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SIZE FRACTIONATION OF BACTERIAL FUNCTIONAL DIVERSITY
WITHIN SOILS

Krista L. Kinneer

THESIS

Submitted to the College of Agriculture, Forestry, and Consumer Sciences
of West Virginia University
in partial fulfillment of the requirements
for the Degree of

Master of Science
in
Plant and Soil Sciences
with a concentration in Environmental Microbiology

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Morgantown, West Virginia
1999

Keywords: Functional diversity, BIOLOG[®], dilution enrichment, 16SrDNA

ABSTRACT

Size fractionation of bacterial functional diversity within soils
Krista L. Kinneer

BIOLOG[®] GN and GP microtitre plates were used to compare functional diversity of large ($>0.45\ \mu\text{m}$, LC) and small ($<0.45\ \mu\text{m}$, SC) cells within A and B horizons of cultivated and forested Guernsey silt-loam soil without enrichment and following enrichment in dilution culture. Without enrichment, SC exhibited limited substrate utilization compared with LC. B horizon SC failed to positively utilize any carbon substrates, which suggests that they may be physiologically inactive and/or metabolically distinct from those in A horizon soils. Following enrichment, A horizon SC from highly diluted enrichment cultures produced substrate utilization patterns distinct both from all enrichments of LC and from SC obtained in less dilute enrichments. At any given inoculum dilution, A horizon SC exhibited greater substrate utilization than B horizon SC, which exhibited positive substrate utilization following enrichment. Amplified ribosomal DNA (ARDRA) analyses of SC dilution culture enrichments demonstrated distinct bacterial sub-populations from a single soil sample.

DEDICATION

This work is dedicated to my parents, Neil and Donna Kinneer.
Thank you for supporting me throughout my academic career
and for teaching me the value of hard work!

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I wish to extend a sincere thank-you to my major advisor, Dr. Alan J. Sexstone, for his guidance throughout my graduate career. Dr. Sexstone, thank you for your encouragement and friendship—you have made my graduate experience a wonderful one!

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GENERAL INTRODUCTION

Historically, biodiversity has been described in terms of macro-organisms with little consideration of microorganisms (Pace, 1997). Lack of confidence among microbiologists in culture/isolation methodology, large variability and diversity among bacterial populations, and difficulty in applying classic taxonomical methodology are among the reasons microorganisms have been largely overlooked (Bakken, 1997). Regardless of these limitations, understanding microbial biodiversity is important if we are to expand our knowledge about the strategies and limits of life (Zlatkin et al., 1996), the importance of microbes to sustainability of life (Kennedy and Smith, 1995), and understanding our own evolutionary history. Commercially, exploring microbial diversity may reveal novel genes and organisms of value to biotechnology (Istock et al., 1996; Rheims et al., 1996) and may enable us to use diversity patterns to monitor and predict environmental change (Borneman and Triplett, 1997; Smit et al., 1997).

One gram of soil can contain more than 10^{10} bacterial cells (Torsvik et al., 1996), of which less than 1% can be isolated and grown in culture (Torsvik et al., 1990). Therefore, isolated bacteria may account for only a minor fraction of the total bacterial diversity in soil, while our knowledge of the dominant part is very scant.

The majority of soil bacteria are “dwarves”, having cell diameters less than 0.3 μm . These numerically abundant cells represent the major part of the nonculturable bacteria in soil (Bakken and Olsen, 1986). These small cells may represent a distinct population (Bakken and Olsen, 1986) or could be derived from larger cells that shrink as a response to starvation (Novitsky and Morita, 1976). Bottomley and Dughri (1989) noticed that the fraction of small cells within the soil bacterial community increased with soil depth, indicating a relationship between nutrient availability and the presence of dwarves. Their data support the hypothesis that

small “dwarf” cells are miniatures of otherwise larger “normal-sized” bacteria that shrink in response to starvation. However, of the few dwarf cells able to form colonies on low-nutrient agar, the majority (80-90%) retains a small cell size during growth (Bakken and Olsen, 1986). Lindahl et al. (1997) demonstrated that small cells ($<0.40\ \mu\text{m}$) contained sufficient phospholipids to support intact cell membranes. Dwarf cell DNA-content ($1.6\text{-}2.4\ \text{fg cell}^{-1}$) is similar to that of larger cells (Bakken and Olsen, 1989), suggesting that a majority of these cells contain intact genomes. These data provide evidence for the hypothesis that dwarf cells may indeed be a distinct community separate from larger-celled bacteria.

The development of molecular techniques for directly characterizing microbial communities without cultivation has greatly increased our knowledge of bacterial community structure (Stahl, 1997; Fuhrman, 1997; Pepper, 1997). Since the mid-1980s, the use of 16S ribosomal ribonucleic acid (rRNA) based techniques has facilitated the identification of a wide variety of as yet uncultivated microorganisms from various environments (Torsvik et al., 1990; Ward et al., 1990; Weller et al., 1991). However, most molecular methods still require culture-based information for interpretation of results and assembling a more complete picture of the organisms’ ecological properties (Liesack et al., 1997; Chandler et al., 1997). Molecular studies can indicate the presence of microorganisms but often fail to tell us what the role of these in the habitat might be.

Garland and Mills (1991) proposed that sole-carbon source utilization could be used as a functionally based measure for classifying diversity of microbial communities. Characterization is based on patterns of substrate utilization exhibited on BIOLOG[®] Gram-negative (GN) and Gram-positive (GP) microtiter plates (Biolog Inc., Hayward, CA), each containing 95 sole-carbon substrates and a redox indicator, tetrazolium dye (Garland, 1996a; Garland, 1996b;

Bossio and Scow, 1995; Lindstrom et al., 1998). By combining GN and GP microplates in an analysis, a large number (128) of distinct carbon compounds can be used to evaluate the functional diversity of microbial communities. While these plates were originally designed to identify pure cultures of single isolates based on their pattern of substrate use, community-level information can be obtained by inoculating the plates with whole environmental samples. Substrate use patterns are then quantified by comparing tetrazolium dye color development in each of the test wells to a control well containing no carbon substrate. Although the exact population densities and taxonomic composition of the communities may not be known, patterns of functional diversity exhibited by BIOLOG[®] plates can provide some insight into the activity of microbial communities.

The BIOLOG[®] method requires that microorganisms be metabolically active under given laboratory conditions, which differ from the natural environmental conditions. It can only detect aerobic respiratory activity—metabolic potential and growth. Therefore, substrate utilization patterns cannot be expected to reflect the metabolic activity of the entire soil community. However, the BIOLOG[®] system is useful in that it may reveal the activity of many culturable and metabolically-active but non-culturable cells. Garland (1997) correlated overall color development to total counts estimated by acridine orange staining, plate counts using R2A agar, and active cell counts estimated by reduction of the fluorescent redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Results indicated that active cell counts obtained from CTC analyses were better predictors of the overall rate of color development, suggesting that active organisms that are not culturable on solid media can respond in the BIOLOG[®] wells. Therefore, it is possible that the BIOLOG[®] system may detect the activity of the largely unculturable dwarf cell community.

With few exceptions, speculations on the role of an organism in its habitat are very difficult without laboratory culture studies. Thus, even with alternative methods available for the detection and characterization of microbial communities, the need to isolate representative strains of microorganisms remains. Recently, dilution culture techniques have been successfully employed to obtain small-celled bacterial isolates in culture (Button et al., 1993; Schut et al., 1993; Janssen et al., 1997). A low-nutrient liquid medium supports the growth of oligotrophic bacteria, while the dilution itself separates different bacterial populations by decreasing competitive exclusion. Janssen et al. (1997) obtained a novel, anaerobic ultramicrobacterium (cell volume $<0.04 \mu\text{m}^3$) through this method, and observed that large bacteria dominated the less-dilute tubes, smaller bacteria dominated the most dilute tubes, and very small cells dominated the tubes in the middle. Use of the dilution culture technique allows for the growth of different bacterial populations from a single soil sample (Jackson et al., 1998). Dilution cultures select for organisms present in high numbers in the sample, if they can grow in the media used. Dilution cultures have advantages for studying oligotrophic bacteria because culture selection favors the most abundant, rather than the most nutrient-tolerant, organisms (Button et al., 1993). Since dwarf cells are the most abundant bacteria in soil, dilution cultures may provide an approach to isolating these numerically significant bacteria.

The overall objective of this research was to assess functional diversity to compare size-fractionated bacterial populations within and among sites of a single soil taxon. Specific objectives included: (1) investigating differences in BIOLOG[®] patterns among small cells ($<0.45 \mu\text{m}$) and large cells ($>0.45 \mu\text{m}$) by comparing A and B horizon soils at cultivated and forested sites (Chapter One); and, (2) performing a dilution culture enrichment in an attempt to isolate and cultivate small cells within and among the study sites. The BIOLOG[®] system and

restriction analysis of PCR-amplified 16S rDNA were employed to evaluate the outcome of this enrichment attempt (Chapter Two).

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CHAPTER 1

Differential response of size-fractionated soil bacteria in BIOLOG[®] microtitre plates

Introduction

Bacterial cells less than 0.4 μm in diameter are numerically abundant in soil, but due to unknown growth requirements rarely are recovered on standard microbiological media (Bakken and Olsen, 1986). Some examples obtained in culture retain their small size during growth and possess a DNA-content cell⁻¹ of 1.5-2.1 fg (Bakken and Olsen, 1989). These small bacteria contain sufficient phospholipid fatty acid content to support intact cell membranes and are predominantly Gram-positive (Lindahl et al., 1997). Populations of small soil bacteria deserve attention, since they may represent species not yet studied in culture whose metabolic activities are still largely unknown.

Direct inoculation of environmental samples into BIOLOG[®] microtitre plates (Biolog Inc., Hayward, CA) containing 95 different sole-carbon substrates produces patterns of potential C source utilization for microbial communities. This method previously has been used to characterize microbial communities within and among different soil types (Garland and Mills, 1991; Winding, 1994; Zak et al., 1994; Bossio and Scow, 1995; Haack et al., 1995; Staddon et al., 1997;) rhizosphere samples (Garland and Mills, 1994; Garland, 1996a; Garland, 1996b), model communities (Haack et al., 1995), and groundwater samples (Fliermans et al., 1997). The community characterizations obtained are inherently selective since they depend on bacterial growth and/or respiration on the various BIOLOG[®] substrates. It is unclear whether small bacterial cells significantly contribute to the substrate utilization pattern observed when BIOLOG[®] plates are inoculated with a mixed microbial community from soil. Garland (1997) suggested that metabolically active bacteria that are not culturable on solid media might respond in the BIOLOG[®] wells. Thus, the BIOLOG[®] method may reveal the metabolic potential of selected small soil bacteria and assist in the enrichment of isolates.

The purpose of this study was to determine if BIOLOG[®] patterns differed between small cells (<0.45 μm) and large cells (>0.45 μm) obtained from a single soil taxon. Differences in the bacterial community from A and B horizons from a cultivated and forested site were evaluated. Our working hypothesis was that BIOLOG[®] patterns would differ between the small and large cell fraction, demonstrating that small cells are metabolically distinct from larger cells.

Materials and Methods

Study sites and sampling procedures

Two sites were studied: a cultivated site, which has undergone regular tillage since 1976; and, a forested site, a naturally wooded area dominated by deciduous trees. The soil type at both sites is a Guernsey silt loam (fine, mixed, mesic Aquic Hapludalfs). The sites are immediately adjacent to each other on the West Virginia University Plant Sciences Farm in Morgantown, WV (39.63⁰N, 79.95⁰W).

Multiple core samples were taken from each site with a split-core soil sampler. Samples from the A and B horizon were separated on site and placed into separate plastic bags for immediate transport to the laboratory, where they were individually bulked and coarse-sieved (4.75 mm mesh size). All samples were stored at field moisture in sealed plastic bags and were refrigerated at 4 C until use.

Sample preparation

For each of the four soil samples, 50 g soil, 200 ml Winogradsky's salt solution (5.0 g K₂HPO₄, 5.0 g MgSO₄ · 7H₂O, 2.5 g NaCl, 0.05 g Fe₂(SO₄)₃ · H₂O, 0.05 g Mn₂(SO₄)₃ · 4H₂O, 2000 ml dH₂O, pH 6; diluted 1/10), and one drop of Tween 80 were blended in a Waring blender for three 1-min intervals with intermittent cooling in an ice bath. This was repeated until six 250

ml centrifuge bottles (Nalgene[®], polyethylene) per horizon and study site were filled with soil homogenate. Large soil particles were removed by centrifugation at 2500 rev min⁻¹ for 15 min at 4 C in a Sorvall[®] RC-5B Refrigerated Superspeed Centrifuge and a Sorvall[®] GSA rotor. The resulting supernatants from each site and soil horizon were pooled and added to 500 ml Winogradsky's salt solution.

To separate bacterial cells according to size, each cell solution was passed through 0.45 µm Millipore HA filters. The filtrate contained bacterial cells smaller than 0.45 µm in diameter and represented the small cell (SC) fraction. Large cells (LC) greater than 0.45 µm in diameter were collected by washing material from the surface of the filters, by placing them into Petri plates containing Winogradsky's salt solution and shaking on a rotary shaker (Lab-line orbit shaker) at 110 rev min⁻¹ for 15-20 min. A sterile paintbrush (5/32" diameter) was used to brush off any remaining cells from the surface of the filters.

To collect cells, LC and SC samples were centrifuged at 10,000 rev min⁻¹, 4 C, for 25 min. Supernatants were discarded, and cells were resuspended in 200 ml Winogradsky's salt solution. The cells were then washed three times, to remove soil particles and dissolved carbon, by centrifugation in the same manner.

Inoculation and reading of BIOLOG[®] plates

Washed cells were resuspended in 80 ml Winogradsky's salt solution to provide enough inoculum for triplicate GN and GP BIOLOG[®] microplates (Biolog Inc., Hayward, CA). Before inoculation, the turbidity of all samples was equalized ($A_{595}=0.01$) by dilution based on spectrophotometric (Bausch and Lomb Spectronic 20) readings. Plates were inoculated with 100 µL sample/well to reduce the risk of cross-contamination from well to well during plate reading (Zak et al., 1994). Plates were stored in a plastic container with moist paper towels to maintain

humidity, and were incubated at 25 C. Plates were read on an automated ELISA reader (Dynatech MR4000) equipped with a 595 nm filter at 12 h intervals for 120 h. Absorbance values for the wells containing carbon substrate were blanked against the control well. Negative values were considered as 0 in subsequent data analyses.

Enumeration of soil bacteria with FITC

Soil bacteria were enumerated for samples taken before centrifugation at 2500 rev min⁻¹, after centrifugation at 2500 rev min⁻¹, and from the LC and SC fractions before cell collection and washing. To prepare slides for fluorescence microscopy, 10 µL of sample was evenly smeared in a defined area (254 mm²) on a microscope slide, air dried, and heat fixed. Smears were stained with FITC staining solution (1 mg FITC, 0.25 ml 0.5 M carbonate-bicarbonate buffer pH 9.6, 1.1 ml 0.01 M phosphate buffer pH 7.2, 1.1 ml 0.85% NaCl) for three min. Slides were washed for 10 min in 0.5 M carbonate-bicarbonate buffer (pH 9.6), two min in 5% sodium pyrophosphate, and rinsed in distilled water. Slides were allowed to air dry and were mounted in buffered glycerol (5 ml 1 M Na₂CO₃ in 45 ml glycerol, pH 9.6) for observation with a Nikon Optiphot epifluorescence microscope, equipped with a filter set for blue light (BP 450-490 nm exciter filter, 510 nm beam splitter, and LP 520 nm barrier filter). Bacteria numbers were determined by counting the number of bacteria observed in ten random fields of view from each triplicate slide. Bacteria were enumerated according to the following equation:

$$N_g = N_f * A * D * W_w / a_m * v_s * W_d,$$

where N_g = number of bacteria gram⁻¹ (dry weight) soil, N_f = average number of bacteria per field of view, A = area of smear (in this case, 254 mm²), D = dilution factor, W_w = wet weight of

soil (g), a_m = area of microscope field in mm^2 , v_s = volume spread on slide (0.01 ml), and W_d = dry weight of soil (g) (Bottomley, 1994).

Data analysis

All statistical analyses were performed using the JMP statistical software package (SAS Institute, Inc.). Principal component analysis was performed using data normalized by dividing the absorbance value for each substrate by the average well color development (AWCD) for the entire plate (Garland and Mills, 1991; Garland, 1996a). AWCD for plates was calculated as the mean of all blanked absorbance values for all wells containing a carbon substrate. Substrate diversity (H) was calculated as $H = - \sum p_i (\ln p_i)$, where p_i is the ratio of the absorbance at a particular substrate to the sum of absorbances over the entire plate. Average sum of activities was calculated by averaging the sum of absorbance values from triplicate plates. Substrate richness was determined as the average number of positive wells with an absorbance value greater than 0.25 (Garland, 1997). Substrate richness per guild was determined by averaging the number of positive wells from triplicate plates within each substrate guild.

Results

Direct bacterial counts determined by fluorescence microscopy ranged from 2.6 to 5.3 x 10^{10} cells gram^{-1} dry weight soil (Table 1). Mean counts were slightly higher in forested compared with cultivated soil and decreased with depth at both sites. Utilizing a direct extraction procedure, bacterial cells were separated from the soil matrix and recovered by centrifugation. Approximately 0.8 to 1.8 % of the original direct bacterial counts in soil were recovered by this procedure (Table 1). Extracted cells from each sample were partitioned into a large ($>0.45 \mu\text{m}$) and small ($<0.45 \mu\text{m}$) size fractions by passage through a membrane filter.

Appropriate bacterial sizes in each fraction were confirmed by negative staining the filtrates followed by cell measurements ($n > 100$) with an ocular micrometer (data not shown).

Approximately twice as many small cells were generally recovered from the soil from both sites and horizons (Table 1).

When BIOLOG[®] GN and GP plates were inoculated with LC bacteria from either the A or B horizon, average well color development (AWCD) values increased rapidly in the interval from 24 to 84 hours (Figure 1). Despite the fact that optical density of the LC and SC inocula were equalized, AWCD increased slower and reached a much lower final average OD when plates were inoculated with the SC fraction. Appreciable increases in AWCD were apparent in A horizon samples but not B horizon samples when SC were used as inocula (Figure 1). GN and GP BIOLOG[®] control plates inoculated with Winogradsky salt solution alone did not exhibit color development during the 120 h incubation period.

Functional diversity can be operationally determined from the number and type of BIOLOG[®] substrates used by a bacterial community (Zak et al., 1994). Typically, BIOLOG[®] data are compared after a fixed incubation time when absorbance values for most substrates are increasing, but have not approached saturation (Bossio and Scow, 1995; Garland, 1996a). After inspection (Figure 1), data obtained at 60 h (cultivated site) and 72 h (forested site) were chosen for further analysis. Total activity and substrate richness for LC decreased with soil depth at both sites (Table 2). Slightly higher values were observed in the cultivated compared with the forested site. Trends were similar for LC regardless of whether GN or GP BIOLOG[®] plates were employed (Table 2). Little difference in the diversity index (range 4.1 to 4.5) was apparent among samples. In contrast, SC had lower total activity, substrate richness, and diversity (range 3.4 to 3.6) when compared with LC plates (Table 2). In order to eliminate weak positive

responses in the calculation of substrate richness, an average absorbance value greater than 0.25 was required (Garland, 1997) for substrates to be considered as positively utilized. No substrate was positively utilized by the SC community from the B horizon of either study site, despite the low but measurable total activity recorded (Table 2).

Principle-component analysis (PCA) performed using values for adjusted AWCD distinctly separated the LC fraction (A and B horizon) from the SC fraction (A horizon), regardless of the BIOLOG[®] plate utilized (Figure 2). Large cell data grouped similarly with some separation of samples from A and B horizons noted with GP plates. In both cases, SC data were distinctly separated according to site. Among the GP data, PC 1 accounted for 44% of the observed total variation, while PC 2 and PC 3 accounted for 24% and 20%, respectively. PC 1 was most positively correlated with glucose-1-phosphate utilization ($r=0.9735$) and negatively correlated with utilization of D-mannitol ($r=-0.9858$) and L-lactic acid ($r=-0.9985$), while PC 2 correlated with utilization of gentiobiose ($r=-0.9732$), α -D-glucose ($r=0.9903$), and pyruvic acid ($r=0.9679$). With GN plates, PC 1, PC 2, and PC 3, accounted for 49%, 31%, and 12%, of the observed total variation. PC 1 was most positively correlated with utilization of β -hydroxybutyric acid ($r=0.9947$) and L-alanyl glycine ($r=0.9944$), and most negatively correlated with utilization of D-gluconic acid ($r=-0.9958$) and α -keto glutaric acid ($r=-0.9846$). PC 2 correlated with α -D-glucose ($r=0.9879$), methyl pyruvate ($r=0.9721$), and quinic acid utilization ($r=0.9891$).

Indices such as AWCD, substrate richness, and substrate diversity provide information about the numbers, activities, and rates at which substrates are utilized, but provide little insight into the types of substrates utilized by bacterial communities. Carbon sources available on GN and GP BIOLOG[®] plates previously have been divided into six substrate guilds (Zak et al.,

1994). Substrates within each guild differ between GN and GP plates as follows: carbohydrates (30/43/27), carboxylic acids (20/16/12), polymers (5/7/5), amines/amides (6/4/3), amino acids (20/8/8) and miscellaneous (10/17/8); where numbers in parentheses represent the total number of GN substrates, the total number of GP substrates, and the number of substrates shared on both GN and GP plates, respectively. Based on average substrate richness, LC utilized greater than 75% of the available substrates in all guilds (Table 3). This trend was apparent regardless of the type of BIOLOG[®] plate. In contrast, SC positively utilized less than 25% of the available carbohydrates, carboxylic acids, and amino acids with no significant utilization of the remaining three substrate guilds (Table 3). Positive substrate use by SC varied within guilds according to site (Table 3).

Data in Tables 2 and 3 represent averages of three replicate BIOLOG[®] plates. When a substrate was positively utilized by LC (mean OD >0.25) all three replicates generally showed strong color development with relatively little variation among replicates [% coefficient of variation (CV) less than 30%]. Much greater variability existed among SC replicates (CV > 80%). As a result, although an individual replicate might be positive for use of a substrate, examination of the mean of the three replicates would indicate a negative result. These data indicate a greater heterogeneity in the distribution of SC among BIOLOG[®] wells, perhaps due to dilution to extinction of a relatively small number of cells capable of growth on a particular substrate. If substrate richness is scored according to the presence of a single positive replicate, then the number of total substrates positively utilized by SC increases, reaching values of 28 and 31 for cultivated A horizon GN and GP plates, respectively, and reaching a value of 24 for forested A horizon GN and GP plates. However, the number and type of substrates utilized are still greatly restricted in comparison to LC.

Discussion

Comparison of the BIOLOG[®] substrate utilization patterns obtained from LC and SC inocula revealed distinct differences in the bacterial community response from A horizon soils of both the forested and cultivated site. In all cases, the number and extent of substrates utilized was much higher for the LC fraction. Small cells used less than 14% of the available BIOLOG[®] substrates, primarily from the carbohydrate and carboxylic acid guilds. Distinguishable substrate utilization patterns also were produced by LC from B horizon soils, however SC from the B horizon produced no positive reactions ($OD_{595} > 0.25$) on any of the BIOLOG[®] GN or GP substrates employed. The dramatic differences in substrate utilization patterns occurred despite similar numbers of bacteria in the LC and SC inocula.

In microbial diversity studies, preparing an inoculum that is representative of the numbers and types of microorganisms present in the original sample is of primary importance. This has been a problem for soil samples, where the efficiency of releasing cells from the solid matrix is low. Adhesion to soil particles and encasement in soil aggregates are the main reasons for retention of microorganisms in the soil matrix, especially in soils with a high clay or silt content (Riis et al., 1998). Lindahl (1996) extracted <20% of cells by blending soil slurries in pyrophosphate solution. Combining shaking and ultrasonication, Riis et al. (1998) were able to recover 45% of the cell counts from soil. In the present study, our cell recovery was much less efficient; i.e., approximately 0.8 to 1.8% of the original direct bacterial counts were recovered. Inefficient cell recovery has implications on the outcome of microbial functional diversity studies, since it is likely that only the most easily-detachable cells are being recovered. Therefore, the resulting inoculum may not represent the majority of the indigenous soil bacteria that firmly attach to surfaces or are found in aggregates (Lindahl et al., 1996). This has a direct

effect on the substrate utilization patterns obtained on BIOLOG[®] plates, since moribund or physiologically-inactive cells may be among these easily-detachable cells (Riis et al., 1998), and the metabolic potential of many indigenous soil bacteria more firmly attached to soil particles may not be included in the analysis. However, Bakken (1985) has shown, on the basis of cell size distribution, that the fractionation-centrifugation technique releases all bacterial types from soil with equal efficiency through several rounds of homogenation-centrifugation. Holben et al. (1988) suggested that a single round of homogenation-centrifugation results in a soil bacteria fraction representative of the total bacterial population.

The manufacturer recommends that BIOLOG[®] plates be inoculated at a density of 10^8 cells ml^{-1} (Biolog Inc., 1993). However, for most environmental samples the cell density is much lower. Konopka (1998) reported inoculum densities $>10^5$ cells ml^{-1} are required in order to ensure that the communities inoculated into the individual wells are the same. We estimated that our inoculum contained 10^5 and 10^6 cells ml^{-1} for the LC and SC, respectively. Therefore, it is likely that the bacterial community was evenly distributed among the BIOLOG[®] wells and among replicate plates. Collected cells were extensively washed and inoculum densities equalized prior to inoculation into plates. Lack of color development in control wells indicated there was little carry over of exogenous carbon. There was a similar lag period with both inocula (24 h) prior to significant color development in the BIOLOG[®] wells, presumably as the inocula multiplied to a sufficient density (10^8 cells ml^{-1}) to produce a positive response (Haack et al., 1995).

Compared with LC we noted much greater variability among SC replicate responses, which might suggest a small number of viable cells that were unequally distributed among the wells. If the density of physiologically active cells in the inoculum is low, it is likely that the

active community inoculated into the individual wells is not the same, and would therefore result in a variable BIOLOG[®] response. Haack et al. (1995) suggested that variability in substrate use among replicates might indicate the presence of rare community members in the inoculum. Such members may exhibit unique substrate utilization on the plates and may be present in such low numbers that they would not be equally distributed among replicate plates.

Although the bacterial size fractions were distinguishable by functional diversity indices, limitations of the methodology preclude our ability to generalize about the populations present. Konopka et al. (1998) suggested that although environmental samples can be differentiated by their substrate utilization patterns, the data obtained may provide incomplete information concerning the authentic microbial diversity or metabolic potential of the communities present. Fundamentally, BIOLOG[®] is a culture-based enrichment method that depends on the ability of the community inocula to metabolize or grow; which in turn depends on the environmental relevance of the carbon sources provided and the physiological state of the inoculum. It is a well established problem that less than 1% of the total direct microscopic count of soil bacteria are culturable on standard laboratory media (Torsvik et al., 1990). It is probable that many of these bacteria do not contribute to BIOLOG[®] substrate utilization patterns and thus their contribution to community diversity is overlooked by this method.

Small cells often are numerically abundant in soil and represent a distinct bacterial community that is largely unculturable (Bakken and Olsen, 1989). Garland (1997) suggested that metabolically active bacteria that are not culturable on solid media may respond in the BIOLOG[®] wells. Thus, the BIOLOG[®] method may reveal the metabolic potential of small soil bacteria unobtainable in culture. In preliminary work (K. Kinneer, unpublished data) soil bacteria passing a 0.45 μm filter occasionally could be cultured on nutrient agar or R2A agar

(Bacto[®], Difco laboratories), but cells passing a 0.3 µm filter were almost never culturable. Olsen and Bakken (1987) previously found that less than 1% of soil bacteria with diameters less than 0.4 µm could be grown on complex low-nutrient media. In contrast, up to 38% of cells in their study larger than 0.4 µm were culturable and were unaffected by the carbon content of the media. Substrate utilization patterns exhibited on BIOLOG[®] plates are reflective of either the original bacterial community, or selective enrichment of fast-growing microbes. In our study, it is likely that the rich and diverse BIOLOG[®] patterns obtained with the LC inocula is due to the activity of readily culturable, rapidly growing, copiotrophic bacteria. Unsuitable growth conditions in BIOLOG[®] plates for physiologically dormant or oligotrophic autochthonous soil organisms probably resulted in the limited substrate utilization by SC. Fredrikson et al. (1991) previously determined that 25% of their subsurface (presumably oligotrophic) bacterial isolates utilized less than 10% of the available BIOLOG[®] GN substrates. Haack et al. (1995) observed that authentic soil isolates of Bacillus and Arthrobacter sp. produced no positive color changes in BIOLOG[®] GN plates despite multiple substrate usage by a type strain. In our study, although substrate utilization was restricted, SC clearly grew using several substrates from the carbohydrates, carboxylic acids, and amino acids guilds.

Physiologically active bacteria are required to produce a positive BIOLOG[®] response. Autochthonous soil bacteria are thought to be slow-growing and/or subject to starvation conditions due to lack of available carbon substrates. Extended starvation limits the ability of these organisms to be isolated in culture as they become dormant and ultimately non-viable. We found that SC from A horizons, but not B horizons, were able to produce a positive response (O.D. > 0.25) on several BIOLOG[®] substrates. Others (Garland and Mills, 1991; Garland, 1996b) have used BIOLOG[®] to demonstrate that greater phenotypic richness exists in

rhizosphere soils, presumably due to the greater variety and availability of root exudate carbon supporting an actively growing bacterial population. Bossio and Scow (1995) found an increase in the metabolic diversity of soil microbial communities with additional carbon inputs. It may be that greater available soil carbon in A horizons of our sites allow the SC population to maintain greater activity and viability, therefore allowing greater substrate utilization in the BIOLOG[®] system.

It has been proposed that viability may be maintained in the absence of culturability, and viable but non-culturable cells may be able to return to an actively metabolizing state under favorable conditions (McDougald et al., 1998). Cells extracted from the soil may be in a state of metabolic inactivity in order to survive low nutrient availability, especially in B horizon soils. Resuscitation of viable but nonculturable cells may occur when a limited number of viable cells in the dormant population grow after nutrient conditions become more favorable (Morgan et al., 1991; Weichart et al., 1992). Kaprelyants et al. (1994) suggested that viable cells may produce a factor that stimulates the resuscitation of dormant cells. Due to differences in nutrient availability, more viable cells may be present in A compared to B horizon soils. Therefore, following enrichment in the BIOLOG[®] wells, some A horizon cells may be “resuscitated” and regain their metabolic integrity, while B horizon cells remain dormant. It is unclear whether SC in the B horizon are metabolically dormant and/or physiologically distinct from those in A horizon soils.

Indirect characterization of microbial community structure by functional diversity measurements has been made convenient due to the commercial availability of BIOLOG[®] microtitre plates. The lack of substrate utilization by the B horizon SC fraction in the present study emphasizes that such functional diversity measurements incompletely characterize the true

diversity of the actual bacterial populations present. It is probable that most functional diversity studies have characterized only large-celled, fast growing, metabolically-active bacteria. In our study, the BIOLOG[®] system was capable of at least partial characterization of the SC community found in A horizon soils. Future work on this topic should include attempts to enhance potential recovery of SC in the BIOLOG[®] system. It may be possible to partially resuscitate SC prior to inoculation into BIOLOG[®] plates, thus increasing the functional diversity measurements obtained. Resuscitation coupled with enrichment and growth in BIOLOG[®] wells may prove a reasonable approach to obtaining additional isolates of previously uncultured species of soil bacteria.

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Table 1. Bacterial numbers in forested and cultivated Guernsey silt loam soil determined by direct microscopic counts.

Sample	Number of cells gram ⁻¹ dry weight soil			
	A Horizon		B Horizon	
	F ^d	C ^d	F	C
Uncentrifuged ^a	5.31 x 10 ¹⁰	4.56 x 10 ¹⁰	3.25 x 10 ¹⁰	2.56 x 10 ¹⁰
Centrifuged ^b	9.27 x 10 ⁸	4.63 x 10 ⁸	5.83 x 10 ⁸	2.25 x 10 ⁸
LC ^c	2.46 x 10 ⁸	1.27 x 10 ⁸	1.82 x 10 ⁸	6.21 x 10 ⁷
SC ^c	5.46 x 10 ⁸	2.89 x 10 ⁸	3.53 x 10 ⁸	2.26 x 10 ⁸

^a Uncentrifuged = counts made before centrifugation at 2500 rev min⁻¹

^b Centrifuged = counts made following centrifugation at 2500 rev min⁻¹

^c LC, large cells (>0.45 µm); SC, small cells (<0.45 µm)

^d F, forested site; C, cultivated site

Figure 1. Average well color development (AWCD) from GN and GP BIOLOG plates with time for large (LC) and small (SC) cell size fractions of forested and cultivated sites. Solid symbols represent data taken from GN plates, open symbols represent data taken from GP plates. Symbols represent the following: ◇ , A horizon LC; △ , A horizon SC; ○ , B horizon LC; □ , B horizon SC. (Note: solid square symbols are obscured by open symbols along the baseline of the figure.)

Table 2. Bacterial functional diversity indices determined in forested and cultivated Guernsey silt loam soil as determined with GN and GP BIOLOG microtitre plates.

Plate Type	Sample ^a	Sum of Activities ^b		AWCD ^c		Richness ^d		Diversity ^e	
		F ^f	C ^f	F	C	F	C	F	C
GN Plates	A LC	114.6	144.0	1.21	1.52	79	86	4.32	4.44
	B LC	98.5	121.7	1.04	1.28	67	81	4.18	4.35
	A SC	7.4	13.6	0.08	0.14	10	11	3.56	3.55
	B SC	0.5	0.9	0.01	0.01	0	0	NC	NC
GP Plates	A LC	120.1	140.9	1.26	1.48	81	92	4.37	4.46
	B LC	82.8	99.1	0.87	1.04	60	73	4.09	4.27
	A SC	9.7	13.8	0.10	0.14	12	13	3.40	3.48
	B SC	0.8	1.2	0.01	0.01	0	0	NC	NC

^aA, A horizon; B, B horizon; LC, large cells (>0.45 µm); SC, small cells (<0.45 µm)

^bAverage sum of activities = average sum of absorbance values from triplicate plates

^cAverage well color development (AWCD) = average sum of activities / 95

^dSubstrate richness = average number of positive wells (absorbance value > 0.25)

^eSubstrate diversity = $-\sum p_i(\ln p_i)$, where p_i = average absorbance of a single substrate / average sum of activities

^fF, forested site; C, cultivated site

NC = not calculated

Figure 2. Principle component analyses based on adjusted AWCD (average absorbance value for each substrate divided by AWCD) from GN and GP BIOLOG plates. Values in parentheses indicate the proportion of total variation accounted for by each principle component. Symbols are as follows: $\diamond$, A horizon LC; $\triangle$, A horizon SC; $\circ$, B horizon LC. Cultivated site samples are denoted by open symbols, forested site samples are denoted by solid symbols.

Table 3. Average richness (absorbance >0.25) within substrate guilds of GN and GP BIOLOG microtitre plates inoculated with size-fractionated bacteria from forested and cultivated Guernsey silt loam soil.

Substrate Guild	#GN/#GP ^a	LC A horizon ^b				SC A horizon ^b			
		GN		GP		GN		GP	
		F ^c	C ^c	F	C	F	C	F	C
Carbohydrates	30/43	28	30	37	42	5	5	5	5
Carboxylic Acids	24/16	16	20	14	16	2	3	4	3
Polymers	5/7	5	5	7	7	0	0	0	1
Amines/Amides	6/4	4	5	2	4	0	0	0	1
Amino Acids	20/8	18	18	8	8	2	2	2	2
Miscellaneous	10/17	8	8	13	15	1	1	1	1

^aIndicates the number of substrates available per guild in GP and GN plates, respectively

^bLC, large cells (>0.45 µm); SC, small cells (<0.45 µm)

^cF, forested site; C, cultivated site

CHAPTER 2

Characterization of dilution enrichment cultures obtained from size-fractionated soil bacteria by BIOLOG[®] substrate utilization patterns and restriction analysis of 16S rRNA genes

Introduction

Small-celled soil bacteria rarely are obtained in culture, therefore the physiological and ecological function of these numerically abundant organisms is currently a matter of speculation (Bakken and Olsen, 1989). Alternative methods to cultivate and study these fastidious organisms are necessary if we are to gain insight into their metabolic activity in soil. Dilution cultures have proven advantageous in studying small-celled oligotrophic bacteria since dilution of the inoculum retains numerically abundant organisms, while eliminating fast-growing competitors that would typically prevail in enrichment culture (Button et al., 1993). Dilution culture allows the recovery of several types of bacteria from a single inoculum source since shifts in the bacterial population occur, depending on the number and growth-rate of the organisms present at each dilution level. Using a wetland biofilm inoculum, Jackson et al. (1998) detected changes in bacterial genotypes with increasing dilution using denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA. Janssen et al. (1997) isolated a novel anaerobic ultramicrobacterium (cell volume $<0.1 \mu\text{m}^3$) from anoxic rice paddy soil by utilizing a liquid dilution culture technique.

In previous work, small soil bacteria ($<0.45 \mu\text{m}$ in diameter) exhibited limited substrate use on BIOLOG[®] GN and GP microtitre plates compared to larger cells ($>0.45 \mu\text{m}$ in diameter), despite similar inoculum densities (K. Kinneer, 1999, MS Thesis Chapter 1, WVU). Differences in the physiological state of these size-fractionated bacteria may have accounted for the observed differences in substrate utilization. Small cells from A horizon soils were able to utilize several carbon substrates in the BIOLOG[®] microtitre plates, however those from B horizon soils utilized none. It was unclear whether small cells from B horizon soils were metabolically dormant and/or physiologically distinct from those in A horizon soils.

In the present study, the dilution culture technique was employed in an attempt to enrich for small-celled bacteria from A and B horizons of forested and cultivated sites of a Guernsey silt-loam soil. Dilution cultures were used to inoculate BIOLOG[®] GN microtitre plates to investigate whether the activity of small-celled bacteria was stimulated by enrichment. Using BIOLOG[®], we examined changes in functional diversity in the bacterial community among flasks of increasing dilution. In addition, restriction digestion of PCR-amplified 16S rRNA genes was employed to compare the genetic diversity of the bacterial communities in the dilution flasks.

Materials and Methods

Study sites and sampling procedures

Two sites were studied: a cultivated site, which has undergone regular tillage since 1976; and, a forested site, a naturally wooded area dominated by deciduous trees. The soil type at both sites is a Guernsey silt loam (fine, mixed, mesic Aquic Hapludalfs). Both sites are located at the West Virginia University Plant Sciences Farm in Morgantown, WV (39.63°N, 79.95°W).

Multiple core samples were taken from each site by using a split-core soil sampler. Samples from the A and B horizon were collected on site and placed into separate plastic bags for immediate transport to the laboratory, where they were individually bulked and coarse-sieved (4.75 mm mesh size). All samples were stored at field moisture in sealed plastic bags and were refrigerated at 4 °C until use.

Sample preparation

For each of the four soil samples, 50 g soil, 200 ml Winogradsky's salt solution (5.0 g K₂HPO₄, 5.0 g MgSO₄ · 7H₂O, 2.5 g NaCl, 0.05 g Fe₂(SO₄)₃ · H₂O, 0.05 g Mn₂(SO₄)₃ · 4H₂O,

2000 ml dH₂O, pH 6; diluted 1/10), and one drop of Tween 80 were blended in a Waring blender for three one-minute intervals with intermittent cooling in an ice bath. This was repeated until six 250 ml centrifuge bottles (Nalgene[®], polyethylene) per horizon and study site were filled with soil homogenate. Large soil particles were removed by centrifugation at 2500 rev min⁻¹ for 15 min at 4 C in a Sorvall[®] RC-5B Refrigerated Superspeed Centrifuge and a Sorvall[®] GSA rotor. The resulting supernatants from each site and soil horizon were pooled and added to 500 ml Winogradsky's salt solution.

To separate bacterial cells according to size, each cell solution was passed through 0.45 µm Millipore HA filters. The filtrate contained bacterial cells smaller than 0.45 µm in diameter and represented the small cell (SC) fraction. Large cells (LC) greater than 0.45 µm in diameter were collected by washing material from the surface of the filters, by placing them into Petri plates containing Winogradsky's salt solution and shaking on a rotary shaker (Lab-line orbit shaker) at 110 rev min⁻¹ for 15-20 min. A sterile paintbrush (5/32" diameter) was used to brush off any remaining cells from the surface of the filters.

To collect cells, LC and SC samples were centrifuged at 10,000 rev min⁻¹, 4 C, for 25 min. Supernatants were discarded, and cells were resuspended in 200 ml Winogradsky's salt solution. The cells were then washed three times, to remove soil particles and dissolved carbon, by centrifugation in the same manner.

Inoculation of dilution cultures

Two separate experiments were performed: the first included LC and SC from the A horizon of both study sites; and, the second included SC only from A and B horizons of both study sites. Prior to inoculation, washed cells were equalized to 95% T ($A_{595}=0.02$) on a spectrophotometer (Bausch and Lomb Spectronic 20). Ten ml of each sample were transferred

to a 250 ml Erlenmeyer flask containing 90 ml of 1/10 R2A broth (0.05 g Bacto Yeast Extract, 0.05 g Bacto Protease Peptone No.3, 0.05 g Bacto Casamino Acids, 0.05 g Bacto Dextrose, 0.05 g soluble starch, 0.03 g potassium phosphate, dibasic, 0.005 g magnesium sulfate, in 1 L distilled H₂O). A serial ten-fold dilution was performed, resulting in a series of 10⁻¹ through 10⁻⁶ dilutions in this medium.

Following inoculation, all flasks were incubated on a shaker (200 rev min⁻¹; Series 25 Incubator Shaker, New Brunswick Scientific Co.) set at 25 C for one week. Aliquots (1 ml) from each turbid flask were transferred to fresh medium and allowed to incubate for three additional days, at which time they were harvested prior to inoculation of BIOLOG[®] microtitre plates.

BIOLOG[®] inoculation

Prior to cell harvest, the turbidity of each flask was determined. Each dilution culture was then poured into a sterile 250 ml centrifuge bottle (Nalgene) and centrifuged at 10,000 rev min⁻¹, 4 C, for 25 min. The supernatant was discarded, cells were washed and resuspended in fresh Winogradsky's salt solution, and the centrifugation process was repeated two additional times. Recovered cells were then suspended in Winogradsky's salt solution and adjusted to 92% T (A₅₉₅=0.04). Suspensions were then inoculated into GN BIOLOG[®] plates, 100 µL per well. The plates were incubated at 25 C and were read every 12 h to monitor color development.

In the second experiment (SC only, A and B horizon soil), cell suspensions were collected for molecular analysis as well as for BIOLOG[®] inoculation. Cells were washed with saline (0.85% NaCl) instead of Winogradsky's salt solution to eliminate excess magnesium that would interfere with subsequent PCR. Washed and centrifuged pellets were resuspended in 3 ml saline of which 1 ml was transferred to an Eppendorf tube and frozen at -60 C until later use.

The remaining cell suspension was diluted to 92% T ($A_{595}=0.04$) and inoculated into GN BIOLOG[®] plates as described above.

Cell measurement

Cells from each turbid enrichment culture were transferred to a clean microscope slide and combined with an equal volume of nigrosin to prepare negative stains. Measurements were made from ten random fields of view by using a compound microscope (Nikon Eclipse E600) equipped with an ocular micrometer.

Amplified ribosomal DNA restriction analysis (ARDRA)

PCR reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 0.1% Triton X-100, 1.25 mM each of deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP; Promega), 2.5 U Taq DNA polymerase (Promega), 1 μ M each of oligonucleotide primers (GIBCO BRL Custom Primers, described below) and 1 μ L or 5 μ L diluted cells as prepared for BIOLOG[®] inoculation above in a total volume of 50 μ L. In preliminary experiments it was determined that following equalization of turbidity to 92% T, samples containing 1 μ L of cells (among several concentrations tested) provided maximal PCR product (data not shown). However, for six samples, amplifications had to be repeated with 5 μ L of cells to obtain sufficient product to conduct restriction analyses. The sequence of the forward primer pA was 5'-AGA GTT TGA TCC TGG CTC AG-3' and the sequence of the reverse primer pH was 5'-AAG GAG GTG ATC CAG CCG CA-3' (Ovreas and Torsvik, 1998). Together, these prime amplification of nearly full-length 16S rDNA and a portion of 23S rDNA (256f-1930r) (Ovreas and Torsvik, 1998; Weisburg et al., 1991). Reaction mixtures were subjected to a thermal-cycling regime (in a PTC-100 Programmable Thermal Controller, MJ Research, Inc.) consisting of an initial denaturization at 94 C for 3 min, followed by 35 cycles of 94 C for 1 min, 54 C for 1

min, and 72 C for 2 min. Negative controls were included with no addition of template DNA. Eight µL of the PCR products were analyzed on 1% (w/v) agarose gels (Agarose SFR, Midwest Scientific) to check for amplification of a band of the expected size (~1600 bp) exclusively.

Appropriate dilutions of PCR products were digested with Sau 3AI or Rsa I in buffers provided by the manufacturer (New England Biolabs). Products of digestion were separated by electrophoresis through 2% agarose SFR gels in 0.5 x TBE (45mM Tris-borate, 1 mM EDTA), stained with ethidium bromide, and photographed while exposed to ultraviolet light.

Data analysis

For the molecular data, pairwise comparisons of the band patterns were manually performed, and a matrix (presence-absence for each band) constructed. From this matrix, a similarity index was calculated according to the formula

$$S = 2 * n_{ab} / (n_a + n_b)$$

where n_a and n_b represent the total number of bands present in lanes a and b, respectively, and n_{ab} is the number of bands which are shared by both lanes (Weising et al., 1995). From the similarity matrix, a distance matrix was calculated using $D = 1 - S$ as distance values. Data were analyzed with the Phylogeny Inference Package (PHYLIP) (Felsenstein, 1993). The UPGMA (Unweighted Pair Group Maximum Averages) method was used to establish relationships among samples based on the distance matrix data. For all other analyses, data were analyzed with JMP Statistical Software (SAS Institute, Inc.).

Results

SC obtained from more highly dilute enrichment cultures of A horizon soils produced substrate utilization patterns in BIOLOG[®] GN plates that were distinct both from all enrichments

of LC and from SC obtained in less dilute enrichments. LC from the forested and cultivated sites utilized 56 to 89% and 76 to 97% of the available BIOLOG carbon substrates, respectively (Table 1). In contrast, SC from the forested site and cultivated sites positively utilized 5 to 80% and 19 to 78% of the available substrates. Substrate richness decreased with increasing dilution in the SC enrichment cultures. SC in the early dilution flasks (10^{-1}) utilized a similar number of substrates as LC. A similar trend was evident in calculated functional diversity, where SC in the early dilutions exhibited values nearly equal to those of the LC (Table 1). However, as with substrate richness, both the SC diversity index and AWCD decreased with increasing dilution. Differences were noted in the SC response from cultivated compared with forested soils. In general, greater values for substrate richness, AWCD, and diversity were observed at any given dilution of the cultivated compared with forested soil.

Principle-component analysis (PCA) performed using normalized values of AWCD provided further evidence that dilution-enrichment of size-fractionated soil bacteria resulted in metabolically distinct sub-populations. PCA separated samples according to inoculum dilution and cell size at both study sites (Figure 1). Consistent with data in Table 1, SC obtained from the least dilute enrichments usually grouped with LC. All dilutions of LC from the cultivated site grouped similarly, however the most dilute enrichment of LC from the forested site (10^{-6}) grouped with SC (Figure 1). At both sites, SC obtained from the most dilute enrichments were clearly separated from LC and all other SC samples.

Substrate utilization in BIOLOG[®] GN plates by SC isolated from A and B horizon soils of both study sites were compared in a second dilution-enrichment experiment. Results from A horizon soils were similar to those from the preceding experiment, although slightly greater substrate utilization was observed in the most dilute enrichments from the cultivated site. In

general, at any given inoculum dilution SC from A horizon soil utilized a greater number of substrates than did SC from the B horizon (Table 2). Greatest substrate richness, AWCD, and diversity values were observed in the least dilute B horizon enrichments followed by generally decreasing values with increasing inoculum dilution. Positive enrichment (turbidity/growth) of B horizon SC occurred with 100-fold more dilute inoculum in the cultivated compared with the forested site (Table 2).

Consistent with information presented in Table 2, PCA separated data by soil horizon and inoculum dilution (Figure 2). A and B horizons of the cultivated site were clearly separated along PC 2. Soil horizons at the forested site separated along both PC1 and PC2. The least dilute B horizon enrichment generally grouped most closely with A horizon samples. More dilute B horizon samples grouped distinctly from the A horizon at both sites.

Cells from these same SC dilution-enrichment cultures were used as a source of template DNA in PCR to amplify the 16S rRNA genes (rDNA) from the resulting SC bacterial community. The PCR products were then digested with restriction enzymes and their restriction fragment length polymorphisms (RFLP) compared. Examination of the band patterns from Sau3AI and RsaI digestions indicated that the dilution culture technique successfully enriched distinct bacterial sub-populations from individual soil samples (Figure 3). The appearance of novel bands in cultures started with more dilute inocula indicates that, through dilution, certain species were released from competition. Summing and averaging band lengths from the two gels suggests that PCR successfully amplified rDNA from between 1 to 4 bacterial species per dilution flask, based on a 1600 base-pair length for the amplified 16S rRNA region (Hartmann et al., 1997).

The unweighted pair group maximum average (UPGMA) method was used to establish relationships among samples based on a distance matrix calculated from combined Sau3AI and RsaI band patterns. Relationships established by this method indicated that enrichment in dilution cultures resulted in SC populations that varied by study site, soil horizon, and dilution (Figure 4). In general, A and B horizon samples were clustered across sites, with the exception of the cultivated A horizon 10^{-4} and 10^{-5} enrichments, which grouped with the B horizon samples. Two dilution cultures from the cultivated site (A horizon 10^{-2} and B horizon 10^{-2}) were separated from the primary A and B horizon clusters because they were dominated by individual organisms that were not significant components of the other dilution cultures (Figure 3). The forested site A horizon samples were similar to each other but distinct from those of the forested B horizon. Cultivated site A horizon samples of increasing dilution were distinct from each other, and were intermingled with cultivated B horizon samples. Dilution-enrichment resulted in a larger number of distinct RFLP patterns in the cultivated compared with the forested site.

Discussion

In a previous study (K. Kinneer, MS Thesis Chapter 1, WVU), substrate utilization patterns generated in BIOLOG[®] microtitre plates differed between SC and LC bacteria extracted directly from soil. These differences were readily apparent despite a similar inoculum density for both cell size fractions. In general, the rate and extent of substrate utilization was greatly restricted for the SC compared with the LC size fraction. It may be that SC grew less readily in the enrichment conditions of a BIOLOG[®] microtitre plates than did LC. These data are consistent with previous studies demonstrating that SC are difficult to grow in culture (Bakken and Olsen, 1986). Among several possible explanations, difficulty in culturing SC may be due to

dormancy brought on by prolonged starvation of slow-growing autochthonous and sometimes oligotrophic soil bacteria. In this study, we attempted to use dilution of the SC and LC bacterial community to partition inocula according to their numerical prevalence. The diluted inocula were then enriched in a low nutrient medium in an attempt to stimulate growth and recovery of members of the SC bacterial community.

Compared with our previous study, substrate utilization by both LC and SC was stimulated by enrichment in dilution culture. In the present study, we found that following enrichment A horizon SC from the least dilute enrichment cultures utilized a nearly equal number of substrates as LC. However, SC from the most dilute enrichments remained metabolically distinct from LC. Inspection of AWCD with time demonstrated no apparent lag period prior to color development in the positive microtitre wells (data not shown). Presumably, the lag period represents the amount of time needed for cells to reach the 10^8 cells ml^{-1} density required for color development to begin in a BIOLOG[®] well (Garland and Mills 1991, Haack et al., 1995). Konopka et al. (1998) suggested that color development is dominated by bacteria in relatively high numbers initially, or those with high maximum growth rates. Lack of a lag period in our data may indicate that either cells were inoculated at a density near 10^8 cells ml^{-1} , or they were in a state of exponential growth at the time of inoculation.

SC from the A horizon of both study sites were previously shown to provide detectable patterns of substrate utilization on the BIOLOG[®] plates (K. Kinneer, MS Thesis Chapter 1, WVU). However, SC bacteria from B horizon soils failed to utilize any of the available carbon substrates, suggesting that they were metabolically dormant and/or physiologically distinct from small cells of A horizon soils. Following dilution-enrichment in the present study, B horizon SC of both study sites exhibited activity rivaling that of A horizon SC. These data suggest that

enrichment in a low-nutrient medium (1/10 strength R2A broth) allowed some B horizon SC to regain their ability to metabolize carbon substrates. It has been proposed that viability may be maintained in the absence of culturability, and viable but non-culturable cells may be able to return to an actively metabolizing state under favorable conditions (McDougald et al., 1998). This phenomenon may explain why B horizon SC exhibited activity in the BIOLOG[®] wells only after enrichment in dilution culture. Cells extracted from the soil may be in a state of metabolic inactivity in order to survive low nutrient availability, especially in B horizon soils. Following enrichment, these cells may be ‘resuscitated’ and regain their metabolic integrity, resulting in an increase in AWCD and substrate richness in BIOLOG[®] plates. Cell measurements taken after enrichment indicated that SC increased in length compared to measurements taken before enrichment (data not shown). This larger size may be the result of growth and increased activity by SC while in the presence of increased nutrient availability.

Shifts in substrate richness, AWCD, and diversity indicated changes in the bacterial community among dilution flasks. Principle component analysis of A horizon LC and SC samples from both study sites revealed that the lowest SC dilution (10^{-1}) grouped with LC samples, while higher SC dilutions were grouped apart from LC (see Figure 1). These data suggest that SC found in the early dilution flasks belong to a similar population as LC, while the numerically-dominant SC found in the highest dilution flasks were a unique community, metabolically distinct from LC. Differences in the SC bacterial community among A and B horizon were also apparent from PCA (see Figure 2). Data suggest that the most competitive, culturable B horizon SC from the least dilute enrichments are similar to cells from the A horizon. However, the most numerically-dominant B horizon SC found in the most dilute enrichments appear to be distinct from the SC community in the A horizon.

Analysis of PCR-amplified 16S rRNA genes indicated that the dilution culture enriched for genetically distinct populations of SC from A and B horizon soils of both study sites. The number of SC species per dilution flask estimated from *Sau3AI* and *RsaI* band lengths was low, ranging from 1 to 4 species per flask. There are several potential explanations for this low species number. It is possible that only a few species of SC bacteria were able to grow in the dilution culture due to nutritional factors, or that only a few species had a sufficient growth rate to prevail in the competitive conditions imposed by enrichment culture. Of the SC able to grow in culture, perhaps only a few species reached population densities high enough to be detected by PCR amplification and EtBr staining. [The detection limit for EtBr-stained PCR products ranges from 10^3 to 10^4 cells (Pepper, 1997).] In this case, only the most competitive members of the community in each dilution flask would be detected, regardless of the actual number of species present. Restriction fragments of nearly equal size are often interpreted as identical bands when band scoring (Weising et al., 1995). Therefore, several species may be contained in each flask, but may give similar restriction fragments, resulting in an underestimation of species content per flask. Moreover, highly conserved regions of the rDNA may give identical-sized fragments from several different species. For example, inspection of the *RsaI* restriction products revealed a 150-bp band common to all SC samples analyzed, regardless of soil horizon or dilution. Because this small fragment is the only fragment in common among all lanes that contain it, we conclude that it represents a highly conserved region of DNA (rather than a species common to all cultures). By counting this fragment only once (as we did) we underestimate the total length of DNA present. Finally, the choice of primer used for PCR amplification can greatly affect the outcome of an RFLP analysis. “Universal” primers do not unequivocally amplify all 16S rRNA genes present in a sample (Fuhrman, 1997). However, the bacterial primers used in this study

were reported to amplify nearly full-length 16S rDNA and 23S rDNA (Ovreas and Torsvik, 1998), and were capable of amplifying a wide variety of bacterial taxa (Weisburg et al., 1991). Differences in annealing temperature and quantity and quality of target DNA may affect the specificity and utility of the primer utilized.

Both the BIOLOG[®] system and ARDRA analysis suggest that enrichment in dilution culture resulted in distinct communities with increasing dilution. Santegoeds et al. (1996) and Jackson et al. (1998) also found changes in the species composition of enrichment cultures throughout the dilution series. Like Jackson et al. (1998), we found bands in higher dilution flasks that were not present in the lowest dilution flasks (see Figure 3) suggesting that dilution cultures select for different types of bacteria by minimizing competitive exclusion.

Analysis of RFLP data by UPGMA resulted in a clear separation of forested A and B horizon samples, while cultivated A and B horizon samples were often grouped together. The overlap in species composition among soil horizons at the cultivated site may be due in part to tillage, which would break up A and B horizon soils and mix them together. At the undisturbed forested site, A and B horizons would not be mixed and therefore, the bacterial communities are more likely to remain distinct from each other.

A great number of as yet uncultured bacterial species exist unstudied in even the most common of soils. Functional diversity measurements by BIOLOG[®] microtitre plates incompletely characterize the true bacterial diversity of soil samples, but have proved useful in operational comparisons between sites (Konopka et al., 1998). Our studies indicate that a limited number of SC bacteria can grow in BIOLOG[®] plates when directly inoculated from soil. Pre-enrichment of the SC bacterial fraction increased growth in BIOLOG[®] plates, particularly of cells from our B horizon samples. Substrate utilization patterns obtained from BIOLOG[®]

microtitre plates proved to be a convenient method to assess the functional diversity of dilution enrichment cultures obtained from size fractionated soil bacteria. Genetic diversity estimates by ARDRA confirmed the community changes indicated by changes in carbon utilization.

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Table 1. Functional diversity indices determined in A horizon forested and cultivated Guernsey silt loam soil using GN BIOLOG plates inoculated with large and small cells enriched in dilution culture.

Sample ^a	Sum of Activities ^b		AWCD ^c		Richness ^d		Diversity ^e	
	F ^f	C ^f	F	C	F	C	F	C
LC 10-1	98.8	135.4	1.04	1.43	78	88	4.32	4.42
LC 10-2	107.2	153.8	1.13	1.62	80	92	4.34	4.23
LC 10-3	106.5	ND	1.12	ND	85	ND	4.37	ND
LC 10-4	102.0	132.2	1.07	1.39	77	86	4.29	4.41
LC 10-5	86.2	94.0	0.91	0.99	70	72	4.17	4.23
LC 10-6	34.1	131.6	0.36	1.39	53	80	4.13	4.35
SC 10-1	100.1	114.9	1.05	1.21	76	74	4.29	4.27
SC 10-2	59.5	48.8	0.63	0.51	56	51	4.01	4.06
SC 10-3	74.8	49.8	0.79	0.52	50	54	3.95	4.18
SC 10-4	8.2	25.6	0.09	0.27	6	21	3.85	4.02
SC 10-5	7.5	41.2	0.08	0.43	8	54	3.23	4.14
SC 10-6	4.6	18.9	0.05	0.20	5	18	3.57	3.96

ND = not determined because of color development in control well.

^aLC, large cells (>0.45 µm); SC, small cells (<0.45 µm); 10-1 to 10-6 indicate dilution

^bAverage sum of activities = average sum of absorbance values from triplicate plates

^cAverage well color development (AWCD) = average sum of activities / 95

^dSubstrate richness = average number of positive wells (absorbance value > 0.25)

^eSubstrate diversity = $-\sum p_i(\ln p_i)$, where p_i = average absorbance of a single substrate/average sum of activities

^fF, forested site; C, cultivated site

Figure 1. Principle component analyses of large small cells extracted from A horizons of forested and cultivated Guernsey silt loam soil and enriched in dilution culture. Numbers in parentheses indicate the percent of variation accounted for by each principle component. Symbols are as follows: $\circ$, 10-1 dilution; $\square$, 10-2 dilution; $\triangle$, 10-3 dilution; ∇ , 10-4 dilution; $\diamond$, 10-5 dilution; $\hexagon$, 10-6 dilution. Solid symbols indicate large cells, open symbols indicate small cells.

Table 2. Functional diversity indices determined in A and B horizon forested and cultivated Guernsey silt loam soil using GN BIOLOG plates inoculated with small cells enriched in dilution culture.

Sample^a	Sum of Activities^b	AWCD^c	Richness^d	Diversity^e
F-A Horizon				
SC 10-1	99.3	1.04	74	4.27
SC 10-2	72.1	0.76	56	4.02
SC 10-3	32.1	0.34	43	4.09
SC 10-4	31.3	0.33	37	3.86
SC 10-5	14.0	0.15	16	3.81
F-B Horizon				
SC 10-1	62.1	0.65	67	4.22
SC 10-3	9.1	0.10	10	3.47
C-A Horizon				
SC 10-1	111.9	1.18	78	4.31
SC 10-2	82.8	0.87	70	4.20
SC 10-3	46.2	0.49	55	4.04
SC 10-4	70.3	0.74	68	4.23
SC 10-5	62.6	0.66	61	4.17
C-B Horizon				
SC 10-1	75.4	0.79	67	4.21
SC 10-2	17.0	0.18	21	4.24
SC 10-3	6.0	0.06	8	3.42
SC 10-4	11.0	0.12	17	3.94
SC 10-5	2.9	0.03	4	2.84

^aF, forested site; C, cultivated site; SC, small cells (<0.45 µm); LC, large cells (>0.45 µm); 10-1 to 10-6, dilution

^bAverage sum of activities = average sum of absorbance values from triplicate plates

^cAverage well color development (AWCD) = average sum of activities / 95

^dSubstrate richness = average number of positive wells (absorbance value > 0.25)

^eSubstrate diversity = $-\sum p_i(\ln p_i)$, where p_i = average absorbance of a single substrate / average sum of activities

Figure 2. Principle component analyses of small cells extracted from A and B horizons of forested and cultivated Guernsey silt loam soil and enriched in dilution culture. Numbers in parentheses indicate the percent of variation accounted for by each principle component. Symbols are as follows: ○ , 10-1 dilution; □ , 10-2 dilution; △ , 10-3 dilution; ▽ , 10-4 dilution; ◇ , 10-5 dilution. Solid symbols indicate A horizon samples, open symbols indicate B horizon samples.

Figure 3. ARDRA analyses of small-celled bacteria extracted from A and B horizons of forested and cultivated Guernsey silt loam soil and enriched in dilution culture. Sau 3AI and Rsa I indicate restriction enzymes used in digestion. Lane markers are as follows: BstEII (digest of bacteriophage lambda) and 50 bp (ladder, GIBCO-BRL), size-markers; F, forested site; C, cultivated site; A, A horizon; B, B horizon; 1 through 5, dilutions.

Figure 4. Relationships among small cells from A and B horizons of cultivated and forested Guernsey silt loam soil established with the UPGMA (Unweighted Pair Group Maximum Averages) method, based on a distance matrix generated from RFLPs in 16S rDNA (combined Sau 3AI and Rsa I data). F, forested site; C, cultivated site; A, A horizon; B, B horizon; 10-1 through 10-5, dilutions

APPENDIX I

METHODS OF CELL EXTRACTION, ENUMERATION, AND BIOLOG[®] INOCULATION

Figure 1. Methods of cell extraction, enumeration, and BIOLOG® inoculation. Denotes points where direct microscopic counts were performed using FITC (fluorescein isothiocyanate) staining.

APPENDIX II

ADDITIONAL DATA NOT PRESENTED IN CHAPTER 2

Figure 1. Average well color development (AWCD) from GN BIOLOG plates with time for large (LC) and small (SC)-celled bacteria extracted from A horizon forested Guernsey silt loam soil and enriched in dilution culture. Symbols are as follows: ◇ , 10⁻¹ dilution; □ , 10⁻² dilution; △ , 10⁻³ dilution; ○ , 10⁻⁴ dilution; ✱ , 10⁻⁵ dilution; ⊕ , 10⁻⁶ dilution. Solid symbols indicate LC, open symbols indicate SC.

Figure 2. Average well color development (AWCD) from GN BIOLOG plates with time for large (LC) and small (SC)-celled bacteria extracted from A horizon cultivated Guernsey silt loam soil and enriched in dilution culture. Symbols are as follows: ◇ , 10-1 dilution; □ , 10-2 dilution; △ , 10-3 dilution; ○ , 10-4 dilution; ✱ , 10-5 dilution; +, 10-6 dilution. Solid symbols indicate LC, open symbols indicate SC.

Figure 3. Average well color development (AWCD) from GN BIOLOG plates with time for small-celled bacteria extracted from A horizon cultivated and forested Guernsey silt loam soil and enriched in dilution culture. Symbols are as follows: $\diamond$, 10^{-1} dilution; $\square$, 10^{-2} dilution; $\triangle$, 10^{-3} dilution; $\circ$, 10^{-4} dilution; $\times$, 10^{-5} dilution. Solid symbols indicate forested site enrichments, open symbols indicate cultivated site enrichments.

Figure 4. Principle component analysis based on adjusted AWCD (average absorbance value for each substrate divided by AWCD) from GN BIOLOG plates inoculated with small-celled bacteria extracted from A and B horizons of forested and cultivated Guernsey silt loam soil. Abbreviations are as follows: F, forested; C, cultivated; A, A horizon; B, B horizon; 10-1 through 10-5, dilutions.

Figure 5. Average well color development (AWCD) per substrate guild from GN BIOLOG plates inoculated with small-celled (SC) bacteria extracted from A and B horizons of forested Guernsey silt loam soil and enriched in dilution culture.

Figure 6. Average well color development (AWCD) per substrate guild from GN BIOLOG plates inoculated with small-celled (SC) bacteria extracted from A and B horizons of cultivated Guernsey silt loam soil and enriched in dilution culture.

Table 1. Estimated width and length of small cells extracted from A and B horizons of forested and cultivated Guernsey silt-loam soil following enrichment in dilution culture.

Sample*	Estimated Width (μm)	Estimated Length (μm)
Forested Site, A Horizon		
SC 10-1	<0.5	>0.5
SC 10-2	<0.5	>0.5
SC 10-3	<0.5	>0.5
SC 10-4	<0.5	>0.5
SC 10-5	<0.5	>0.5
Forested Site, B horizon		
SC 10-1	<0.5	>0.5
SC 10-3	<0.5	>0.5
Cultivated Site, A horizon		
SC 10-1	<0.5	>0.5
SC 10-2	<0.5	>0.5
SC 10-3	<0.5	>0.5
SC 10-4	<0.5	>0.5
SC 10-5	<0.5	>0.5
Cultivated Site, B horizon		
SC 10-1	<0.5	>0.5
SC 10-2	<0.5	>0.5
SC 10-3	<0.5	~1
SC 10-4	<0.5	~1
SC 10-5	<0.5	>0.5

*SC, small cells (<0.45 μm before enrichment); 10-1 through 10-5, dilution

Table 2. Estimated width and length of small (SC) and large (LC) cells extracted from A and B horizons of forested and cultivated Guernsey silt-loam soil without enrichment.

Sample*	Estimated Width (µm)	Estimated Length (µm)
Forested-A LC	>0.5	>1
Forested-B LC	>0.5	>1
Cultivated-A LC	>0.5	~1
Cultivated-B LC	>0.5	>0.5
Forested-A SC	<0.5	<0.5
Forested-B SC	<0.5	<0.5
Cultivated-A SC	<0.5	<0.5
Cultivated-B SC	<0.5	<0.5

Table 3. Average richness per substrate guild determined in GN BIOLOG plates inoculated with small-celled bacteria (<0.45 μm) extracted from A and B horizons of forested and cultivated Guernsey silt loam soil and enriched in dilution culture.

Sample*	Carbohydrates	Carboxylic acids	Polymers	Amines/Amides	Amino Acids	Miscellaneous
F-A Horizon	25	18	4	4	16	7
SC 10-1	15	17	2	2	14	5
SC 10-2	11	14	2	3	10	3
SC 10-3	9	12	2	2	9	4
SC 10-4	4	5	2	1	4	1
SC 10-5						
F-B Horizon						
SC 10-1	21	19	3	3	14	7
SC 10-3	2	4	1	0	2	0
C-A Horizon						
SC 10-1	22	22	5	5	15	9
SC 10-2	21	21	3	3	16	6
SC 10-3	16	16	3	2	12	6
SC 10-4	20	19	3	2	16	8
SC 10-5	25	18	2	2	15	6
C-B Horizon						
SC 10-1	25	18	2	2	15	6
SC 10-2	4	8	1	1	5	2
SC 10-3	0	3	1	0	2	1
SC 10-4	2	5	2	1	5	2
SC 10-5	1	1	1	0	0	0

* F, forested site; C, cultivated site; A, A horizon; B, B horizon; SC, small cells (<0.45 μm); 10-1 through 10-5, dilutions.

Table 4. Specific substrates utilized by selected enrichment cultures of small-celled bacteria extracted from forested A horizon Guernsey silt loam soil.

Sample^a	Carbohydrates	Carboxylic acids	Polymers	Amines/Amides	Amino Acids	Miscellaneous
SC 10-6	D-psicose D-mannose	*β-hydroxybutyric acid succinic acid	*tween 40 *tween 80		*L-glutamic acid L-proline	
SC 10-5	D-melibiose *β-methyl-D-glucoside D-trehalose D-galactose *D-mannitol D-psicose D-sorbitol D-raffinose	citric acid D-galacturonic acid β-hydroxybutyric acid succinic acid D-glucuronic acid p-hydroxy phenylacetic acid quinic acid	dextrin glycogen *tween 40		L-glutamic acid L-asparagine	
SC 10-4	D-fructose D-psicose α-D-lactose	propionic acid β-hydroxybutyric acid citric acid α-keto valeric acid D,L-lactic acid D-saccharic acid	dextrin *tween 40			urocanic acid glucose-1-phosphate
SC 10-5				*succinamic acid putrescine		

*indicates that 2 or more of the triplicate plates had an OD > 0.25 for these substrates

^a SC, small cells (<0.45 μm); 10-4 through 10-6, dilutions

Table 5. Specific substrates utilized by selected enrichment cultures of small-celled bacteria extracted from cultivated A horizon Guernsey silt loam soil.

Sample ^a	Carbohydrates	Carboxylic acids	Polymers	Amines/Amides	Amino Acids	Miscellaneous
SC 10-6	L-arabinose	*acetic acid	*dextrin	succinamic acid	D-alanine	inosine
	*D-galactose	citric acid	*tween 40	*glucuronamide	*L-asparagine	thymidine
	gentiobiose	*β-hydroxybutyric acid	*tween 80	*alaninamide	*L-aspartic acid	*glucose-1-phosphate
	mono-methyl	γ-hydroxybutyric acid		putrescine	*L-glutamic acid	*glucose-6-phosphate
	succinate	L-hydroxyphenylacetic acid			glycyl-L-glutamic acid	
	methyl pyruvate	*D,L-lactic acid			*L-proline	
	sucrose	propionic acid			L-serine	
	D-raffinose	succinic acid			γ-amino-butyric acid	
	β-methyl-D-glucoside					
	D-melibiose					
	cellobiose					
	SC 10-5				succinamic acid	
SC 10-4	*citric acid	α-cyclodextrin	glucuronamide	*L-aspartic acid		bromo succinic acid
	D-galactonic acid lactone	glycogen	putrescine	*L-glutamic acid		urocanic acid
	D-galacturonic acid	*tween 40	2-amino ethanol	*glycyl-L-glutamic acid		2,3-butanediol
	*β-hydroxybutyric acid			L-histidine		D,L-α-glycerol phosphate
	γ-hydroxybutyric acid			L-leucine		*glucose-1-phosphate
	itaconic acid			L-ornithine		glucose-6-phosphate
	α-keto-glutaric acid			L-phenylalanine		
	α-keto-valeric acid			L-serine		
	malonic acid			γ-amino butyric acid		
	propionic acid					
	quinic acid					
	D-saccharic acid					
	succinic acid					

* indicates that 2 or more of the triplicate plates had an OD > 0.25 for these substrates

^a SC, small cells (<0.45 μm); 10-4 through 10-6, dilutions