Soil Diffusion System Enriches the Growth of Diverse and Previously Uncultivated Bacterial Taxa

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Recent studies have demonstrated that culturing microorganisms in association with their native habitat promoted the growth of diverse and previously uncultivable bacteria. We developed a soil diffusion system (SDS) where soil microorganisms were inoculated and allowed to grow on regenerated cellulose filters (RCF) that were tightly coupled with the soil habitat. The objective of the study was to assess the influence of the native habitat and the biotic (microbe–microbe) and abiotic soil effects on the selective growth of microbial communities. Regenerated cellulose filters were used as support for the growing microbial community. A polycarbonate membrane (0.003-μm pore size) was inserted between the RCF and the soil to prevent the movement of bacteria and larger organisms. Three different SDS treatments were used to differentiate biotic and abiotic growth effects along with a control-SDS treatment and a traditional culturing medium. The treatments were: (i) inoculated RCF on unsterilized soil ($Biotic_{RCF}$), (ii) inoculated RCF on autoclave-sterilized soil ($Abiotic\text{-}HN_{RCF}$), (iii) inoculated RCF without soil but amended with sterilized soil extracts ($Abiotic\text{-}LN_{RCF}$), and (iv) sterilized cellulose–Congo red agar medium (CCRA). A fifth uninoculated RCF ($Uninoc_{RCF}$) treatment on unsterilized soil was included as control to check for contamination from the soil, and all the treatments were replicated thrice. Following 20 d of incubation, the developing communities from all treatments were characterized using 16S rRNA gene clone libraries. Our results showed that nutrient levels had a small effect on the growing communities, and as hypothesized, the community growing in association with the unsterilized soil ($Biotic_{RCF}$) was the richest and most unique among all treatments. Previously uncultured members of the phyla *Proteobacteria*, *Bacteroidetes*, and few members of *Verrucomicrobia*, *Planctomycetes*, and OP10 were detected on the $Biotic_{RCF}$ treatment. Members of the phylum *Firmicutes* were the dominating bacteria in abiotic treatments, followed by the members of phylum *Planctomycetes*. The CCRA medium supported the growth of a less diverse community, with ~91% of the sequences closely related to isolates of the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. The presence or absence of a living microbial community appears to have a significant impact on the richness and structure of bacterial community growth and development.

Abbreviations: FAME, fatty acid methyl ester; NMS, nonmetric multidimensional scaling; OTU, operational taxonomic unit; PC, polycarbonate; RCF, regenerated cellulose filters; SDS, soil diffusion system.

The explosion of molecular descriptions of microbial life in soil has resulted in the characterization of ~1.9 million bacterial 16S rRNA gene sequences (Cole et al., 2011) in the environment. These exciting discoveries have been a boon to understanding the diversity of life but have added to the challenge of describing the genetic and physiological potential of the microbial world. Studies of microbial function using state-of-the-art transcriptomics and

Soil Sci. Soc. Am. J. 76:463–474
Posted online 6 Feb. 2012
doi:10.2136/sssaj2011.0227
Received 16 June 2011.
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proteomics are highly reliant on genomic sequences of isolated organisms to interpret the dynamics of microbial processes in situ; however, very few cultured representatives exist to represent the vast microbial diversity. For example, while members of the phylum *Acidobacteria* account for 20 to 70% of the 16S rRNA gene sequences derived from soils, there are only eight cultured genera so far to represent this broad phylum (Barns et al., 1999; Janssen, 2006; Männistö et al., 2011). Hence, the isolation of microbial representatives is needed to interpret the potential and functional activities of microbial life in soil.

The cultivation of new and novel microorganisms has been relatively slow, perhaps a reflection of microbial diversity and associated habitat requirements for successful growth. Novel bacterial taxa have been cultivated by modifying the inoculum size, increasing the incubation time, and altering the nutrient status (Aagot et al., 2001; Bruns et al., 2003; Davis et al., 2005; Stott et al., 2008; Zengler et al., 2002). Janssen et al. (2002) reported success in isolating soil bacteria belonging to novel lineages within divisions of *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, and *Verrucomicrobia* by using highly diluted nutrient broth media. Similarly, higher dilutions and low nutrient concentrations also resulted in the isolation of a number of marine bacteria (Button et al., 1993; Eilers et al., 2001; Rappé et al., 2002).

Other studies have had success in cultivating and isolating novel bacteria by coupling their growth to simulated natural environments (Ferrari et al., 2005; Kaeberlein et al., 2002). While the simulation of the biotic environment does not provide information on specific microbial needs for growth, it does allow an assessment of how microbial activities, including signaling and metabolite production, might be important for bacterial growth and the cultivation of soil microorganisms. Indeed, several studies recently emphasized how signaling molecules, the presence of neighboring microbes, and cell–cell communication stimulate the growth of certain bacterial species (Bollmann et al., 2007; Bruns et al., 2002, 2003; D'Onofrio et al., 2010; de Bruyn et al., 1990; Diggle et al., 2007; Kato et al., 2005). The addition of a helper microbe and signaling molecules, for example, supported the growth of a previously uncultivated bacterium *Psychrobacter* sp. strain MSC33 (Nichols et al., 2008). Likewise, Bruns et al. (2002) reported the growth of many previously uncultured bacteria by adding signaling compounds such as homoserine lactones and cAMP in the growth media. These studies point to the relevance of biotic and abiotic habitat characteristics for growing microorganisms.

An SDS was designed to test the role of biotic and abiotic environments on the selection and growth of bacterial communities. The objective was to grow bacteria in close association with their native and biologically rich soil habitat and to compare this biotic system with those grown in association with biologically inactivated or abiotic systems. The abiotic systems were comprised of bacterial communities growing in association with sterilized soil, sterilized soil extract amendment, and traditional CCRA medium. It was hypothesized that the biologically active SDS would support the growth of a very diverse bacterial community having many previously uncultivated members compared with the autoclaved abiotic SDS and traditional growth plate treatments.

Regenerated cellulose filters were selected as a supporting matrix and a nutrient source to represent the most common substrate for microbial growth in soils (Mullings and Parish, 1984; Ulrich and Wirth, 1999). Cellulose, being the most abundant organic molecule on earth, acted as a key energy resource for a potentially diverse group of microorganisms. The capacity to degrade and metabolize cellulose is a characteristic that defines diverse phylogenetic groups across the bacterial kingdoms. Numerous bacterial representatives have been cultured using cellulosic media, but a large proportion of the cellulolytic community remains uncultured (de Boer et al., 2005; Lynd et al., 2002; Ulrich et al., 2008) and thus provides an opportunity to test the hypothesis and discover novel bacterial taxa.

MATERIALS AND METHODS

Sample Collection and Residue Incubation

Soil samples were collected from the A horizon of the Marietta series (a fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudept) located on the University Farm adjacent to the Mississippi State University campus. The site was forested with >50-yr-old deciduous vegetation dominated by pecan [*Carya illinoinensis* (Wangenh.) K. Koch]. The Marietta series is a deep alluvial soil in the Blackland Prairie region of Mississippi. The organic matter content is close to 4% and the pH is neutral. Large plant material and rocks were removed and the soil was passed through a 5-mm, sterile, brass sieve. Approximately 2 g of sterile rice (*Oryza sativa* L.) straw was mixed thoroughly in 100 g of soil and incubated at 25°C for >3 mo. The water content of the soil was maintained at field capacity throughout the incubation period.

Preparation of Bacterial Inoculum

One gram of decomposing rice straw residues, along with the adhering soil particles, was collected and dispersed into 100 mL of 0.9% sterile NaCl solution. The aliquot was vortexed repeatedly for about 10 min and allowed to settle for 5 min, which was considered to be 10^{-2} dilution. The supernatant was further diluted to obtain 10^{-3} dilution, which was used as the inoculum in all the treatments. The 10^{-3} dilution was found to be an ideal dilution in our preliminary tests, resulting in 50 to 200 colonies per plate or RCF filter.

Soil Diffusion System and Traditional Treatments

Regenerated cellulose filters (18407-47-N, Sartorius Mechatronics, Bohemia, NY) with a pore size of 0.2 μm were used as a support matrix and major C source for microbial growth in the SDS. One hundred microliters of the bacterial inoculum (10^{-3}dilution) was mixed with 5 mL of sterile physiological saline solution (0.9% NaCl) and filtered onto RCFs using sterile polycarbonate filter holders (29550-44, John Morris Scientific, Chatswood, NSW, Australia). The treatments were: (i) inoculated RCF on unsterilized soil ($Biotic_{RCF}$), (ii) inoculated RCF on autoclave-sterilized soil ($Abiotic\text{-}HN_{RCF}$), (iii) inoculated

RCF without soil but amended with soluble soil organics (Abiotic-LN_{RCF}), (iv) uninoculated RCF on unsterilized soil ($Uninoc_{RCF}$), and (v) traditional cellulose–Congo red agar (CCRA) medium. In total, there were four SDS and one non-SDS treatments, and each treatment was replicated three times. Biotic and abiotic RCF treatments varied in terms of the presence and absence of microbial activity, respectively, in the associated soil substrate (Table 1). Autoclave sterilization of soil results in the release of high concentrations of nutrients, so to better assess the effects of the abiotic treatment and nutrients, a low-nutrient abiotic treatment was also included.

Inoculated Regenerated Cellulose Filters on Unsterilized Soil

Unsterilized Marietta soil was used as the native habitat for microbial growth. Approximately 35 g of air-dried soil was placed in a petri plate (100 by 15 mm), and a small circular mound of approximately 42 mm wide and 10 mm high was raised in the center. A thin layer of soil was spread and placed in contact with the soil mound. Autoclaved water was added to the base of the soil mound to achieve a water potential of approximately −0.03 MPa at the top of the mound. The soil mound was then covered with a sterile, 47-mm-diameter, polycarbonate (PC) membrane (PCT00347100, Sterilitech Corp., Kent, WA) with a pore size of 0.003 μm and lightly dripped with water to initiate contact with the underlying soil. The PC membrane is the key piece of the SDS and prevents the migration of bacteria and larger organisms across the membrane but allows the diffusion of nutrients and other large molecules that may support microbial growth. Inoculated RCFs were then placed on the sterile PC membrane and the moisture from the inoculant solution helped to initiate contact between the membranes (Fig. 1). The cellulose in the RCF mimics the natural form of cellulose in plants and acts as a major C source for the growing bacteria.

Inoculated Regenerated Cellulose Filters on Autoclaved Soil

The $Abiotic_{RCF}$ treatments were designed to determine the effect of the microbe-free soil environment on the composition of the microbial community growing on the associated RCFs.

Table 1. Description of treatments.

Treatment†	Medium	Growth support	Microbial activity in associated soil or solution	Available nutrients
$Biotic_{RCF}$	unsterilized soil	RCF	present	low
Abiotic-HN_{RCF}	sterilized soil	RCF	undetectable	high
Abiotic-LN_{RCF}	sterile soil organics	RCF	undetectable	low
$Uninoc_{RCF}$	unsterilized soil	RCF	present	low
CCRA	CCRA	CCRA	NA‡	NA

† $Biotic_{RCF}$, inoculated regenerated cellulose filter (RCF) on unsterilized soil; Abiotic-HN_{RCF}, inoculated RCF on sterilized soil; Abiotic-LN_{RCF}, inoculated RCF without soil but amended with sterilized soil extract; $Uninoc_{RCF}$, uninoculated RCF on unsterilized soil; CCRA, cellulose–Congo red agar medium.

‡ NA, not applicable.

The Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments varied in the amount of nutrients available to support microbial growth. The HN and LN designations represent high nutrients and low nutrients, respectively.

The Abiotic-HN_{RCF} treatment was created identically to that of $Biotic_{RCF}$ treatment but utilized sterilized rather than unsterilized soil. Soil sterilization was achieved by autoclaving three times at 121°C for 1 h, with 2 d of incubation between each autoclave event (Wolf and Skipper, 1994). The sterilization process was considered successful because (i) extracts of the autoclaved soil showed no growth on LB agar plates and (ii) there was no respiratory CO_2 production during aerobic incubation (28°C) of the autoclaved soil. It is notable that sterilization of soil through autoclaving results in a large flush of N and other nutrients into the soil solution.

To determine the importance of nutrients for the selection of the cultivable community in the autoclaved high-nutrient soil, a corresponding sterile treatment with low nutrients was also devised (Abiotic-LN_{RCF}). This treatment was without soil but was amended with a sterile solution of soil extract. The

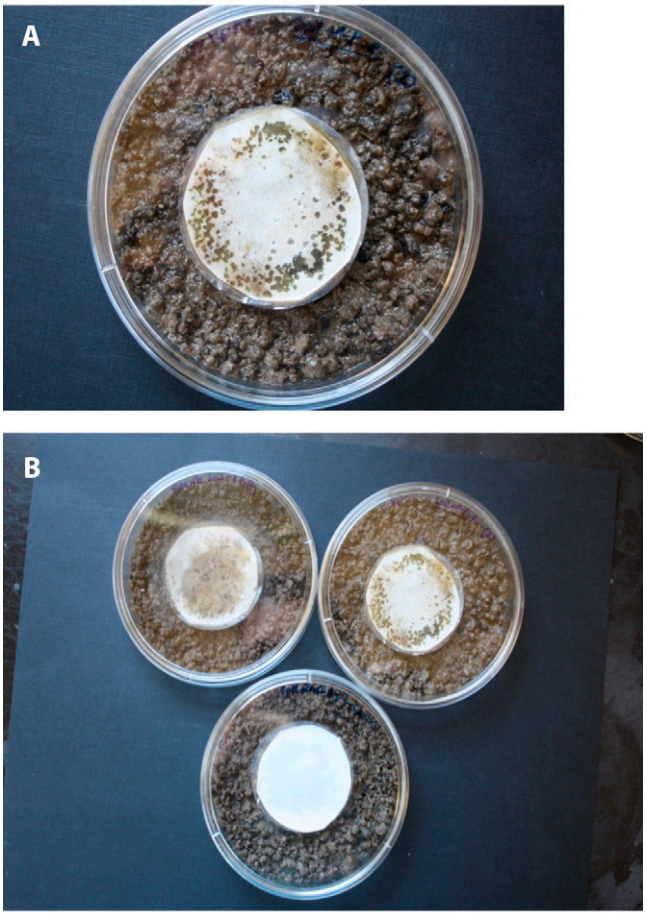

Fig. 1. Photographs of (A) representative inoculated regenerated cellulose filter on unsterilized soil ($Biotic_{RCF}$) treatment after 20 d of incubation (the dark spots on the filter paper represent the degradation of filter paper); and (B) representative inoculated regenerated cellulose filter (RCF) on autoclaved soil (Abiotic-HN_{RCF}; top left), inoculated RCF on unsterilized soil ($Biotic_{RCF}$; top right) and uninoculated RCF on unsterilized soil ($Uninoc_{RCF}$; bottom) treatments after 20 d of incubation.

inoculated RCF membrane was placed in sterile petri plates without soil and amended with 0.3 μL of soluble organics from soil. Soluble organics were derived from autoclaving (121°C for 1 h at 103 kPa) 100 g of soil with 150 mL of tap water. The soil was allowed to settle overnight and the liquid was poured into a 250-mL bottle and centrifuged at 3500 × *g* for 10 min. The supernatant was poured into a 250-mL flask, autoclaved again for 30 min, and frozen at −20°C until use. All the RCF treatments were maintained at optimum moisture during the incubation period of 20 d at 28°C.

Uninoculated Control Treatment

This treatment was set up in the same way as the $Biotic_{RCF}$ but the cellulose filter remained uninoculated and was used as a negative control to assess the growth of contaminating microbes. After 20 d of incubation, there was no conspicuous growth on the top of the cellulose filters; however, light yellowish growth was observed under the RCF membrane (the interphase between the PC membrane and cellulose filter). The RC filters from the control treatment were also analyzed for DNA and fatty acids similar to the other RCF treatments.

Cellulose–Congo Red Agar Medium

Cellulose–Congo red agar medium has cellulose as the major C source, gelatin, and Noble agar as solidifying agents (Hendricks et al., 1995). It consists of 0.25 g $MgCl_2$, 0.5 g of K_2HPO_4, 1.88 g of acid-washed cellulose powder, 2 g of gelatin, 0.2 g of Congo red, 5.0 g of Noble agar, 100 mL of soil extract, and 900 mL of tap water. The medium was autoclaved for 20 min at 121°C. The plates were inoculated with 100 μL of 10^{-3} dilution solution and incubated for 20 d at 28°C.

Collection of Microorganisms and DNA Extraction

The microorganisms from the cellulose filters were collected by the method adapted from Ferrari et al. (2005) with some modifications. Briefly, the cellulose filters were carefully removed from the petri plates and cut into small pieces using sterile scissors. The pieces were placed in sterile, 15-mL centrifuge tubes and 1.5 mL of sterile physiological saline was added. To dislodge microorganisms from the filters, the samples were vortexed vigorously for 2 min. The suspension was transferred to microcentrifuge tubes and centrifuged for 5 min at 15,000 × *g* to pelletize the microorganisms. The extraction and pelletizing process was repeated once again using 1 mL of sterile solution. The DNA was extracted from the pellet using the Ultraclean DNA isolation kit (12224-50, MO BIO Laboratories, Carlsbad, CA) as recommended by the supplier.

The collection of microbial colonies from the traditional cellulose agar plates was done using a plate wash technique (Stevenson et al., 2004). The bacteria from the aggregate of colonies was obtained by flooding the surface of the CCRA medium with 2 mL of sterile physiological saline solution and dislodging the colonies using a sterile glass spreader to get as many colonies as possible. The suspension was collected in microcentrifuge tubes and centrifuged for 5 min at 15,000 × *g* to pelletize the microorganisms. The DNA was extracted from the pellet using the Ultraclean DNA isolation kit as per the manufacturer's protocol. The extracted DNA was divided into multiple tubes and stored at −80°C until use for clone library preparation.

Preparation and Analysis of 16s rRNA Clone Libraries

To obtain 16S rRNA gene products for cloning, the bulk microbial DNA recovered from the different treatments was amplified by 15-cycle polymerase chain reaction (PCR) using the 27F (5′-GAGTTTGATCMTGGC TCAG-3′) and 1492R (3′-GGTTACCTTGTTACGACTT-5′) primers. Cloning of target genes was done using the PCR2.1 vector and TOPO TA cloning kit (K4500-01, Invitrogen, Carlsbad, CA) from as per the supplier's protocol. Clones from each treatment were randomly picked and placed in separate 96-well plates, i.e., one clone library was made from sequences obtained from two replications of each treatment. The clone libraries were then stored in freezing medium (LB agar with 10% glycerol added and 25 g/L ampicillin and 12.5 g/L kanamycin) and sent to the synthesis facility of the USDA-ARS, Stoneville, MS, for sequencing. Before statistical analysis, the sequences were edited using CodonCode Aligner software (CodeonCode Corp., Dedham, MA) and were checked for chimera using the Mallard and Pintail programs (Ashelford et al., 2006, 2005). The chimera-free sequences were aligned using green genes (DeSantis et al., 2006) and analyzed by the LIBSHUFF and DOTUR software. The LIBSHUFF program (D. Singleton, Univ. of North Carolina, Chapel Hill, whitman.myweb.uga.edu/libshuff.html [verified 13 Jan. 2012]) was used to determine whether two clone libraries were significantly different, whereas assigning the sequences into different operational taxonomic units (OTUs; evolutionary distance $D = 0.03$) was done using DOTUR (Schloss and Handelsman, 2005a). An average neighbor algorithm was used in DOTUR to construct randomized rarefaction and collector's curves of the observed OTUs, diversity indices, and richness estimators (Schloss and Handelsman, 2005b). The taxonomic assignment for the clones was done using the online tools at the Ribosomal Data Project (rdp.cme.msu.edu; verified 13 Jan. 2012) and at GenBank (www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide [verified 21 Jan. 2012]; GenBank accession no. JF489234–JF489571).

Fatty Acid Methyl Ester Analysis

The total microbial community structure was assessed using fatty acid methyl ester (FAME) analysis (Sasser, 1990; Williams et al., 2010). Briefly, at the end of incubation period, the filter papers were cut into small pieces and placed in 20-mL glass tubes with polytetrafluoroethylene caps. The FAME analysis was done in four steps: (i) saponification: 1.25 mL of solution containing 3.75 mol/L NaOH in aqueous methanol was added to the tube, vortexed, and heated to 100°C for 30 min for lysing the microbial cells and saponifying the fatty acids; (ii) methylation: FAMEs were formed by adding 2 mL of HCl and methanol and heating to 80°C; (iii) extraction: the FAMEs were extracted by

adding 1.25 mL of methyl tertiary butyl ether (MTBE) and hexane (1:1) solution; and (iv) base wash: the lower aqueous phase was discarded and the leftover MTBE and hexane solution was washed with 3 mL of 0.3 mol/L NaOH solution. The upper organic phase was collected in separate tubes and dried under ultra-high-purity N_2. The FAMEs were redissolved in 110 μL of hexane and analyzed using MIDI Sherlock gas chromatograph (MIDI Inc., Newark, DE). Fatty acid methyl esters were separated and detected by an Agilent 6890 Series gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector and an Ultra-2 column (19091B-102, 0.2 mm by 25 m) and controlled by a computer loaded with ChemStation and Sherlock software. The carrier gas included ultra-high-purity H_2 at a column head pressure of 20 kPa, septum purge of 5 mL/min, a split ratio of 40:1, injection temperature of 300°C, injection volume of 2 μL, and a column temperature that ramped from 170 to 288°C at 28°C/min. Peak identification was performed by the Sherlock microbial identification system (MIDI Inc.) following calibration with a standard mixture of 17 FAMEs (1300A calibration mix). The upper organic phase was transferred to a test tube and evaporated under 99.999% ultra-high-purity N_2 gas. Standard nomenclature is used to describe the fatty acids. Microbial biomass C was calculated based on the total amount of FAMEs extracted from each treatment (Haack et al., 1994).

Statistical Analysis

The mole percentage of the 47 dominant FAMEs and the relative abundance of the 38 most common OTUs were separately analyzed using PCord software (MJM Software, Gleneden Beach, OR). Nonmetric multidimensional scaling (NMS), a nonparametric method, was used to provide graphical ordination of FAMEs and OTUs. The fatty acids i15:0, a15:0, 15:0, i16:0, 16:0, 16:1ω7, i17:0, a17:0, cy17:0, 18:1ω7, and 18:0 were chosen as bacterial fatty acid biomarkers, and 18:2ω6 was used as the fungal biomarker. The fungal/bacterial ratio was represented by the ratio of 18:2ω6 to bacterial phospholipid fatty acids. The relative abundance of bacterial and fungal fatty acids was expressed as a percentage of the total FAMEs.

RESULTS

Phylogenetic Assignment of Sequences

Bacterial communities were characterized using 16S rRNA gene analysis. The negative control treatment, $Uninoc_{RCF}$, as expected, showed the lowest degree of richness and diversity, with ~60% of the clones most closely related to *Cellvibrio fulvus* (EF692635.1). *Rhizobium* spp. made up the remainder of the identified sequences (data not shown). Because the bacterial communities that grew on the $Uninoc_{RCF}$ were very simple in structure and clearly different from those of the inoculated systems, the focus of the data reported here is on the four inoculated systems.

Of the total 384 clones sequenced in the four inoculated treatments, 341 sequences remained following chimera check. The $Biotic_{RCF}$ and CCRA treatments were dominated by bacteria belonging to the phyla *Proteobacteria* and *Bacteroidetes*, whereas members of *Firmicutes* were predominant in Abiotic-HN_{RCF} and Abiotic-LN_{RCF} (Table 2). However, representatives of eight different phyla, including members of rarely cultivated groups like *Planctomycetes*, *Acidobacteria*, and *Verrucomicrobia*, uncultured bacterial division OP10, and unclassified bacteria were detected in the RCF treatments. All clones from the CCRA treatment were distributed among three phyla, the *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Table 2). Interestingly, members of the rarely cultivable group *Planctomycetes* occupied up to 13% of the total clones in the Abiotic-HN_{RCF} treatment. Nonetheless, the differences among the treatments were more pronounced at finer levels of taxonomic resolution. For instance, the members of *Proteobacteria* in the $Biotic_{RCF}$ treatment were relatively evenly distributed into nine different orders, whereas such even distribution into a wide range of taxonomic groups was not obvious in the other treatments (Table 2).

LIBSHUFF Analysis of 16S rRNA Gene Sequences

The community composition of the bacteria growing on RCFs was significantly different from that of traditional plates (using the LIBSHUFF program). A significant difference was also noticed between the bacterial communities growing on the $Biotic_{RCF}$ treatment and communities grown on the Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments; however, the difference between bacterial communities growing on the Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments was insignificant.

Diversity Indices and Community Composition

To assess bacterial diversity, clones were binned into OTUs with <0.03 evolutionary distance (Table 3). The number of OTUs obtained was greater in the SDS than the CCRA treatments. For instance, in the $Biotic_{RCF}$ treatment, a total of 77 sequences were placed into 41 OTUs, whereas in the CCRA treatment, 91 sequences were distributed into 14 OTUs. The diversity indices also showed that the bacterial community richness and evenness were considerably greater in the RCF than the CCRA media. Among the three inoculated RCF treatments, the Simpson reciprocal and Shannon index were greatest in the $Biotic_{RCF}$ treatment, followed by Abiotic-HN_{RCF} and Abiotic-LN_{RCF}. It is also clear that evenness was considerably higher in the $Biotic_{RCF}$ than the other treatments. The rarefaction analysis supported the findings of the indices (data not shown). The five most abundant OTUs accounted for 72% of the clones in the CCRA treatment, whereas they accounted for only 2% in $Biotic_{RCF}$ (Table 4). Although there was some overlap in the 95% confidence interval of the Chao1 estimator among SDS treatments, no overlap was observed between CCRA and the SDS treatments.

The compositional and structural distribution of the bacterial community within the treatments reflected the results of LIBSHUFF and the diversity indices. The community composition of the $Biotic_{RCF}$ treatment was very different from that of the abiotic treatments. Moreover, the Abiotic-HN_{RCF} and Abiotic-

Table 2. Phylogenetic distribution of sequences among different phyla.

Phylum	Class	Order	Distribution†			
			Biotic$_{RCF}$	Abiotic-HN$_{RCF}$	Abiotic-LN$_{RCF}$	CCRA
Acidobacteria			1	1	0	0
	Acidobacteria		1	1	0	0
		Acidobacteriales	1	1	0	0
Verrucomicrobia			3	1	1	0
	Verrucomicrobiae		3	1	1	0
		Verrucomicrobiales	3	1	1	0
Proteobacteria			43	18	26	82
	Deltaproteobacteria		4	1	1	0
		Myxococcales	4	1	1	0
	Gammaproteobacteria		18	2	0	23
		Altermonadales	8	0	0	0
		Legionellales	2	1	0	0
		Xanthomonadales	4	1	0	4
		Pseudomonadales	0	0	0	19
		unclassified	4	0	0	0
	Betaproteobacteria		3	9	10	57
		Burkholderiales	3	8	10	57
		unclassified	0	1	0	0
	Alphaproteobacteria		18	6	13	2
		Sphingimonadales	6	1	2	1
		Caulobacterales	2	1	1	0
		Rhizobiales	7	3	6	1
		Rickettsiales	0	0	2	0
		unclassified	3	1	2	0
	unclassified		0	0	2	0
Firmicutes			1	54	39	2
	Bacilli		1	54	39	2
		Bacillales	1	54	39	2
Planctomycetes			2	12	7	0
	Planctomycetacia		2	12	7	0
		Planctomycetales	2	12	7	0
Bacteroidetes			23	3	10	7
	Flavobacteria		0	0	0	1
		Flavobacteriales	0	0	0	1
	Sphingobacteria		22	3	10	6
		Sphingobacteriales	22	3	10	6
	unclassified		1	0	0	0
OP10			2	0	0	0
unclassified bacteria			2	0	1	0

† The distribution of sequences (based on 16s rRNA gene analysis) in different treatments when matched to the Ribisomal Database Project database. Each number indicates the number of clones in the respective treatment matched to the respective phylum, class, or order. Treatments: Biotic$_{RCF}$, inoculated regenerated cellulose filter (RCF) on unsterilized soil; Abiotic-HN$_{RCF}$, inoculated RCF on sterilized soil; Abiotic-LN$_{RCF}$, inoculated RCF without soil but amended with sterilized soil extract; Uninoc$_{RCF}$, uninoculated RCF on unsterilized soil; CCRA, cellulose–Congo red agar medium.

Table 3. Diversity indices calculated based on operational taxonomic units (OTUs) formed using DOTUR at an evolutionary distance of <0.03 for the following treatments: inoculated regenerated cellulose filter (RCF) on unsterilized soil (Biotic$_{RCF}$), inoculated RCF on sterilized soil (Abiotic-HN$_{RCF}$), inoculated RCF without soil but amended with sterilized soil extract (Abiotic-LN$_{RCF}$), uninoculated RCF on unsterilized soil (Uninoc$_{RCF}$), and cellulose–Congo red agar (CCRA) medium.

Diversity index	Biotic$_{RCF}$	Abiotic-HN$_{RCF}$	Abiotic-LN$_{RCF}$	CCRA
Clones, no.	77	89	84	91
OTUs, no.	44	41	39	14
Simpson (1/*D*)†	33.63	9.25	10.53	5.03
Shannon (*H*)	3.49	3.01	3.02	1.94
Evenness (H/H_{max})	0.92	0.81	0.82	0.73
Chao 1	102.12	113.5	82.63	16.5
95% CI	66.65–193.12	67.93–236.21	55.41–156.26	14.36–30.98

† Simpson (1/*D*), Simpson Reciprocal Index.

LN$_{RCF}$ treatments were structurally similar (Table 4; Fig. 2A). In this regard, clones most closely related to *Bacillus megaterium* were strongly dominant members of both the Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments. This dominance played a huge role in the patterns that developed in the NMS analysis (Fig. 2A). In contrast, such strong domination was not found in the Biotic$_{RCF}$ treatment (Table 4). Indeed, the distribution of taxa across a wide range of taxonomic groups explains the high evenness in the Biotic$_{RCF}$ treatment.

Growth of Previously Uncultured Bacteria

The bacterial community composition of the treatments was unevenly distributed among different phyla (Table 4). A search for similar sequences using the Ribosomal Data Project revealed that the majority of clones were closely related to environmental DNA instead of bacterial isolates. Hence, the taxa we have grown are most closely related to previously uncultivated bacterial taxa. This was particularly true with the Biotic$_{RCF}$ treatment, where the majority of taxa showed low (90–96%) sequence homology to isolated bacterial strains. The Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments shared great similarity in community composition. More than 80% of their sequences were shared among 10 OTUs and were dominated by clones closely resembling *Bacillus megaterium*.

Fatty Acid Methyl Ester Analysis

The FAME profiles of the microbial communities growing on the four main treatments indicated that the largest differences were between the SDS treatments and the CCRA plates. The Abiotic-HN$_{RCF}$ treatment had the greatest abundance of FAMEs followed by the Biotic$_{RCF}$, CCRA, and Abiotic-LN$_{RCF}$ treatments (Table 5). The relative abundance of fungal fatty acids was significantly greater in the Biotic$_{RCF}$ and Abiotic-HN$_{RCF}$ treatments than the Abiotic-LN$_{RCF}$

Table 4. Distribution and phylogenetic affiliation of the most abundant operational taxonomic units (OTUs) in Genbank for the inoculated regenerated cellulose filter (RCF) on unsterilized soil (Biotic$_{RCF}$), inoculated RCF on autoclaved soil (Abiotic-HN$_{RCF}$), inoculated RCF without soil but amended with sterilized soil extracts (Abiotic-LN$_{RCF}$), and sterilized cellulose–Congo red agar (CCRA) treatments.

Sequences per OTUs	Distribution				Phylum	Closest match†	Closest cultured organism‡
	Biotic$_{RCF}$	Abiotic-HN$_{RCF}$	Abiotic-LN$_{RCF}$	CCRA			
52		28	24		*Firmicutes*	*Bacillus megaterium*; AceR-2; FJ605385 (99.2)§	*Bacillus megaterium*; AceR-2; FJ605385 (99.2)
42		4	5	33	*Proteobacteria*	*Achromobacter insolitus* (T); LMG 6003; AY170847(99)	*Achromobacter insolitus* (T); LMG 6003; AY170847(99)
20	1	1	2	16	*Proteobacteria*	*Ralstonia* sp. MCT1; DQ232889 (98.7)	*Ralstonia* sp. MCT1; DQ232889 (98.7)
17				17	*Proteobacteria*	*Pseudomonas* sp. MG1; AF326378 (99.3)	*Pseudomonas* sp. MG1; AF326378 (99.3)
14		5	9		*Firmicutes*	*Bacillus megaterium*; PC IW 13; AM992177 (99.9)	*Bacillus megaterium*; PC IW 13; AM992177 (99.9)
10	2	7	1		*Planctomycetes*	uncultured bacterium; OTUc55; EU834799 (98.5)	*Gemmata*-like str. CJuql4; AF239693 (90.3)
8	8				*Proteobacteria*	uncultured bacterium; nbw778f06c1; GQ009721(97.3)	*Saccharophagus degradans* 2–40; CP000282 (90.9)
7	5	2			*Bacteroidetes*	*Chitinophaga soli*; Gsoil 219; AB267723 (95.7)	*Chitinophaga soli*; Gsoil 219; AB267723 (95.7)
7	7				*Bacteroidetes*	uncultured bacterium; 28RHF48; AJ863367(96.8)	*Terrimonas lutea* (T); DY; AB192292 (95.2)
6		5	1		*Firmicutes*	*Paenibacillus pocheonensis*; Gsoil 1138; AB245386(99.5)	*Paenibacillus pocheonensis*; Gsoil 1138; AB245386(99.5)
6		2	1	3	*Proteobacteria*	*Cupriavidus* sp. A2; EU363682(99.2)	*Cupriavidus* sp. A2; EU363682(99.2)
6	6				*Bacteroidetes*	uncultured bacterium; WC2_183; GQ263931(99.3)	*Niastella* sp. Gsoil 221; GQ339899 (96.5)
6				6	*Bacteroidetes*	uncultured bacterium; FW1_a34; GQ263287(98.2)	Bacteroidetes bacterium CK32 5.3; FJ688408(98.2)
5	1			4	*Proteobacteria*	uncultured bacterium; S1–3-CL17; AY725259(99)	*Pseudoxanthomonas* sp. RN402; FJ032195(98.9)
4		1	2	1	*Proteobacteria*	*Rhizobium* sp. Cg-A3; AB456621(99.3)	*Rhizobium* sp. Cg-A3; AB456621(99.3)
4		2	2		*Firmicutes*	*Bacillus megaterium*; ZFJ-14; EU931553 (99.4)	*Bacillus megaterium*; ZFJ-14; EU931553 (99.4)
4			4		*Bacteroidetes*	*Chitinophaga* sp. 37C1; GQ281771(96.5)	*Chitinophaga* sp. 37C1; GQ281771(96.5)
3		3			*Firmicutes*	*Bacillus muralis*; REG126; GQ844961(99.4)	*Bacillus muralis*; REG126; GQ844961(99.4)
3		2	1		*Planctomycetes*	uncultured bacterium; p26m12ok; FJ478560 (98.5)	*Planctomyces* sp.; Schlesner 658; X81954(94.8)
3		1		2	*Firmicutes*	*Bacillus* sp. P05; AY822613(98.9)	*Bacillus* sp. P05; AY822613(98.9)
3	2		1		*Proteobacteria*	*Caulobacter* sp.; FWC33; AJ227772(97.6)	*Caulobacter* sp.; FWC33; AJ227772(97.6)
3	3				*Proteobacteria*	uncultured bacterium; UWL_CL-080514_OTU-34; EU809244(98.6)	Rhodospirillaceae bacterium KNA-P; AB539973(98.9)
3			2	1	*Proteobacteria*	*Cupriavidus campinensis*; LMG 20576; AY040355(99.7)	*Cupriavidus campinensis*; LMG 20576; AY040355(99.7)
2		2			*Planctomycetes*	uncultured bacterium; 1–2D; EU289425(92.6)	*Nostocoida limicola* III; Ben223; AF244750(92.4)
2	1	1			*Proteobacteria*	*Nordella oligomobiliz*; N21; AF370880(98.6)	*Nordella oligomobiliz*; N21; AF370880(98.6)
2	1	1			*Firmicutes*	*Bacillus thuringiensis*; DQ286358(99.5)	*Bacillus thuringiensis*; DQ286358 (99.5)
2	2				*Verrucomicrobia*	uncultured Opitutales bacterium; B15-Capima; AB479055(97.3)	Opitutaceae bacterium TAV1; AY587231(94.1)
2	2				*Proteobacteria*	*Legionella quinlivanii*; sreogroup 2, nctc 12433; Z49733(98.3)	*Legionella quinlivanii*; sreogroup 2, nctc 12433; Z49733(98.3)
2	2				*unclassified bacteria*	uncultured bacterium; FFCH4309; EU134282 (98.3)	*Vampirovibrio chlorellavorus*; ICPB 3707; HM038000(93.4)
2	2				*Proteobacteria*	*Pseudoxanthomonas* sp. D7–5; AM403203(99.5)	*Pseudoxanthomonas* sp. D7–5; AM403203(99.5)
2	2				*Proteobacteria*	*Sphingomonas mali* (T); IFO 10550-T; Y09638(98)	*Sphingomonas mali* (T); IFO 10550-T; Y09638(98)
2			2		*Bacteroidetes*	*Sphingoterrabacterium composti*; TR6–03; AB267719(97.6)	*Sphingoterrabacterium composti*; TR6–03; AB267719(97.6)
2			2		*Proteobacteria*	*Rickettsia conorii* str. Malish 7; AE008647(81.5)	*Rickettsia conorii* str. Malish 7; AE008647(81.5)
2			2		*Proteobacteria*	uncultured bacterium; c5LKS72; AM086142(94)	bacterial symbiont of *Diophrys* sp.; AJ630204(86.3)
2			1	1	*Proteobacteria*	*Sphingomonas* sp. MN57.2a; AM159534(98.8)	*Sphingomonas* sp. MN57.2a; AM159534(98.8)
2				2	*Proteobacteria*	*Pseudomonas* sp. LAB-18; AB051696 (100)	*Pseudomonas* sp. LAB-18; AB051696 (100)
2				2	*Proteobacteria*	*Ralstonia* sp. MCT1; DQ232889 (98.9)	*Ralstonia* sp. MCT1; DQ232889 (98.9)

† The closest match identified among environmental and cultured organisms in Genbank, with their corresponding accession number.

‡ The closest match identified among the cultured bacteria in GenBank, with their corresponding accession number.

§ Maximum identity of sequences in our experiment to their closest match in GenBank in parentheses.

and CCRA treatments. Overall, the amount of fatty acid, used as an index of microbial biomass, was positively related to the amount of available nutrients in the respective media (Table 6).

The clustering of Biotic$_{RCF}$ and Abiotic-HN$_{RCF}$ treatments in the NMS plot indicated that although the total microbial biomass was different, the community structure tended to be very similar. Individual fatty acids 18:2ω6,9 and 18:1ω9c were highly correlated with the separation of the Biotic$_{RCF}$ and Abiotic-HN$_{RCF}$ treatments on Axis 1 (Fig. 2; Table 5; $r > 0.75$). The FAME analysis of the Uninoc$_{RCF}$ treatment also showed the presence of fatty acids, of which 60% were short-chain fatty acids. The remaining 40% were dominated by 16:0, 16:1ω7c, and 18:1ω7c.

DISCUSSION

The fastidious nature of soil microbes and their close ties to the biotic conditions of their native habitat may explain past difficulties in growing the majority of bacteria and why there has been recent success in the growth of previously uncultivated bacteria when in situ conditions have been mimicked (Bollmann et al., 2007; Ferrari et al., 2008; Kaeberlein et al., 2002). Intra- and interspecific interactions among microbial populations influence microbial growth, which in turn can have direct consequence for microbial cultivation. The present study was conducted to test the importance of the soil habitat and its co-occurring microbial community on the composition and structure of a newly developing soil bacterial community. To do this, an SDS was developed to enrich microorganisms growing in association with both living, unsterilized and nonliving, autoclave-sterilized soil (Fig. 1). For each of the four SDS treatments, inoculated RCFs were placed in close association with (i) unsterilized soil (Biotic$_{RCF}$), (ii) sterilized soil (Abiotic-HN$_{RCF}$), (iii) sterilized soil extract amendment but no soil (Abiotic-LN$_{RCF}$), and (iv) uninoculated RCF coupled to unsterilized soil (Uninoc$_{RCF}$), which is an SDS control for contamination. Sterilized traditional cellulose media plates (CCRA) were used as a traditional plating method that utilized an abiotic approach to cultivate bacteria. The Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments differed based on the pool of available nutrients, such as N, that can support microbial growth, and thus represent high- and low-nutrient treatments, respectively. The three abiotic treatments provided different ways to test the effect of the abiotic environment and how strongly it contrasted with the community that developed when grown in association with the living biotic soil community. Except for the low- and high-nutrient treatments, the range of conditions provided by both biotic and abiotic treatments resulted in the selection of different bacterial communities.

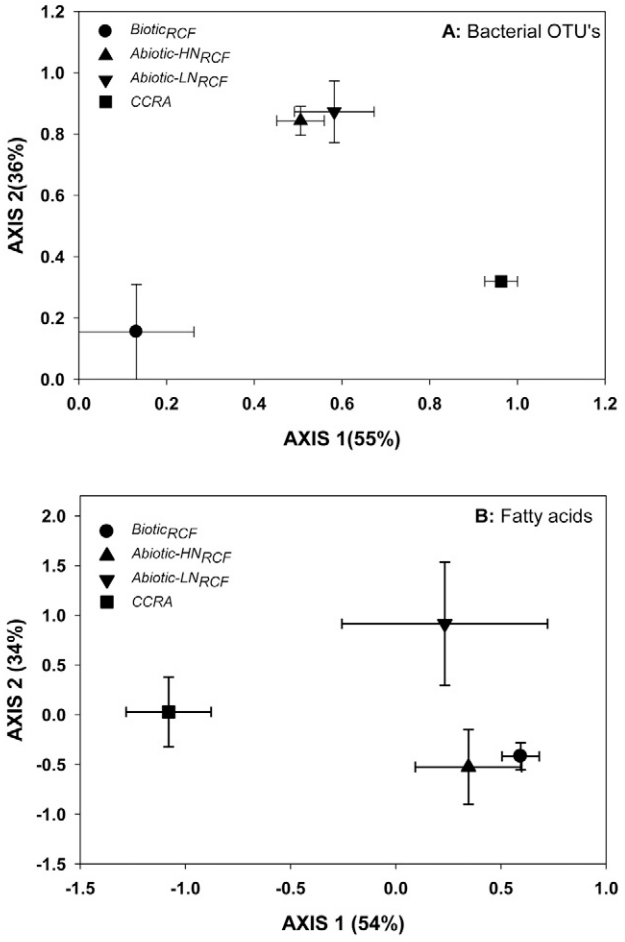

Fig. 2. Differences in the structure of the microbial community associated with the different treatments using nonmetric multidimensional scaling based on (A) bacterial operational taxonomic units (OTUs; *D* = 0.03) and (B) mole percentage of fatty acid methyl esters (FAMEs) for the inoculated regenerated cellulose filter (RCF) on unsterilized soil (Biotic$_{RCF}$), inoculated RCF on autoclaved soil (Abiotic-HN$_{RCF}$), inoculated RCF without soil but amended with sterilized soil extracts (Abiotic-LN$_{RCF}$), and sterilized cellulose–Congo red agar (CCRA) treatments. Percentages denote the amount of variability associated with each axis. The standard errors of the treatments are noted for each symbol.

Effect of Microbe-Rich Habitat on Bacterial Growth

The development of the SDS was inspired by the idea of growing microbes in a simulated natural environment. Simulating the native habitat (Kaeberlein et al., 2002) and using soil as the substrate (Ferrari et al., 2005; Svenning et al., 2003) has been shown to be important for growing previously uncultivated microorganisms; however, this approach, as far as we know, has not been used to differentiate biotic and abiotic effects of the native soil habitat on bacterial growth. Considering the enormous heterogeneity in chemical and physical features that describe soil habitats at microbial scales, and the enormous possibilities for biological interaction, it was expected that some microorganisms that are fastidiously dependent on their environment for survival and growth would benefit from growth in close association with their native habitat. Even though it was hard to specify the exact mechanism of influence, the bacterial community grown on Biotic$_{RCF}$ was clearly very different from those found in the abiotic treatments.

The presence of the living soil microbial community, although separated by ~100 μm from the inoculum, strongly impacted the bacterial community composition, richness, and diversity on cellulose filters. These results support the hypothesis that the biotic soil community provides important conditions that aid in the growth of a larger array of bacterial types. Our

Table 5. Abundance of fatty acid methyl esters (FAMEs) from the inoculated regenerated cellulose filter (RCF) on unsterilized soil ($Biotic_{RCF}$), inoculated RCF on autoclaved soil (Abiotic-HN_{RCF}), inoculated RCF without soil but amended with sterilized soil extracts (Abiotic-LN_{RCF}), uninoculated RCF on unsterilized soil ($Uninoc_{RCF}$), and sterilized cellulose–Congo red agar (CCRA) treatments.

Property†	$Biotic_{RCF}$	Abiotic-HN_{RCF}	Abiotic-LN_{RCF}	$Uninoc_{RCF}$	CCRA
Total FAMEs, nmol	65.9 (5.3)‡	131 (3.2)	48.3(1.4)	16.5(0.17)	70.90 (14.1)
Bacterial FAMEs, mol%	30.7(1.9)	32.2 (1.8)	23.5 (3.9)	38.2 (2.4)	30.41 (4.5)
Fungal FAMEs, mol%	12.8(3.4)	22.9 (7.5)	1.50 (0.59)	0	0.098 (0.01)
Fungal/bacterial ratio	0.41(0.09)	0.72 (0.27)	0.06 (0.01)	0	0.002 (0.0001)

† Total FAMEs were calculated using 16:0 as the abundance standard. Bacterial and fungal FAMEs were expressed in mol% of the total FAMEs. The fungal/bacterial ratio is the mol% ratio of 18:2ω6 to total bacterial fatty acids.

‡ Means and standard errors in parentheses.

results are on a par with other studies that have reported greater diversity and richness of microbial types when cultured in a simulated natural environment (Bollmann et al., 2007; Ferrari et al., 2005; Kaeberlein et al., 2002) and support the concept of the SDS as a tool to enrich and potentially cultivate unique microbes and microbial communities.

The results presented here are also similar to other simulated natural environment studies by having a large cadre of uncultured members within *Proteobacteria* and *Bacteroidetes*. In contrast, there was a different impact of growth conditions on the fatty acid composition of the microbial communities among the treatments. The bulk of the differences can be attributed to fungal growth on the plates. Moreover, the growth of fungi was much greater in the Abiotic-HN_{RCF} than the Abiotic-LN_{RCF} treatment, and yet there were similar effects of both nutrient treatments on bacterial community composition. Spatial separation on the plates may have played a role in allowing the fungi to grow without showing any obvious influence on the bacterial community composition.

The precise role that biotic activity played on the selection of the bacterial community in the $Biotic_{RCF}$ treatment is not known; however, the presence of autoinducer molecules in the growing media might have impacted the growth of a diverse bacterial community on the RCFs (Bruns et al., 2003; Kaeberlein et al., 2002; Williams et al., 2007). For instance, in soil, acyl homoserine lactones (AHL) were detected in 24% of isolates recovered from soil bacterial communities, suggesting that a number of organisms in soil utilize AHL for communication (DeAngelis et al., 2008). Nevertheless, microbial interactions appear to be occurring across relatively long distances and thus have relevance for the debate about the importance of physical and spatial isolation of microbial populations in soil (Carson et al., 2010; Zhou et al., 2002).

The conditions in the $Biotic_{RCF}$ treatment favored the growth of members of bacterial phyla that have only a few cultured representatives. At 97% evolutionary distance, approximately 40% of the bacterial taxa from the $Biotic_{RCF}$ treatment and 91% from the CCRA treatment were most similar to previously cultured bacterial isolates documented in Genbank. As such, the majority of taxonomic units in the $Biotic_{RCF}$ treatment were related to uncultivated environmental sequences. This result is notable for the high degree of novelty in the growth of rarely cultivable bacteria associated with a simulated biotic environment but also from the standpoint that rather common cultivation media such as CCRA may not have been fully probed for their diversity of bacterial types. For instance, Joseph et al. (2003) reported success in culturing 350 bacterial isolates on simple solid media, out of which 27% of isolates belonged to yet-unnamed families.

The rich and even distribution of bacterial taxa in the $Biotic_{RCF}$ treatment is in contrast with traditional culturing media, which tends to favor the growth of few dominant species. Indeed, the degree of diversity associated with the $Biotic_{RCF}$ treatment is on a par with estimates of diversity found in soil environments, perhaps suggesting that there was little competition among populations (Zhou et al., 2002). Members of hard-to-culture phyla like *Verrucomicrobia* and OP10 were fairly well represented in $Biotic_{RCF}$. It has been speculated that the cultivation of *Verrucomicrobia* may be enhanced when the abundance of other microbes in the culture are low (Sangwan

Table 6. Microbial demand and supply for N among the inoculated regenerated cellulose filter (RCF) on unsterilized soil ($Biotic_{RCF}$), inoculated RCF on autoclaved soil (Abiotic-HN_{RCF}), inoculated RCF without soil but amended with sterilized soil extracts (Abiotic-LN_{RCF}), and sterilized cellulose–Congo red agar (CCRA) treatments.

Treatment	Microbial C†	Microbial N demand‡	Estimated N supply in cultures§	N index¶
		μg		
$Biotic_{RCF}$	370.3(33.77)#	61.72(5.62)	12.79 (0.26)	0.21
Abiotic-HN_{RCF}	744.06(24.54)	124.01(4.09)	141.67 (2.92)	1.14
Abiotic-LN_{RCF}	268.81(15.78)	44.80 (2.63)	4.51 (0.16)	0.10
CCRA	360.41(33.35)	60.07 (5.55)	30.06 (0.01)	0.50

† Calculated based on total fatty acids

‡ Calculated based on a microbial C/N ratio of 6:1 and used as an indicator of total nutrient demand.

§ N available in each cultivation treatment. The N supply for $Biotic_{RCF}$ and Abiotic-HN_{RCF} calculated based on water-soluble soil N; N available in Abiotic-LN_{RCF} and CCRA based on added N amendments. Ninhydrin analysis was used to estimate N pools.

¶ Ratio of N supply to microbial N demand.

Means and standard errors in parentheses.

et al., 2005). Other approaches to cultivation of previously uncultured biota from soil, however, such as diluting the nutrient content of growth media and especially the extension of incubation times, have also successfully grown bacteria considered cultivation resistant, such as *Acidobacteria*, *Actinobacteria*, and *Verrucomicrobia* (Janssen, 2003; Janssen et al., 2002). In this regard, the absence of any dominant bacterial group associated with the $Biotic_{RCF}$ treatment would have lowered the likelihood of a strong antagonist taking over the culture and thus increase the potential for the growth of many other bacterial taxa.

It has been observed that *Verrucomicrobia* have been found in a wide array of environments and that their activity and abundance in nature may be partially linked to the available water resources (Buckley and Schmidt, 2001; Tarlera et al., 2008). The abundance of *Verrucomicrobia* in soil can range spectacularly, from 0 to 21% of the division-level census, so it is clear that these organisms are sensitive to environmental and habitat conditions. From these studies, it is difficult to speculate on the conditions that allow growth of *Verrucomicrobia* in culture. The proximity of other microorganisms in the associated soil habitat next to the culture with *Verrucomicrobia*, however, may have allowed diffusion and exchange of important metabolites for their growth (Bollmann et al., 2007; Greene, 2002).

Selection of Bacteria in Abiotic Treatments with Different Nutrient Levels

In the case of the two $Abiotic_{RCF}$ treatments, it was expected that the nutrient concentrations would influence the composition of the bacterial community. Indeed, fatty acid abundance (index of microbial biomass) indicated that nutrients probably limited bacterial growth in the Abiotic-LN_{RCF} treatment. Even though the Abiotic-LN_{RCF} treatment received only a portion of the soil solution and its associated nutrients compared with what would be found in the Abiotic-HN_{RCF} treatment, the bacterial community members residing in both $Abiotic_{RCF}$ systems were very similar. This result suggests that, across the range utilized for these experiments, nutrient availability did not differentially influence the composition of the cultivated bacterial communities.

The most obvious resemblance between the high- and low-nutrient treatments comes from the observation that ~50% of clones showed high sequence similarity (> 99%) to *Bacillus megaterium*. *Bacillus megaterium* is a fairly well described bacterium with a large genome and wide industrial utilization (Vary et al., 2007). While it is not known whether the specific clones that we have identified have novel ecological roles or metabolic capacities compared with those strains previously isolated, there are numerous methods already available for the cultivation of *Bacillus megaterium*. Nevertheless, it is an intriguing outcome that *Bacillus megaterium* was able to dominate growth in the presence of RCF habitat in the abiotic treatments but that this growth was completely muted when its RCF habitat was associated with a soil containing an active microbial community ($Biotic_{RCF}$). Clearly, the existence of a microbial community impacts, across relatively long micrometer-scale distances, the direction of new microbial community development.

In spite of having greater dominance of *Bacillus megaterium* related clones, the high-nutrient medium (Abiotic-HN_{RCF}) was also represented by ~13% of clones most closely related to taxa in *Planctomycetes*. *Planctomycetes* are a group with sparse representation in culture that have an array of unusual traits that include the production of rare fatty acids and the lack of peptidoglycan in the cell wall (Wagner and Horn, 2006). *Planctomycetes* are typically rare but are widespread inhabitants in numerous soil and aquatic environments (Bauld and Staley, 1976; Stackebrandt et al., 1993). Recently, however, it has been shown that they often dominate the intestinal tracts of various animals, especially termites (Kohler et al., 2008). Termite hindguts are zones that receive periodic influxes of nutrients and cellulose-rich organic matter. This habitat may be mimicked by the Abiotic-HN_{RCF} habitat. It must be acknowledged, however, that the exact phylogenetic relationship between the clone groups in our work and those of Tholen and Brune (2000) are not known. Even though autoclaving the soil and sterilization removed the biotic activity from the $Abiotic_{RCF}$ treatments, there is the possibility that biologically active molecules remained behind and could have had impact on the developing community. The chemistry of the solutions in the two abiotic treatments may have been similar and contributed to the related response in the Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments; however, the nature of the effect, if present, was nevertheless very different from that of the $Biotic_{RCF}$ treatment.

Soil Diffusion System vs. Traditional Plating Medium

Bacterial communities cultivated on cellulose filters in the SDS treatments were obviously very different from those growing on traditional carboxycellulose agar medium (CCRA). The CCRA medium resulted predominantly in the growth of fairly well described members of the *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, many of which have been reported as cellulose-degrading bacteria (Danon et al., 2008; Lynd and Zhang, 2002; Schellenberger et al., 2010; Ulrich et al., 2008; Yang and Zhang, 2007). These results agreed well with other studies and further show that this traditional plating method is good for redundantly selecting certain bacterial groups (Böckelmann et al., 2000; Bollmann et al., 2007; Kopke et al., 2005).

From the standpoint of ubiquity, cellulose is a good source of C that dominates soil ecosystems and can thus be rationalized as a strong selective force in shaping the evolution of microbial catabolism across numerous taxonomic groups. It would thus be interesting to know how many of the bacteria in our experiments are capable of decomposing cellulose. In contrast to the obvious clearing zones that are indicative of cellulase activity when using CCRA, the observation of cellulose degradation using RCFs is less easily attributed to a single colony. It is also difficult to ascertain whether the cellulose decomposition is related to bacterial rather than fungal activity. When we tried to isolate and regrow the colonies from the RCFs (SDS treatments)

onto low-nutrient soil extract media, bacterial growth was noticed on approximately 70% of the plates (data not shown). When these bacteria were transferred to CCRA media, ~50% of them formed clearing zones, indicating the capability to degrade cellulose (Hendricks et al., 1995; Ulrich et al., 2008). The capacity to regrow many of the RCF-cultivated bacteria on CCRA also indicates that once these organisms are coaxed into culture, they may then be grown in isolation using standard methods and thus further characterized for their ecological relevance and metabolic functioning.

Impacts of Contamination on Bacterial Cultivation

During incubation, very small amounts of bacterial growth were observed on the control SDS treatment. It is thought that bacterial movement from the soil and onto the surface of the uninoculated RCF may have occurred via aerial transport or through the movement of mites around the 0.003-μm polycarbonate filter. Nevertheless, the impact of this influence was small and does not alter the major findings of the study. Indeed, the bacterial communities that grew were again very different from those of the other treatments, probably the result of selection of specific bacteria capable of transport. Hence, even with the potential for unintended bacterial inoculation, the cultivation systems selected for unique communities unlike the bacterial contaminants, which were most closely related to cellulolytic bacteria in the genera *Cellvibrio* and *Rhizobium*.

CONCLUSIONS

In conclusion, this study has shown that the coupling of an SDS to the biologically active soil supports the growth of a unique and diverse bacterial community that is very different from the abiotic SDS and traditional abiotic-based cultivation systems. It was notable, however, that the richness of the bacterial communities in the SDS treatments, regardless of abiotic or biotic treatment, were taxa-rich compared with the traditional CCRA medium. The huge variations we found in the microbial communities grown on different treatments suggests that microbial growth is sensitive to abiotic and biotic factors associated with the growth medium. Indeed, the impact of growing bacteria in association with a living soil environment speaks to the power of microbial interactions for shaping microbial communities, even across distances of ~100 μm. The impacts that microbial interactions have on the development of microbial community structure in soil need further investigation.

ACKNOWLEDGMENTS

Funds from the Department of Energy and National Science Foundation supported the research presented here.

REFERENCES

Aagot, N., O. Nybroe, P. Nielsen, and K. Johnsen. 2001. An altered *Pseudomonas* diversity is recovered from soil by using nutrient-poor *Pseudomonas*-selective soil extract media. Appl. Environ. Microbiol. 67:5233–5239. doi:10.1128/AEM.67.11.5233-5239.2001

Ashelford, K.E., N.A. Chuzhanova, J.C. Fry, A.J. Jones, and A. J. Weightman. 2005. At least one in twenty 16S rRNA sequence records currently held in public repositories estimated to contain substantial anomalies. Appl. Environ. Microbiol. 71:7724–7736. doi:10.1128/AEM.71.12.7724-7736.2005

Ashelford, K.E., N.A. Chuzhanova, J.C. Fry, A.J. Jones, and A.J. Weightman. 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl. Environ. Microbiol. 72:5734–5741. doi:10.1128/AEM.00556-06

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

Bauld, J., and J.T. Staley. 1976. *Planctomyces maris* sp. nov.: A marine isolate of the *Planctomyces–Blastocaulis* group of budding bacteria. J. Gen. Microbiol. 97:45–55.

Böckelmann, U., W. Manz, T.R. Neu, and U. Szewzyk. 2000. Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. FEMS Microbiol. Ecol. 33:157–170.

Bollmann, A., K. Lewis, and S.S. Epstein. 2007. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl. Environ. Microbiol. 73:6386–6390. doi:10.1128/AEM.01309-07

Bruns, A., H. Cypionka, and J. Overmann. 2002. Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl. Environ. Microbiol. 68:3978–3987. doi:10.1128/AEM.68.8.3978-3987.2002

Bruns, A., U. Nubel, H. Cypionka, and J. Overmann. 2003. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. 69:1980–1989. doi:10.1128/AEM.69.4.1980-1989.2003

Buckley, D.H., and T.M. Schmidt. 2001. Environmental factors influencing the distribution of rRNA from Verrucomicrobia in soil. FEMS Microbiol. Ecol. 35:105–112. doi:10.1111/j.1574-6941.2001.tb00793.x

Button, D., F. Schut, P. Quang, R. Martin, and B.R. Robertson. 1993. Viability and isolation of marine bacteria by dilution culture: Theory, procedures, and initial results. Appl. Environ. Microbiol. 59:881–891.

Carson, J.K., V. Gonzalez-Quinones, D.V. Murphy, C. Hinz, J.A. Shaw, and D.B. Gleeson. 2010. Low pore connectivity increases bacterial diversity in soil. Appl. Environ. Microbiol. 76:3936–3942. doi:10.1128/AEM.03085-09

Cole, J.R., Q. Wang, B. Chai, and J.M. Tiedje. 2011. The Ribosomal Database Project: Sequences and software for high-throughput rRNA analysis. p. 313–324. *In* F.J. de Bruijn (ed.) Handbook of molecular microbial ecology I: Metagenomics and complementary approaches. John Wiley & Sons, Hoboken, NJ.

Danon, M., I.H. Franke-Whittle, H. Insam, Y. Chen, and Y. Hadar. 2008. Molecular analysis of bacterial community succession during prolonged compost curing. FEMS Microbiol. Ecol. 65:133–144. doi:10.1111/j.1574-6941.2008.00506.x

Davis, K.E., S.J. Joseph, and P.H. Janssen. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. 71:826–834. doi:10.1128/AEM.71.2.826-834.2005

DeAngelis, K.M., S.E. Lindow, and M.K. Firestone. 2008. Bacterial quorum sensing and nitrogen cycling in rhizosphere soil. FEMS Microbiol. Ecol. 66:197–207. doi:10.1111/j.1574-6941.2008.00550.x

de Boer, W., L. Folman, R. Summerbell, and L. Boddy. 2005. Living in a fungal world: Impact of fungi on soil bacterial niche development. FEMS Microbiol. Rev. 29:795–811. doi:10.1016/j.femsre.2004.11.005

de Bruyn, J.C., F.C. Boogerd, P. Bos, and J.G. Kuenen. 1990. Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. Appl. Environ. Microbiol. 56:2891–2894.

DeSantis, T.Z., P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G.L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72:5069–5072. doi:10.1128/AEM.03006-05

Diggle, S.P., A.S. Griffin, G.S. Campbell, and S.A. West. 2007. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450:411–414. doi:10.1038/nature06279

D'Onofrio, A., J. Crawford, E. Stewart, K. Witt, E. Gavrish, S. Epstein, J. Clardy, and K. Lewis. 2010. Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem. Biol. 17:254–264. doi:10.1016/j.chembiol.2010.02.010

Eilers, H., J. Pernthaler, J. Peplies, F.O. Glockner, G. Gerdts, and R. Amann. 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. Appl. Environ. Microbiol. 67:5134–5142. doi:10.1128/AEM.67.11.5134-5142.2001

Ferrari, B.C., S.J. Binnerup, and M. Gillings. 2005. Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. Appl. Environ. Microbiol. 71:8714–8720. doi:10.1128/AEM.71.12.8714-8720.2005

Ferrari, B.C., T. Winsley, M. Gillings, and S. Binnerup. 2008. Cultivating previously uncultured soil bacteria using a soil substrate membrane system. Nat. Protoc. 3:1261–1269. doi:10.1038/nprot.2008.102

Greene, K. 2002. New method for culturing bacteria. Science 296:1000. doi:10.1126/science.296.5570.1000a

Haack, S.K., H. Garchow, D.A. Odelson, L.J. Forney, and M.J. Klug. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl. Environ. Microbiol. 60:2483–2493.

Hendricks, C.W., J.D. Doyle, and B. Hugley. 1995. A new solid medium for enumerating cellulose-utilizing bacteria in soil. Appl. Environ. Microbiol. 61:2016–2019.

Janssen, P.H. 2003. Selective enrichment and purification of cultures of *Methanosaeta* spp. J. Microbiol. Methods 52:239–244. doi:10.1016/S0167-7012(02)00181-1

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72:1719–1728. doi:10.1128/AEM.72.3.1719-1728.2006

Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl. Environ. Microbiol. 68:2391–2396. doi:10.1128/AEM.68.5.2391-2396.2002

Joseph, S.J., P. Hugenholtz, P. Sangwan, C.A. Osborne, and P.H. Janssen. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Environ. Microbiol. 69:7210–7215. doi:10.1128/AEM.69.12.7210-7215.2003

Kaeberlein, T., K. Lewis, and S.S. Epstein. 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129. doi:10.1126/science.1070633

Kato, S., S. Haruta, Z.J. Cui, M. Ishii, and Y. Igarashi. 2005. Stable coexistence of five bacterial strains as a cellulose-degrading community. Appl. Environ. Microbiol. 71:7099–7106. doi:10.1128/AEM.71.11.7099-7106.2005

Kohler, T., U. Stingl, K. Meuser, and A. Brune. 2008. Novel lineages of *Planctomycetes* densely colonize the alkaline gut of soil-feeding termites (*Cubitermes* spp.). Environ. Microbiol. 10:1260–1270. doi:10.1111/j.1462-2920.2007.01540.x

Kopke, B., R. Wilms, B. Engelen, H. Cypionka, and H. Sass. 2005. Microbial diversity in coastal subsurface sediments: A cultivation approach using various electron acceptors and substrate gradients. Appl. Environ. Microbiol. 71:7819–7830. doi:10.1128/AEM.71.12.7819-7830.2005

Lynd, L.R., P.J. Weimer, W.H. van Zyl, and I.S. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66:506–577. doi:10.1128/MMBR.66.3.506-577.2002

Lynd, L.R., and Y. Zhang. 2002. Quantitative determination of cellulase concentration as distinct from cell concentration in studies of microbial cellulose utilization: Analytical framework and methodological approach. Biotechnol. Bioeng. 77:467–475. doi:10.1002/bit.10142

Männistö, M.K., S. Rawat, V. Starovoytov, and M.M. Häggblom. 2011. *Terriglobus saanensis* sp. nov., an acidobacterium isolated from tundra soil. Int. J. Syst. Evol. Microbiol. 61:1823–1828. doi:10.1099/ijs.0.026005-0

Mullings, R., and J.H. Parish. 1984. Mesophilic aerobic Gram negative cellulose degrading bacteria from aquatic habitats and soils. J. Appl. Bacteriol. 57:455–468. doi:10.1111/j.1365-2672.1984.tb01411.x

Nichols, D., K. Lewis, J. Orjala, S. Mo, R. Ortenberg, P. O'Connor, C. Zhao, P. Vouros, T. Kaeberlein, and S.S. Epstein. 2008. Short peptide induces an "uncultivable" microorganism to grow in vitro. Appl. Environ. Microbiol. 74:4889–4897. doi:10.1128/AEM.00393-08

Rappé, M.S., S.A. Connon, K.L. Vergin, and S.J. Giovannoni. 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418:630–633.

Sangwan, P., S. Kovac, K.E. Davis, M. Sait, and P.H. Janssen. 2005. Detection and cultivation of soil verrucomicrobia. Appl. Environ. Microbiol. 71:8402–8410. doi:10.1128/AEM.71.12.8402-8410.2005

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids: Identification of bacteria by gas chromatography of cellular fatty acids. Microbial ID, Newark DE.

Schellenberger, S., S. Kolb, and H.L. Drake. 2010. Metabolic responses of novel cellulolytic and saccharolytic agricultural soil *Bacteria* to oxygen. Environ. Microbiol. 12:845–861. doi:10.1111/j.1462-2920.2009.02128.x

Schloss, P.D., and J. Handelsman. 2005a. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71:1501–1506. doi:10.1128/AEM.71.3.1501-1506.2005

Schloss, P.D., and J. Handelsman. 2005b. Metagenomics for studying unculturable microorganisms: Cutting the Gordian knot. Genome Biol. 6:229. doi:10.1186/gb-2005-6-8-229

Stackebrandt, E., W. Liesack, and B.M. Goebel. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J. 7:232–236.

Stevenson, B.S., S.A. Eichorst, J.T. Wertz, T.M. Schmidt, and J.A. Breznak. 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. 70:4748–4755. doi:10.1128/AEM.70.8.4748-4755.2004

Stott, M.B., M.A. Crowe, B.W. Mountain, A.V. Smirnova, S. Hou, M. Alam, and P.F. Dunfield. 2008. Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. Environ. Microbiol. 10:2030–2041. doi:10.1111/j.1462-2920.2008.01621.x

Svenning, M.M., I. Wartiainen, A.G. Hestnes, and S.J. Binnerup. 2003. Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. FEMS Microbiol. Ecol. 44:347–354. doi:10.1016/S0168-6496(03)00073-4

Tarlera, S., K. Jangid, A.H. Ivester, W.B. Whitman, and M.A. Williams. 2008. Microbial community succession and bacterial diversity in soils during 77,000 years of ecosystem development. FEMS Microbiol. Ecol. 64:129–140. doi:10.1111/j.1574-6941.2008.00444.x

Tholen, A., and A. Brune. 2000. Impact of oxygen on metabolic fluxes and in situ rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ. Microbiol. 2:436–449. doi:10.1046/j.1462-2920.2000.00127.x

Ulrich, A., G. Klimke, and S. Wirth. 2008. Diversity and activity of cellulose-decomposing bacteria, isolated from a sandy and a loamy soil after long-term manure application. Microb. Ecol. 55:512–522. doi:10.1007/s00248-007-9296-0

Ulrich, A., and S. Wirth. 1999. Phylogenetic diversity and population densities of culturable cellulolytic soil bacteria across an agricultural encatchment. Microb. Ecol. 37:238–247. doi:10.1007/s002489900146

Vary, P.S., R. Biedendieck, T. Fuerch, F. Meinhardt, M. Rohde, W.D. Deckwer, and D. Jahn. 2007. Bacillus megaterium: From simple soil bacterium to industrial protein production host. Appl. Microbiol. Biotechnol. 76:957–967. doi:10.1007/s00253-007-1089-3

Wagner, M., and M. Horn. 2006. The *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr. Opin. Biotechnol. 17:241–249. doi:10.1016/j.copbio.2006.05.005

Williams, M.A., E.B. Taylor, and H.P. Mula. 2010. Metaproteomic characterization of a soil microbial community following carbon amendment. Soil Biol. Biochem. 42:1148–1156. doi:10.1016/j.soilbio.2010.03.021

Williams, P., K. Winzer, W.C. Chan, and M. Camara. 2007. Look who's talking: Communication and quorum sensing in the bacterial world. Philos. Trans. R. Soc. London B 362:1119–1134. doi:10.1098/rstb.2007.2039

Wolf, D.C., and H.D. Skipper. 1994. Soil sterilization. p. 41–51. *In* R.W. Weaver et al. (ed.) Methods of soil analysis. Part 2. Microbiological and biochemical properties. SSSA Book Ser. 5. SSSA, Madison, WI.

Yang, M.X., and H.B. Zhang. 2007. Diversity of microorganisms in decaying maize stalks revealed by a molecular method. J. Microbiol. 45:367–370.

Zengler, K., G. Toledo, M. Rappé, J. Elkins, E.J. Mathur, J.M. Short, and M. Keller. 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. 99:15681–15686. doi:10.1073/pnas.252630999

Zhou, J., B. Xia, D.S. Treves, L.Y. Wu, T.L. Marsh, R.V. O'Neill, A.V. Palumbo, and J.M. Tiedje. 2002. Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol. 68:326–334. doi:10.1128/AEM.68.1.326-334.2002