation by the induced Variovorax isolates (Fig. 26). Post induction sonication and vortexing were attempted. However, this led to disruption of cellular material which took up the fluorescent dye and masked the stained phage. Use of flow cytometry may be hampered by the dense flocculates as well.

Induction and host growth rate

Based on this limited study, the incidence of lysogeny cannot be attributed to host growth rate, however the number of induction assays will need to be dramatically increased before a true assessment can be made. Host growth rates may have been influenced by a variety of factors, including nutrient level, dilution rate, or atmospheric CO_2/O_2 concentration during initial plating. An optimal method of predicting growth rate may be to quantify the rRNA operon copy number for each host, with higher copy numbers generally correlating to faster growth rates (Klappenbach et al., 2000). Other factors, such as land management and media substrate influence, should also be

FIG. 26. One set of Variovorax sp. culture flasks after 15 hours of mitomycin C (mitC) and acyl homoserine lactone (AHL) induction. The control flask is on the far left, mitC is in the middle with obvious flocculation, and AHL is on the right.

analyzed. Unfortunately, during the induction assays for this study, Anodisc filters became unavailable due to manufacturing issues. Other methods, such as Flow Cytometry or QC PCR (Lunde et al., 2000), could replace or supplement the use of vacuum filtration in the future; however, these will require standardization.

Spontaneous induction

Williamson et al. (2008) induced 20 isolates and found that 6 were mitC induced, and only one was spontaneously induced (5%). However, none of the hosts were closely related to the *Variovorax sp*. utilized in this study. Even so, we attained similar results for mitC induction (29%), though we had a higher proportion of spontaneously induced isolates (5 out of 21). Several reasons could explain the high spontaneous induction rate, including growth phase at time of induction, temperature fluctuations, or stress caused by growth changing from solid to liquid medium (Lunde et al., 2003; Lunde et al., 2005). Lunde et al. (2003) found that up to 9% of *lacticoccus lactis* used in dairy processes were spontaneously induced when early stationary phase was reached. In a second study, Lunde et al. (2005) showed the spontaneous induction rate to be between 0.082 and 1.76, and found that spontaneous induction varied based on nutrient level, pH, osmolarity, and temperature, along with interactions between dilution rate and osmolarity, temperature and osmolarity, and acidity and temperature.

Several of the induced isolates had low levels of phage present in control flasks, even though there was a significant induction response in mitC flasks. It is likely that this is due to the continued growth of control cell cultures during the 15 hour induction period. Since induction occurred at early log phase, it is reasonable that control cells moved into early stationary phase within the next 15 hours of incubation. For this reason, future studies might include the incubation of control flasks 4℃ to reduce growth until the induction period ends, or control flasks could be sampled and counted at the initiation of induction.

Studies to determine the optimal growth conditions for the isolates in this study should be conducted to reduce spontaneous induction. The assays in this study were all

conducted at pH 5.5, low nutrient levels, and temperatures maintained at 25°C, except where noted.

Burst size

An attempt was made to count induced host bacterial cells utilizing Sybr Gold dye and epifluorescence microscopy. However, we were deterred by the growth characteristics of the host when exposed to mitC. MitC interrupts normal cell division, leaving cells highly segmented and elongated, and rendering them nearly impossible to count. In the future, a sample of the host culture could be removed just prior to the addition of the inducing agent, stained and counted. Since mitC interrupts cell division, there should be minimal host population increase beyond the induction point.

Conclusions

This study found that lysogeny occurs commonly in soil bacterial *Variovorax* sp.; 29% of all hosts and 46% of *Variovorax* sp. hosts contained mitC inducible prophage. Furthermore, we verified that prophage can be induced by AHL, as reported by Ghosh et al. (2009). The influence of host colony formation rate and land management on lysogeny, however, could not be determined.

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Vita

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