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Members of soil bacterial communities sensitive to tillage and crop rotation

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ABSTRACT

Pyrosequencing was used to study the effect of rotation and tillage on total bacterial communities. We designed primers to the bacterial 16s rDNA and amplified DNA from soil samples from a long-term tillage/rotation trial in Kansas for two seasons. The 2 × 2 factorial trial had two rotation treatments (wheat–wheat and wheat–soybean) and two tillage treatments (conventional and no-till). A total of 20,180 16s rDNA sequences were generated and 2337 operational taxonomic units (OTUs) were assembled using a 97% similarity cut-off. The phylum Proteobacteria represented 38% of 299 identified taxa. The second most abundant phylum was Acidobacteria, making up 20% of the sequences, the majority of which were Acidobacteria Group 1. The phyla Actinobacteria and Gemmatimonadetes comprised 12% and 3.5% of the sequences. Other groups detected included TM7, Nitrospira, Verrucomicrobia, and Bacteroidetes. Some clusters of Acidobacteria Group 1 were more frequent in continuous wheat versus wheat–soybean rotation, some Acidobacteria Group 2 were more frequent in no-till, and some Acidobacteria Group 4 were more frequent in wheat–soybean rotation. These results were validated by quantitative real-time PCR. Pyrosequencing provided taxonomic information about the overall bacterial community, and detected community shifts resulting from different cropping practices.

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1. Introduction

Soil microorganisms play important roles in many soil processes (Abawi and Witmer, 2000; Garbeva et al., 2004; Kennedy, 1999; Van Bruggen and Semenov, 2000; Van Elsas et al., 2002). They regulate carbon and nitrogen cycling and provide nutrition to plants. Bacteria and fungi are critical for the production of soil aggregates and the conversion of plant residue to soil organic matter (SOM). SOM increases aggregate stability, cation exchange capacity, water holding capacity, water infiltration and soil porosity (Nannipieri et al., 2003). Soil microbial communities are also part of the food web that supports populations of invertebrates and protozoans. Furthermore, the abundance of specific antagonistic microbes and the diversity of soil microbial communities are important to the capacity of soil to suppress soilborne plant diseases via antibiosis, competition or stimulation of plant host defenses (Abawi and Witmer, 2000; Cook et al., 1995; Van Bruggen and Semenov, 2000; Van Elsas et al., 2002).

Many studies have been performed to determine the ecological and environmental factors regulating microbial community structure (Baek and Kim, 2009; Högberg et al., 2007; Nagy et al., 2005). Several recent studies have focused on the effects of agricultural practices on the community diversity of soil microorganisms (Buckley and Schmidt, 2003; Clegg et al., 2003; Johnson et al., 2003; Salles et al., 2006; Steenwerth et al., 2002; Vepsäläinen et al., 2004; Yao et al., 2000). No-till or reduced tillage systems can reduce the erosion of soil, which is not a renewable resource and is declining on much of our agricultural land (Kabir, 2005; Papendick, 2004). Reduced tillage can also increase soil organic matter which is generally thought to increase soil quality through better structure, drainage and ion exchange (Dao, 1993; Douglas and Goss, 1982; Drijber et al., 2000; Woods, 1989). Microbial biomass increases have been observed in no-till plots compared to conventional tillage (Drijber et al., 2000). Soils in no-till farming systems, also called direct-seeded systems, where the soil is disrupted only at

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planting, also differ from conventionally tilled soils in several other respects. For example, no-till soils may warm more slowly and remain wetter in the spring. The lack of soil disruption also reduces oxidation and physical disruption of mycelia that affect soil community composition. However, one important weakness of these conservation tillage systems is that certain soilborne plant pathogens can increase to damaging levels (Conway, 1996). Management practices such as crop rotation can sometimes prevent this problem, particularly for pathogens with limited host range.

A thorough analysis of microbial communities requires methodologies for analyzing thousands of microorganisms in a cost-effective and timely manner. Soil microbial diversity has historically been studied by substrate utilization or plating (Hill et al., 2000; Joergensen and Emmerling, 2006). These methods provided an incomplete assessment because they only detect culturable organisms, but recent work suggests that up to 97% of the organisms detected microscopically cannot be cultured (Amann et al., 1995; Pace, 1997). Newer biochemical and molecular analytical tools have recently been used to describe the composition of soil microorganism. These include phospholipid fatty acid (PLFA) or fatty acid methyl ester (FAME) profiles that distinguish broad groups of fungi and bacteria, based on signature fatty acids in cell membranes (Zelles et al., 1992, 1995); denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) which target 16S rDNA and produce electrophoretic bands characteristic of particular groups of microbes (De Oliveira et al., 2006). Although these methods do not rely on culturing of microorganisms, they cannot provide high-resolution taxonomic information. In addition, they detect large-scale population components or activities that are assumed to be important *a priori*, but they miss some small-scale community components that may be also important. Furthermore, DGGE and T-RFLP techniques require extensive cloning and sequencing, which is time consuming and expensive.

Recent advances in DNA sequencing methodology, such as pyrosequencing (Margulies et al., 2005), have made sequence analysis sufficiently affordable to examine population structure in soil (Liu et al., 2007; Sogin et al., 2006). Amplification of a target sequence, such as a fragment of the 16s rDNA, with conserved primers, followed by high-throughput sequencing of amplification products generates taxonomic information for each product, provided that the amplified fragments carry sufficient variation in the sequences. However, no study to date has looked at tillage and rotation effects using this technique. In this study, we examined microbial populations in four cultivation and crop rotation treatments from a long-term field experiment in Kansas. The frequencies of specific sequences generated from each treatment were compared to determine if differences could be detected among the cultural practices. Estimated differences in the abundance of various taxa were validated by quantitative real-time PCR.

2. Materials and methods

2.1. Field plots

Field plots with different cultural and rotation practices were established and cropped annually since 1974 at the Kansas State University Ashland Bottoms Farm near Manhattan. Kansas (McVay et al., 2006). The soil type was a Muir silt loam (fine-silty, mixed, mesic Pachic Haplustoll). Further soil characteristics of these plots were reported by Godsey et al. (2007). All plots were fertilized before planting each year by broadcasting 112 kg N ha^{-1} and 11.3 kg P ha^{-1}. We evaluated a subset of the treatments included in the complete experiment, analyzing the effects of two rotations, continuous wheat (*Triticum aestivum*) and wheat alternated with soybeans (*Glycine max*). Each rotation in our analysis was treated either by no-till, where the seed was drilled directly into the previous year's residue, or by conventional tillage, which consisted of annual cultivations with a chisel plow, disk and field cultivator. The experimental design was a split-plot with four replications at the sub-plot level, with rotation applied in adjacent plots at the whole plot level and tillage as applied to the sub-plot.

2.2. Isolation of DNA from soil and amplification and sequencing of rDNA fragments

In May of 2005 and 2007, when all the plots were sown to wheat, nine soil cores from the top 15 cm of soil were collected from transects within each of the 16 sub-plots sampled in each year. The soil cores from each sub-plot were thoroughly mixed. Ten grams of each bulk sample were then used for DNA extraction. DNA was extracted using the UltraClean™ Mega Soil DNA Isolation Kit (MO BIO Laboratories, Solana Beach, CA) according to the manufacturer's instructions. DNA was visualized by gel electrophoresis and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham MA).

DNA was amplified from purified soil DNA by PCR using the primers listed in Table 1. Each reaction included U342F-FC-B as the forward primer and one of 16 different reverse primers. Primers were designed to amplify the V3 hyper-variable region of bacterial 16S rDNA. The oligonucleotide design included 454 Life Science's A or B sequencing adapter (shown in bold letters in Table 1) fused to the 5′ end of reverse and forward primers. Unique barcode sequences (underlined) were added between the A sequencing adapter and the reverse 16S primer U529R to differentiate between samples. PCR reactions consisted of 2 ng of soil DNA, 1× reaction buffer, 0.2 mM deoxynucleotides, 0.25 μM of each primer, 5 μl DMSO and 0.125 U of Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA) in a total volume of 25 μL. PCR was performed using the DNA Engine thermocycler (Bio-Rad, CA) using a hot start program: 80 °C 3 min (1 cycle); 94 °C 5 min (1 cycle); 94 °C 30 s, 55–69 °C 30 s, 72 °C 30 s (30 cycles); 72 °C 7 min (1 cycle). Each soil

Table 1
Primers used to amplify bacterial sequences from soil DNA for sequence analysis and real-time amplification to verify population differences.

primer	Sequence (5′–3′)
U341F-FC-B	**GCCTTGCCAGCCCGCTCAG**CCTACGGGRSGCAGCAG
U529R-FC-A3	**GCCTCCCTCGCGCCATCAG**ACTCAACCGCGGCKGCTGGC
U529R-FC-A9	**GCCTCCCTCGCGCCATCAG**AGCAGACCGCGGCKGCTGGC
U529R-FC-A11	**GCCTCCCTCGCGCCATCAG**TATCAACCGCGGCKGCTGGC
U529R-FC-A14	**GCCTCCCTCGCGCCATCAG**AGTATACCGCGGCKGCTGGC
U529R-FC-A16	**GCCTCCCTCGCGCCATCAG**CTACGACCGCGGCKGCTGGC
U529R-FC-A18	**GCCTCCCTCGCGCCATCAG**ACTAGACCGCGGCKGCTGGC
U529R-FC-A20	**GCCTCCCTCGCGCCATCAG**TCTCTACCGCGGCKGCTGGC
U529R-FC-A22	**GCCTCCCTCGCGCCATCAG**ACTCGACCGCGGCKGCTGGC
U529R-FC-A24	**GCCTCCCTCGCGCCATCAG**ACTCTACCGCGGCKGCTGGC
U529R-FC-A25	**GCCTCCCTCGCGCCATCAG**TGTCAACCGCGGCKGCTGGC
U529R-FC-A27	**GCCTCCCTCGCGCCATCAG**CTACTACCGCGGCKGCTGGC
U529R-FC-A29	**GCCTCCCTCGCGCCATCAG**AGCTGACCGCGGCKGCTGGC
U529R-FC-A33	**GCCTCCCTCGCGCCATCAG**TGATGACCGCGGCKGCTGGC
U529R-FC-A35	**GCCTCCCTCGCGCCATCAG**AGCGCACCGCGGCKGCTGGC
U529R-FC-A40	**GCCTCCCTCGCGCCATCAG**TCACTACCGCGGCKGCTGGC
U529R-FC-A42	**GCCTCCCTCGCGCCATCAG**TGTGCACCGCGGCKGCTGGC
RT-64ww-F1	ATCCCGCCGAAGCAGGAGTTT
RT-64ww-R1	GAGGTAACGGCTCACCAAGG
RT-74ww-F2	TTCCACCTGCCTCTACAGTGCTC
RT-74ww-R2	ACGAATGTGCGCTTGAGAGCGT
RT-Gp2-F1	TCAAGCCCTTGCGGGCATTCGT
RT-Gp2-R1	TGATCGGTAGCTGGTCTGAGAGG
RT-GP4-F2	GCATTTCACTGCTACACCGAGA
RT-GP4-R2	AAGTAAGGGTTAATATCTCTTACG

DNA template was amplified in 5–6 separate individual reactions. Each PCR product was checked on 2% agarose gels and only specific amplification product (180–200 bp) was pooled. The amplicons in each reaction pool were adjusted to equal concentration after electrophoresis on 2% agarose gels. Samples were then pooled (100 ng of each amplicon), precipitated and suspended in 10 μl ddH_2O for 454 sequencing.

DNA amplicons were sequenced by 454 Life Sciences (Branford, CT). Clusters were assembled with the CAP3 program (Huang and Madan, 1999) using a 97% cut-off. Only clusters that contained a total of 8 or more duplicate sequences were chosen for taxonomic identification. Each cluster was considered to be an operational taxonomic unit (OTU). Taxonomic assignments of the clusters were determined using Ribosomal Database Classifier, a Baysian classifier used to compare known sequences to those in the Ribosomal Database Project Database (RDP, Michigan State University http://rdp.cme.msu.edu/).

2.3. Statistical analysis

The counts of the number of sequences of each cluster or OTU within each sample were converted to frequencies by dividing the number of counts of each cluster by the total number of sequences generated within each sample. A factorial 2-way ANOVA (with cut-off for significance $P < 0.05$) was used to determine if the frequency of an individual OTU was affected by rotation or tillage. Rotation and tillage were main effects and the interaction between rotations × tillage was also determined. There were four replicates for each treatment (wheat–wheat/conventional tillage; wheat–wheat/no-till; wheat–soybean/conventional tillage; and wheat–soybean/no-till). The diversity and richness indices for each sample were also compared using a 2-way ANOVA. The Shannon diversity index and richness indices of OTUs were calculated for each sample, using all clusters, including those represented by just one sequence. The ANOVA was performed using JMP Version 4.0 (SAS Inc, Cary, NC).

2.4. Real-time PCR analysis

Real-time PCR was conducted for four groups of bacteria (Acidobacteria Groups 2 and 4 and two *Gemmatimonas* clusters) to confirm the results of the sequence analysis. To make a standard curve, plasmids containing each of four amplified fragments were constructed. Approximately 200-bp fragments were amplified by PCR from one of the soil samples by using the primer pairs (RT-64ww-F1/RT-64ww-R1; RT-74ww-F2/RT-74ww-R2; RT-Gp2-F1/RT-Gp2-R1 and RT-Gp4-F2/RT-Gp4-R2) (Table 1). The real-time PCR primers were designed to amplify the specific regions of four groups of bacteria respectively. The PCR products were cloned into the pGEM-T easy vector (Promega). Standard curves, based on threshold cycles (Ct), were created using this plasmid DNA with each primer pair. Plasmid DNA was quantified using a Nanodrop ND-1000 spectrophotometer and diluted to 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg or 200 ag in each 5-μL sample. Sterile water was used as a negative control to replace template DNA in PCR reactions. The standard curves were obtained by plotting the Ct value, defined by the crossing cycle number, versus the logarithm of the quantity of the different plasmid DNA samples. Real-time PCR was carried out using the Bio-Rad iQ5 Real-time PCR system. The 20-μl master mixture contained 5 μl of 25-fold diluted DNA from each of the 16 soil samples from 2005 to 2007, 10 μl of SYBR Green PCR master mix (Bio-Rad), 0.5 μM final concentration of each primer and 4 μl ddH_2O. PCR conditions were 95 °C for 3 min, followed by 50 cycles of 95 °C for 20 s, 45.6–65.1 °C for 30 s and 72 °C for 30 s and by a melting curve program. The amount of template DNA was calculated by interpolating the cycle threshold with the standard curve, determined by the Bio-Rad iQ5 software program. All reactions were carried out in triplicate.

3. Results

3.1. Bacterial taxa

From the two years of sampling, a total of 20,180 DNA sequences were generated from the 32 samples (2 years × 4 treatments × 4 replicate sub-plot) included in this study. The number of sequences per sample ranged from 227 to 871 in the 2005 run and from 215 to 1574 in the 2007 run. A total of 2337 clusters were assembled from the combined sequences of both years, using a 97% identity cut-off. The number of sequences in each OTU (or the number of times each OTU appeared in the total pool of sequences from a given year) showed a highly skewed frequency distribution (Figs. 1 and 2). Nine-hundred and seven of the clusters (38%) did not appear in 2005 (Fig. 1), and only 152 of the 2337 clusters (6.5%) were represented by 8 or more sequences. Twenty-nine percent of the clusters (677) did not appear in 2007, and only 196 (8.4%) were represented by 8 or more sequences (Fig. 2).

Among all the phyla, Proteobacteria represented 38.3% of the 299 identified taxa (Fig. 3). The second most abundant taxonomic group among the 20,180 sequences was the phylum Acidobacteria, which made up 20.4% of the sequences. Acidobacteria Group 1 was the most common genus, making up 14.6% of the sequences (Table 2). The phylum Actinobacteria comprised 12% of the sequences. The genus *Gemmatimonas* comprised 3.5% of the sequences. All the phyla were identified with greater than 90% similarity to the sequences in the Ribosomal Database Project, except for Gemmatimonadetes, Firmicutes, and Chloroflexi.

Within the phylum Proteobacteria, the class Alphaprotobacteria made up over half of the OTUs (Fig. 4). This class was composed primarily of the orders Rhizobiales, Rhodospiralles, and Sphingomonadales. The order Burkholderiales made up the bulk of the Betaproteobacteria and the order Myxococcales made up the bulk of Deltaproteobacteria. Pseudomonadales and Xanthomodadales made up the majority of the Gammaproteobacteria. Within the order Actinomycetales, the family Frankinae was the most common, followed by Micrococcineae, Micromonosporineae, and Pseudonocardineae. A similar trend was observed for families within the orders Rhizobiales and Burkholderiales, with some families identified with high levels of confidence (Bradyrhizobiaceae, Oxalobacteraceae), but others were not (Methylobacteriaceae, Comamonadaceae). On the other hand, the families within the

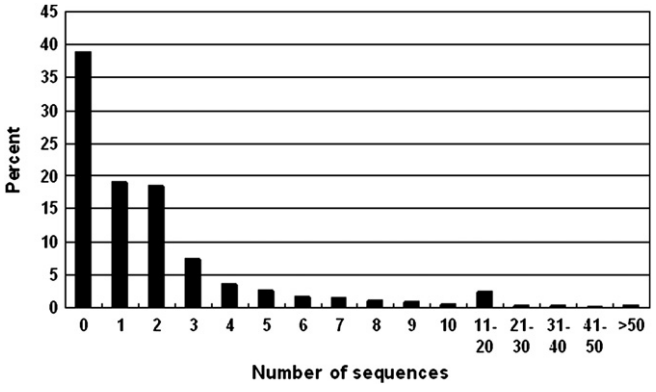

Fig. 1. Frequency distribution of the number of sequences in each cluster or OTU in samples from 2005.

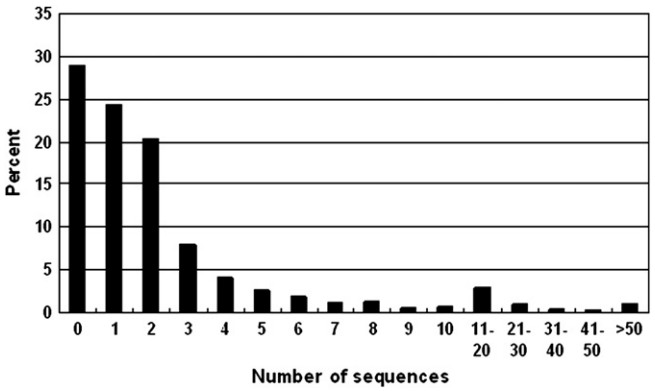

Fig. 2. Frequency distribution of the number of sequences in each cluster or OTU in samples from 2007.

order Verrrucomicrobiales were all identified with similarities of 96%–100%.

3.2. Effect of tillage and rotation on community diversity and Acidobacteria

Cluster diversity was evaluated based on Shannon's Diversity and Richness Indices. For Shannon's Diversity Index, there was no significant difference in the diversity of clusters in 2005 between the four tillage/rotation treatments (Table 3), however, in 2007, there was significantly higher diversity in the no-till treatments ($P < 0.05$). For cluster richness, in 2005, rotation had an effect at $P = 0.07$, with an interaction between tillage and rotation ($P = 0.066$) (Table 3) and the wheat-conventional tillage treatment had the highest richness level. However in 2007, there were no significant differences.

Each OTU (152 in 2005 and 196 in 2007) that had more than 8 sequences was analyzed in a factorial two-way ANOVA to determine whether rotation or tillage had a significant main effect on the frequency of the OTU, and whether there was an interaction between rotation and tillage. Because we defined statistical significance based on a $P = 0.05$ cut-off, 5% of each taxonomic group would be expected to appear to respond to the experimental

Table 2
Abundance of sequences within the Phyla Acidobacteria and Gemmatimonadetes.

Major Groups	No. OTUs	No. sequences	% of total sequences
Acidobacteria Group 1	43	2214	14.6
Acidobacteria Group 2	8	258	1.7
Acidobacteria Group 3	20	544	3.6
Acidobacteria Group 4	9	77	0.5
Gemmatimonas	24	538	3.5

treatments even if there were no true treatment effects. For Acidobacteria Group 1, 48% of the clusters showed no treatment effect, but 40% were more frequent in the wheat–wheat rotation, compared to only 2% that were significantly more frequent in the wheat–soybean rotation (Table 4). For Acidobacteria Group 2, half of the clusters were more frequent in the no-till treatment, but none were more frequent in the conventional tillage treatment (Table 4). Seventy percent of the clusters of Acidobacteria Group 3 showed no treatment effect, but 22% were more frequent in the wheat–wheat rotation (Table 4). Unlike the other Acidobacteria groups, 75% of Group 4 clusters were more frequent in the wheat–soybean rotation (Table 4). The genus *Gemmatimonas* showed a treatment response similar to Acidobacteria Group 1 (Table 4). Thirty-eight percent of the clusters showed no treatment response, but 34% were more frequent in the wheat–wheat rotation, compared to only 10% that were more frequent in the wheat–soybean rotation.

3.3. Real-time PCR

To confirm these trends, real-time PCR primers were constructed to determine if the DNA corresponding to the specific OTUs was more abundant in the plots in which more sequences were found or if the differences were imposed by errors due to poor sampling by the sequencing methodology. The DNA sequences of the OTUs in each of these major groups were compared to see if primers could be designed to amplify specific OTUs, or groups of closely related OTUs. Primers were designed that were predicted to be specific for OTUs 64 and 74, which belonged to the genus *Gemmatimonas*. It was also possible to design a primer pair predicted to specifically amplify four OTUs from Group 2 of the Acidobacteria and another pair specific for three OTUs from Group 4 of the Acidobacteria. The

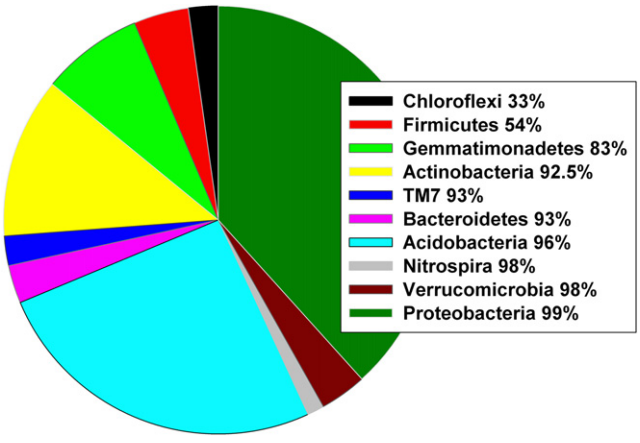

Fig. 3. Frequency of bacterial phyla from clusters of both years. Numbers after taxa represent median percent similarity values calculated using Naive Bayesian rDNA Classifier Version 1.0, November 2003 (Ribosomal Database Project). The nomenclatural taxonomy of Garrity and Lilburn, release 6.0 was used. $N = 299$ clusters in pie chart. Phyla are ordered counterclockwise on pie chart, starting with Chloroflexi (black) at the 12:00 position.

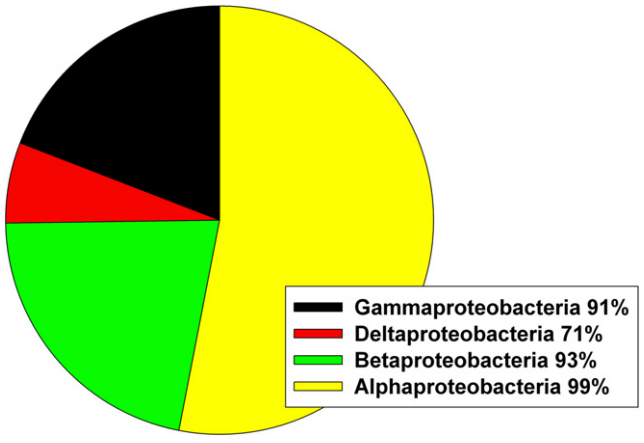

Fig. 4. Frequency of classes within the Phylum Proteobacteria from clusters of both years. Numbers after taxa represent percent similarity values calculated using Naive Bayesian rDNA Classifier Version 1.0, November 2003 (Ribosomal Database Project). The nomenclatural taxonomy of Garrity and Lilburn, release 6.0 was used. $N = 115$ clusters in pie chart. Classes are ordered counterclockwise on pie chart, starting with Gammaproteobacteria (black) at the 12:00 position.

Table 3
Diversity and richness indices for different tillage and rotation treatments in each of two years.

	Shannon Diversity Index		Richness Index	
Treatment*	2005	2007	2005	2007
Wheat–wheat, CT	108.3 A	63.3 A	284	306
Wheat–wheat, NT	115.5 A	102.9 B	223	293
Wheat–soybean, CT	99.1 A	76.2 A	151	265
Wheat–soybean, NT	118.9 A	103.6 B	224	228

*CT = conventionally tilled, NT = no-tilled.
For 2005, rotation effect significant at $P = 0.07$, interaction between rotation and tillage significant at $P = 0.066$. No significant effect in 2007.

highest concentration of DNA from the four Acidobacteria Group 4 OTUs was found in the wheat–soybean no-till treatment, almost 10-fold more than in the wheat–wheat no-till rotation, confirming the trend found from the frequencies in the sequence data (Table 5). For the four OTUs in Acidobacteria Group 2, the highest concentration of DNA was found in the wheat–wheat rotation with no-till, which also was consistent with the cluster frequency results. For OTU 64, in the genus *Gemmatimonas*, the highest level of DNA was found in the wheat–wheat no-till treatment, while for OTU 74, the highest level was found in the wheat–wheat-conventional tillage treatment, also confirming the sequence frequency results.

4. Discussion

Pyrosequencing has been used to evaluate differences in bacterial communities among different land management practices such as pasture, CRP (Conservation Reserve Program) or a cotton and wheat rotation (Acosta-Martinez et al., 2008). Pyrosequencing analysis was sufficiently sensitive to detect these differences and provide taxonomic information on the microbes whose frequencies vary in the different treatments (Liu et al., 2007). However, no study to date has looked at tillage and rotation effects using this technique. The two tillage and rotation treatments examined had noticeable effects on the abundance of different bacterial taxa. Examination of the relatively small numbers of sequences (300–1000) per plot enabled detection of differences in some of the more abundant taxa, most of which have not been cultured. The sequence tags of approximately 150–200 nucleotides (nt) that were generated were sometimes sufficient to assign genus designations to the sequences. In other cases, sequences could only be assigned to higher order taxonomic designations, such as orders. The main reason for the differences in classification accuracy appeared to be the information content of the variable region sequences in the different taxa. Those which had insertions had many highly informative nucleotides while those with deletions, and thus fewer polymorphic nucleotides, provided less taxonomic information. In the future, pyrosequencing projects will have more refined taxonomic abilities. The present analysis was sufficient to derive the sequence of the V3 (variable 3) region of the 16s rDNA, but more recent technology advances in 454 sequencing allow sequence reads of approximately 400 nucleotides, which would allow single reads to derive the V2 and V3 regions together. Sequencing technology advances are also decreasing sequencing costs and will allow larger numbers of sequences to be obtained. The ability to generate several thousands of sequences for each soil treatment or plot will allow researchers to identify responses in minor components of microbial populations and not just the most abundant taxa.

4.1. Experimental design

The design of the experiment requires extra attention for interpretation of our experimental results. The long-term experiment we sampled was a great resource for addressing our experimental question about the effects of tillage because of the continuous maintenance of the cropping system treatments for over 30 years. However, the experimental design was suboptimal in the sense that the rotation treatment was in effect included without 'true' replication (Hurlbert, 1984). This was due to the agronomic design of keeping the rotations in single strips for ease of planting and crop maintenance. The split-plot design had a single replicate at the whole plot level where the rotation treatment was applied. It had four replicates at the sub-plot level where the tillage treatment was applied. Therefore we suggest that the analysis of the effects of tillage treatments be interpreted as a hypothesis test, while the analysis of the effects of rotation might more appropriately be treated as generating hypotheses for more complete analysis in the future. This takes into account that unknown differences between the whole plot to which the wheat–wheat rotation was applied and the whole plot to which the wheat–soybean rotation was applied could explain the observed differences between those whole plots. It might be possible to argue that the relative homogeneity of the soil at the beginning of the experiment precludes that issue and justifies treating the rotation treatments as replicated (Oksanen, 2001). Instead we take the more conservative approach of viewing the rotation analysis as hypothesis generating rather than hypothesis testing. Another point for interpretation of the results is that, even with four replicates, the statistical power for detecting tillage effects on communities has limits. Despite this limitation, differences in several taxa were observed in response to the treatments. The differences observed are likely to be the largest, while more

Table 4
Numbers of bacterial OTUs that were affected by rotation and tillage treatments.

Taxa	No effect	Rotation effect		Tillage effect		Rotation tillage interaction			
		Wheat[a]	Soybean[b]	CT[c]	NT[d]	WCT[e]	SCT[f]	WNT[g]	SNT[h]
Acidobacteria Gp1	28	13	1	4	2	7		3	
Acidobacteria Gp2	4	1			3			2	
Acidobacteria Gp3	16	3		1	1	2			
Acidobacteria Gp4	3		2				1		5
Gemmatimonas	11	3	3	3	2	5		2	

[a] OTU found in significant excess in continuous wheat plots over wheat/soybean plots.
[b] OTU found in significant excess in wheat/soybean rotation plots over continuous wheat plots.
[c] Cluster found in significant excess in conventional tillage plots.
[d] Cluster found in significant excess in no-till plots.
[e] Cluster found in significant excess in conventionally tilled, continuous wheat plots.
[f] Cluster found in significant excess in conventionally tilled, wheat/soybean rotation plots.
[g] Cluster found in significant excess in no-till, continuous wheat plots.
[h] Cluster found in significant excess in no-till, wheat/soybean rotation plots.

Table 5
Quantification of the target DNA of bacteria from soil samples by real-time PCR.

OUT	Rotation, tillage treatment	Estimated DNA (fg/ng soil DNA ± SD)	Number of sequences, 2007[a]	Number of sequences, total[b]
Gemmatimonas OUT 64	wheat–wheat, conv. till	3.19 ± 1.78	13	20
	wheat–wheat, no-till	12.71 ± 10.04	59	74
	wheat–soybean, conv. till	2.28 ± 1.22	9	9
	wheat–soybean, no-till	4.83 ± 2.97	20	20
Gemmatimonas OUT 74	wheat–wheat, conv. till	5.58 ± 3.31	21	32
	wheat–wheat, no-till	0.50 ± 0.27	1	1
	wheat–soybean, conv. Till	2.69 ± 1.24	10	10
	wheat–soybean, no-till	0.57 ± 0.36	0	2
Four Acidobacteria GP2 OTUs[c]	wheat–wheat, conv. till	4.89 ± 2.88	6	19
	wheat–wheat, no-till	14.54 ± 7.54	86	115
	wheat–soybean, conv. till	2.56 ± 1.62	11	11
	wheat–soybean, no-till	7.54 ± 5.78	19	22
Three Acidobacteria GP4 OTUs[d]	wheat–wheat, conv. till	0.18 ± 0.08	0	1
	wheat–wheat, no-till	0.42 ± 0.46	3	4
	wheat–soybean, conv. till	1.09 ± 0.55	1	12
	wheat–soybean, no-till	3.84 ± 1.56	9	52

[a] Corresponds to the number of sequences summed from all four replicated plots from the soils collected in 2007.
[b] Corresponds to the number of sequences summed from all four replicated plots from each of the soils collected in both 2005 and 2007.
[c] The numbers of DNA sequences are pooled for four OTUs (91, 110, 1468 and 2021) that belong to Acidobacteria Group 2.
[d] The numbers of DNA sequences are pooled for three OTUs (2, 867 and 566) that belong to Acidobacteria Group 4.

subtle differences or differences in taxa which exhibit high spatial variability are less likely to be detected.

4.2. Bacterial phyla

Pyrosequencing was successfully used to detect a wide range of bacterial phyla. Of the 25 recognized phyla, we found 10 represented in the bulk soil of an agricultural ecosystem. The phylum Firmicutes was not well represented and most of the clusters identified within this group did not have a high degree of similarity with known sequences. This is surprising, since most culture-based studies have yielded a predominance of *Bacillus* and *Clostridium*, which can produce spores and survive in soils for long time periods (Zhang and Xu, 2008). The low representation of species within these phyla may be due to inefficiencies of our DNA extraction method with bead beating, which may not have extracted DNA from thick-walled spores. Other poorly represented phyla include Chloroflexi, Bacteroidetes, and TM7, a non-cultured group. The latter two were identified with a high similarity to known sequences. However, we were able to detect the four groups that are ubiquitous in most soils-types: class α-proteobacteria and phyla Actinobacteria, Acidobacteria and Verrucomicrobia (Zhang and Xu, 2008).

4.3. Proteobacteria

The Proteobacteria was the most abundant taxonomic group among the 20,180 sequences (Fig. 3). This group is frequently the most numerous in soil samples. Janssen (2006) reported that the most abundant bacterial phyla were Proteobacteria (39%) and Acidobacteria (19%) from a variety of soils with meta-analyses of 16S rRNA sequences. The Proteobacteria was also dominant phylum in Atlantic Forest soil samples (Faoro et al., 2010). Compared with the Acidobacteria lineage, the phylum Proteobacteria was more stable and did not seem to differ in response to the tillage and crop rotation.

4.4. Acidobacteria

After the Proteobacteria, the other abundant taxonomic group was the phylum Acidobacteria, which accounted for more than 20% of the sequences. Acidobacteria Groups 1–4 were the most abundant, similar to the results of Jones et al. (2009). Acidobacteria have been found in a diverse array of soils sampled throughout North and South America (Jones et al., 2009; Lauber et al., 2009). Acidobacteria have been found in wheat fields (Smit et al., 2001), associated with the later stages of take-all decline (Sanguin et al., 2009) and found to be active in the rhizosphere of chestnut trees (Lee et al., 2008). Members of this phylum were also abundant in deep-sea plankton (Quaiser et al., 2008). Acidobacteria are not only ubiquitous, but they are also phylogenetically diverse. Many of the Acidobacteria in this study appeared to be more frequent in particular tillage regimes or crop rotations. For example, approximately half of the Acidobacteria Group 2 OTUs were more frequent in the no-till plots. The reduced physical mixing of the soils in no-till fields can cause localized zones with unique properties, such low pH (Limousin and Tessier, 2007; Matowo et al., 1999; Tong and Naramngam, 2007). However, in our study, tillage did not have a significant effect on soil pH (Godsey et al., 2007), so some other environmental factor may be responsible.

4.5. Soil pH and Acidobacteria

The soil pH is a strong indicator of Acidobacterial community composition. The abundances of Groups 1, 2, 3, 12 and 13 had negative relationships with pH, while groups 4, 6, 7, 10, 11, 16, 17, 18, 22 and 25 had positive correlations with pH (Jones et al., 2009; Lauber et al., 2009). In our study, crop rotation had a major influence on soil pH. In the top 6 cm, the pH of the wheat–wheat rotation was 4.6–4.7, while it was 5.5 in the wheat–soybean rotation (Godsey et al., 2007). Acidobacteria in Groups 1 and 3 and several OTUs in the genus *Gemmatimonas* showed a preference for the wheat–wheat rotation, while Acidobacteria Group 4 showed a preference for the wheat–soybean rotation in this study (Table 4, 5). Our results confirmed the results of Jones et al. (2009) and Lauber et al. (2009), that subgroups 1 and 3 are more abundant at lower pHs, while group 4 is more abundant at higher pHs. However, the mechanism for this is unknown.

The predominance of the Phylum Gemmatimonadetes was not surprising. Since this phylum was erected in 2003 (Zhang et al., 2003), it has been found in diverse soils ranging from extreme environments such as permafrost in Antarctica and gold mines (Rastogi et al., 2009; Shivaji et al., 2004; Steven et al., 2007), to forest and agricultural soils (Ge et al., 2008; Tsai et al., 2009). The

wide range of taxa in the Actinobacteria was also not surprising. This group of gram positive bacteria with a high G + C content is metabolically diverse and common in agricultural soils (Zhang and Xu, 2008).

The mechanisms of how soil microbial communities are affected by agricultural practices remain poorly understood and appear to be highly complex. Changes in microbial communities in response to crop rotations have been documented in previous work (Johnson et al., 2003; O'Donnell et al., 2001). But in some cases rotation has been found to have a smaller effect that other aspects, such as farmyard manure, bioorganic and conventional management of the cropping system (Esperschütz et al., 2007; Hartmann et al., 2006). Experiments examining the effects of specific soil attributes (soil organic carbon, soil C/N ratio) on microbial taxa should help reveal what drives their population changes. Ultimately, a better understanding of how cropping systems affect soil microbial ecology will support development of more productive, sustainable systems.

5. Conclusions

A wide range of bacterial taxa, many unculturable, were detected in the soil of long-term rotation/tillage agricultural plots in Kansas. Many of these taxa could be identified with a high degree of certainty, to the level of family or genus. One of the most numerous taxa was the Phylum Acidobacteria, which could be identified to the group level. The frequency of sequences of some Acidobacteria groups was affected by the wheat/soybean crop rotation, which was confirmed by real-time quantitative PCR. Soil pH differed significantly between these two rotations, which may explain these community differences.

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Appendix. Supplementary material

Supplementary material associated with this paper can be found, in the online version, at doi:10.1016/j.soilbio.2010.08.006

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