Soil microbes in organic vegetable production: Capturing the active players

The response of soil microbial communities to organic and conventional fertilization

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Summary

Soil microbial communities play fundamental roles in the productivity of agricultural systems. Organic methods may foster more diverse soil microbial communities beneficial for crop production that may reduce losses to pathogens. We evaluated active bacterial community responses, in established long-term experimental systems, to organic vs. conventional nutrient management. This graduate research project complements our main Ceres project, allowing comparisons of total resident microbial pools via sequencing the rDNA (main project for The Ceres Trust) and the active microbial pools via sequencing the RNA present in the ribosomes (this project) that are responsible for translation of the transcribed RNA into proteins. In other words, we can compare the general pool of bacteria with the bacteria that are actively metabolizing. We evaluated the microbial communities in an experiment comparing organic and conventional fertilizer systems, with low and high levels of fertility. Using one measure of diversity, Inverse Simpson's Dominance, we found higher bacterial diversity under organic management for high fertility treatments. We recovered a number of bacterial genera that have important agroecological roles. There were more bacterial phyla that increased in frequency in DNA samples compared to RNA samples than vice versa.

Analyses in the next year

As part of the main Ceres Trust project, we will build on these results with systematic analysis of the effects of the treatments on all taxa. We will also develop a similar analysis for fungi. For DNA we will also evaluate changes in the microbial community over the course of the season.

Introduction

Microbial communities in soil help to maintain the productivity and health of agricultural systems (Pankhurst et al., 1996). Soil microbiota play key roles in most of the functional processes that support terrestrial ecosystems, including nutrient acquisition and recycling, degradation of agrochemicals, and nutrient cycling (Pankhurst et al., 1996; van der Heijden et al., 2008). This capacity of soil to function as a vital living system able to fulfill all these functions defines 'soil quality' (Karlen et al., 1997). Microbial diversity represents the repertoire of genetic diversity that supports the health of soil (Jain et al., 2005). However, despite the importance of soil microorganisms, very little is known about their diversity and community structure (Fierer et al., 2007). In this regard, taxonomic approaches to estimating diversity of soil microbial communities have been limited by the traditional methods and the non-culturability of the majority of the microbial species present in soil (Fierer et al., 2007; Rondon et al., 2000).

Extraction of the total RNA from soil has been used to quantify the abundance of Proteobacteria, Actinobacteria, Bacteria and Eukarya under different field management regimes (fertilization, tillage, and the effect of historical cultivation on microbial community) before pyrosequencing was available for characterization (Buckley and Schmidt, 2001). Most recently, metagenomic and small subunit rRNA-based sequence analysis techniques have measured the genetic diversity of Bacteria, Archaea, Fungi and Viruses in soils collected from different ecosystems (prairie, desert and rainforest) (Fierer et al. 2007).

The use of 454-sequencing techniques allows analysis of millions of microorganisms, bypassing culturing (Roesch et al., 2007), and the recent development of sample-specific sequence tags (DNA tagging) for this technique allows simultaneous analysis of large numbers of individual samples, making DNA sequencing and analysis more efficient (Acosta-Martinez et al., 2008; Jumpponen and Jones, 2009; Lauber et al., 2009; Roesch et al., 2007). We used this technique for characterizing the soil bacterial community composition in organic agriculture compared to conventional management, for two fertility levels (high and low) in a tomato crop. The graduate grant allowed us to include an additional analysis. We also compared the microbial communities recovered when sampling DNA (which may include dormant microbes) and the microbial

communities recovered when sampling RNA, later processed as cDNA (which includes those microbes that are more actively metabolizing).

Materials and Methods

Study location

The field study was at the K-State Horticulture Center in Olathe, KS, in an experiment comparing organic and conventional fertilization. These field plots have been maintained in place for six years and consist of three replicate plots for each combination of management treatment (organic and conventional) and three levels of fertilization (high, medium, and low nitrogen, with levels as identified by organic growers). Including the comparison of DNA and cDNA gives a split-split plot design with whole plots (organic vs. conventional treatment) in a randomized complete block design. Each subplot to which fertilizer treatments were applied was 10.6 ft by 20 ft. Our analysis includes the three replicate plots for two levels of fertilization (high and low nitrogen), for a total of 12 experimental units. We have chosen the two extreme levels of fertilizer to compare the soil bacterial community responses, and to compare bacterial community DNA and cDNA. We sampled during the tomato part of the crop rotation in the system, following pac choi 'Mei Qing Choi'. Buckwheat was used as a cover crop between plantings.

Fertilizer treatments

High: Pre-plant fertilizer, and liquid fertilizer.

- High conventional: soluble fertilizer of KNO_3 and $Ca(NO_3)^2$ + inorganic pre-plant fertilizer
- High organic: fish emulsion, and compost pre-plant fertilizer

Low (or control): No added fertilizer, for either conventional or organic treatments.

Soil sampling

Soil samples were collected September 23, 2010, after harvest of the tomato plants. From each experimental unit, four 15 cm deep 5 cm dia. cores were collected in a systematic sampling scheme that avoided the edges of the plots. The subsamples from within each experimental unit were bulked, homogenized and stored on a cooler with

blue -ice until 2.0g was transferred into a 15 ml bead tube that had 1.5 g beads (these bead tubes are components of the kit: RNA PowerSoil, Total RNA Isolation, MoBio, Carlsbad, CA, USA), additionally we first added 5 ml of LifeGuard solution (LifeGuard Soil Preservation solution, Mo Bio, Carlsbad, CA, USA) into each bead tube to protect the RNA integrity while the samples were transported from the field to the lab.

RNA and DNA isolation from the soil samples

RNA was isolated from the soil samples used the Total RNA Isolation kit (MoBio, Carlsbad, CA, USA), following the instructions from the manufacturer. The extracted samples were eluted in 100 µl of solution SR7 (which is RNase/DNase free water and comes with the Total RNA Isolation kit) with 100 U of RNaseOUT (40 U/µl; Invitrogen, Carlsbad, CA, USA) to inhibit RNA degradation before reverse transcription and first-strand complementary DNA (cDNA) synthesis. The RNA extracts were stored in -80 C until further processed. DNA was isolated from the soil samples used the RNA PowerSoil DNA Elution Accessory kit (Mo Bio, Carlsbad, CA, USA), following the manufacturer's instructions. The extracted DNA samples were eluted in 100 µl of RNase/DNase free water, and stored in -80 C until further processed.

Reverse transcription of soil samples

The extracted rRNAs were quantified with an ND 1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA), and 100 ng for each extracted rRNA sample along with the blank extraction control were reverse –transcribed using the Thermoscript reverse transcription PCR (RT-PCR) two-step system (Invitrogen, Carlsbad, CA, USA). Universal bacterial primers 27f (Lane, 1991) and 338R were used for PCR amplification of the V1-V2 hypervariable regions of the 16S rRNA genes. To denature the rRNAs before the cDNA synthesis, 100 ng of RNA for each one of the samples was combined with 1µl of nuclease-free 10 µM 338R primer, 2 µl of 10 mM dNTPs, and the corresponding volume (in µl) of nuclease free H_2O for a 12 µl final volume. Samples were incubated at 65 °C for 5 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The denatured RNAs were transferred to ice and combined with 4 µl of 5 x cDNA buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT (Invitrogen, Carlsbad, CA, USA),

1 µl nuclease free H_2O, and 1 µl ThermoScript Reverse Transcriptase or Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) which was used as the control for DNA contamination. The cDNAs were synthesized in the Eppendorf Mastercycler at 50°C for 60 min, and the synthesized cDNAs were returned to ice until PCR amplification.

PCR amplification

The reverse-transcribed cDNAs were PCR-amplified with Platinum *Taq* polymerase with bacterial-specific primers (27F and 338R) that target a ca. 311 bp region of the SSU. The PCR reactions were conducted in a 25 µl volume with 12.5 µl of Amplitaq Gold 360 PCR Master Mix (Applied Biosystems, USA), 2.5 µl of 10 µM forward and reverse primers, and 2 µl of the cDNA template plus 5.5 µl of nuclease free water. The PCR reactions were carried out with initial 10 min denaturation at 95 °C followed by 34 cycles of 1 min at 95 °C, 1 min at 50 °C, 2 min at 72°C, and a terminal elongation at 72°C for 7 min. Longer extension steps were chosen to minimized the chimeric PCR products (Jumpponen, 2007). After this first amplification, a second PCR reaction was conducted to add the A and B adapters required for direct 454 sequencing of the variable regions V1-V2 of the bacterial small subunit of the ribosome (16S rRNA) amplicons using massively parallel sequencing (MPS) (Margulies et al., 2005). For this purpose, two new primer constructs were synthesized where the 454-sequencing primer (A-primer) and the forward primer (27F) with a ten base pair (bp) DNA tag for post-sequencing sample identification in between, or the DNA capture bead anneal primer (B-primer) for the emulsion PCR (emPCR) and the reverse primer (338 R) to make the single strands on beads as required for 454 pyrosequencing (Margulies et al., 2005). The resulting sequences were as follows: 27 F- 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG NNNNNNNNNN**AGAGTTTGATCCTGGCTCAG**-3', and 338R- 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG**TGCTGCCTCCCGTAGGAGT**-3' where the underlined sequences are the 454 primers A and B, respectively, and the bold letters denote the universal 16S rRNA primers 27 F and 338 R. The 10 bp barcode within primer 27 F is denoted by 10 Ns. The second PCR reaction was carried out with initial

10 min denaturation at 95 °C followed by 5 cycles of 1 min at 95 °C, 1 min at 50 °C, 2 min at 72°C, and a terminal elongation at 72°C for 7 min.

The amplification of target-sized amplicons was confirmed by horizontal gel electrophoresis. The PCR products were purified with Agencourt AMPure PRC purification system (AgenCourt Bioscience, Beverly, MA, USA) following the manufacturer's instructions. This clean-up system was selected because it discriminates against fragments of less than 100 bp in size, removes salts, enzymes and effectively eliminates dimers of the fusion primer constructs that exceed 40 bp in size. The clean PCR products were again quantified with the ND1000 spectrometer.

Control reactions

To account for contaminating nucleic acids in the samples, three controls were included. First, to account for RNA/DNA contamination from the extraction system, a blank extraction without sample was carried through the extraction protocol. Second, to account for PCR reagent-borne contaminants, a PCR control without template DNA was included in the PCR. Third, to account for DNA carryover through the RNA extraction, a control where Thermoscript reverse transcriptase was replaced with Platinum *Taq* polymerase was included. All these controls remained free of contaminants and yielded no visible PCR amplicons.

Analysis of the sequence data

The cDNA and DNA bacterial sequences were submitted to the computational pipeline PyroTagger v. 1.0 (Kunin and Hugenholts, 2010). This pipeline removed low quality bases and shorter sequences based on a quality filtering and length trimming. Using the algorithm 'pyroclust', the sequences were grouped in clusters at 97% similarity. The sequences were assigned to operational taxonomic units (OTUs) using RDP (Ribosomal Database Project) tools (Cole et al., 2009).

Diversity indices

From the derived OTU (Operational Taxonomic Unit) frequency data, we calculated two common estimators of diversity: Inverse Simpson's dominance, and Pielou's evenness.

- Inverse Simpson's index, expressed as 1/D, where D is a dominance measure (Magurran, 1988,). Higher values of 1/D indicate higher diversity. Inverse Simpson's index is calculated as:
  $1/D = 1/\sum p_i^2$,where p_i is the frequency of occurrence of each OTU
- Pielou's evenness, J, was calculated as the ratio of Shannon's index and the natural logarithm of species richness (S): $J = H' / \ln (S)$

These diversity indices were used to test hypotheses about diversity responses among the treatments in an analysis of variance using SAS (SAS Institute Inc., Cary, NC). Hypotheses of interest include (a) Organic management results in higher microbial diversity than conventional management, and (b) microbial communities detected through RNA (cDNA) will be less diverse than those detected through DNA.

Results and Discussion

Diversity responses among the treatments

The bacterial community showed higher diversity as measured by Inverse Simpson's Index for organic (O) management compared to conventional (C) management ($p = 0.08$, Fig. 1). There was some evidence for a three-way interaction (management type * fertilizer level * DNA vs cDNA) ($p = 0.10$, Fig.1). The main difference in Inverse Simpson's Index appears to be the higher levels of diversity for the organic high fertility treatment compared to other treatments (Fig. 1). There was not evidence for an effect of these treatments on Pielou's evenness.

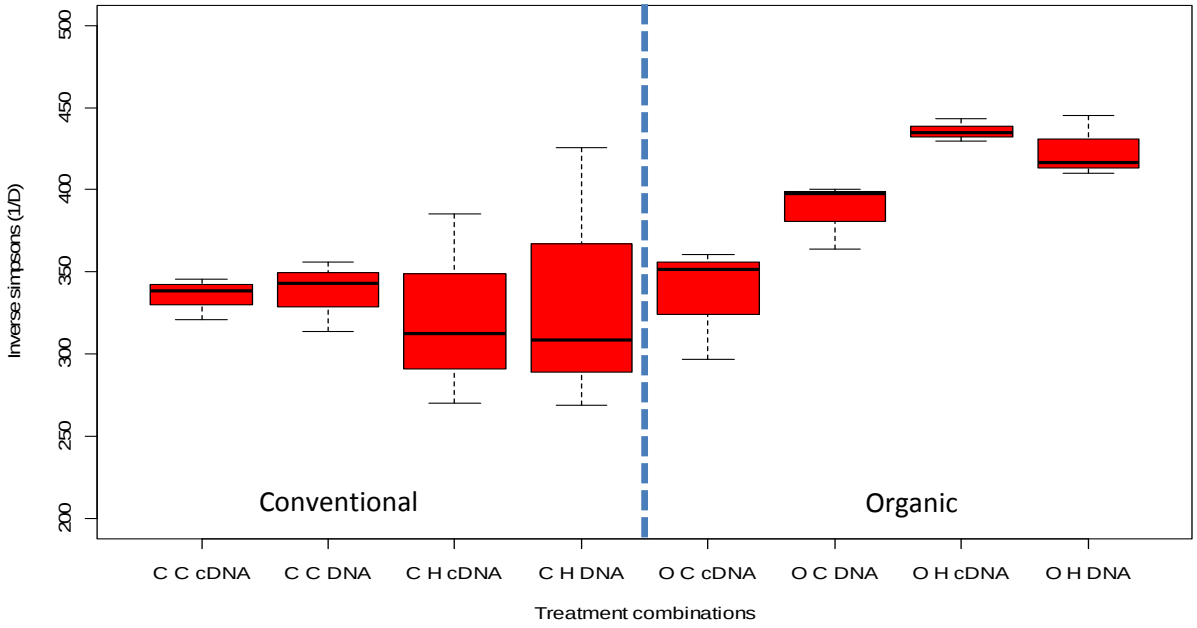

Fig 1. Box plots showing Inverse Simpson's index for the bacterial cDNA/DNA community in the field plots under different treatment combinations. The blue dashed-line separates the results for: conventional (C) and organic (O) management. The second treatment was the fertilizer level, control (C) or high (H). The third treatment was the type of DNA, DNA or cDNA (RNA). Each entry on the x-axis is labeled as follows: management type, fertilizer level, and DNA type. An AOV gives some evidence for a three-way interaction (P = 0.10).

Most abundant bacterial phyla among the treatments

The total number of sequences obtained across the field plots was 780,266. More bacterial and archaeal phyla were found to be more frequent in soil samples where DNA was extracted compared to RNA extraction (cDNA) (Fig.2). This result is consistent with the fact that soil DNA extraction allows detection of a broad group of microorganisms that, at the moment of sampling, can be recently dead, moribund, dormant, or alive. The samples included two phyla (*Crenarchaeota and Euryarchaeota*) that are classified in the archaea domain. The phylum *Dictyoglomi,* only present in the cDNA samples, usually grows at high temperatures (Euzeby, 2011).

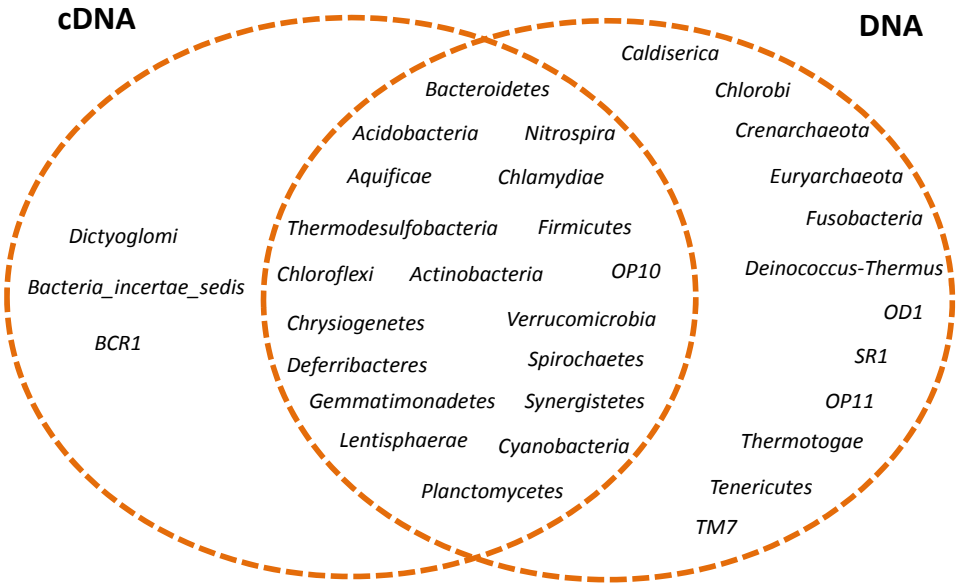

Figure 2. Venn diagram showing the most abundant bacterial and archaeal phyla for cDNA and DNA samples. The identity of phyla was inferred using RDP (Ribosomal Database Project) tools. There were more bacterial phyla that were more frequent in DNA samples compared to phyla more frequent in cDNA.

Bacterial genera sampled

A total of 991 genera of bacteria were obtained from the RDP classifier. Some of the genera include taxa with important ecological roles in the nitrogen cycle or in the production of antibiotics. Others contain taxa that are plant pathogens causing severe losses in crops. We recovered a number of genera of bacteria that have roles in agroecological systems (Table 1).

Genus	
Streptomyces	Plant pathogens, and taxa used for biological control that, like many actinomyces, produce a range of antibiotics
Bacillus	Ubiquitous in nature, some being plant pathogenic species
Nitrospira	Nitrite-oxidizing bacteria, important for wastewater treatment
Ferrimicrobium	Iron-oxidizing actinobacteria
Pseudomonas	Plant pathogens and biocontrol agents
Rhizobium	Bacteria that fix nitrogen, forming associations with legume roots
Azotobacter	Playing an important role in the nitrogen cycle
Erwinia	Usually plant pathogenic species
Desulfovibrio	Sulfate reducing bacteria, used for bioremediation

Table 1. Examples of genera of bacteria recovered in our experiments that have important ecological roles.

References

Acosta-Martinez, V., Dowd, S., Sun, Y. and Allen, V., 2008. Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology & Biochemistry, 40(11): 2762-2770.

Buckley, D.H. and Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology, 42(1): 11-21.

Cole, J.R. et al., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research, 37: D141-D145.

Euzeby, J., 2011. List of new names and new combinations previously effectively, but not validly, published. International Journal of Systematic and Evolutionary Microbiology, 61: 1011-1013.

Fierer, N. et al., 2007. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Applied and Environmental Microbiology, 73(21): 7059-7066.

Jain, R.K. et al., 2005. Microbial diversity: Application of microorganisms for the biodegradation of xenobiotics. Current Science, 89(1): 101-112.

Jumpponen, A., 2007. Soil fungal communities underneath willow canopies on a primary successional glacier forefront: rDNA sequence results can be affected by primer selection and chimeric data. Microbial Ecology, 53(2): 233-246.

Jumpponen, A. and Jones, K.L., 2009. Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate Quercus macrocarpa phyllosphere. New Phytologist, 184(2): 438-448.

Karlen, D.L. et al., 1997. Soil quality: A concept, definition, and framework for evaluation. Soil Science Society of America Journal, 61(1): 4-10.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: S.a.M. Goodfellow (Editor), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY, pp. p. 115-147.

Lauber, C.L., Hamady, M., Knight, R. and Fierer, N., 2009. Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. Applied and Environmental Microbiology, 75(15): 5111-5120.

Margulies, M. et al., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature, 437(7057): 376-380.

Pankhurst, C.E., OphelKeller, K., Doube, B.M. and Gupta, V., 1996. Biodiversity of soil microbial communities in agricultural systems. Biodiversity and Conservation, 5(2): 197-209.

Roesch, L.F. et al., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. Isme Journal, 1(4): 283-290.

Rondon, M.R. et al., 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Applied and Environmental Microbiology, 66(6): 2541-2547.

van der Heijden, M.G.A., Bardgett, R.D. and van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters, 11(3): 296-310.