The Identification of Bacteria Species in Vermicompost

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Abstract Standard microbial culture-based methods were used to study the composition and diversity of three vermicompost samples (Terra Vesco, Sonoma, CA, USA). Bacterial colonies were isolated from vermicompost using serial dilutions and plated onto general growth media. The amplification and sequence analysis of the 16S rDNA gene from 39 isolates showed a high percentage (79%) of *Actinobacteria* sp., especially *Arthrobacter* sp. (54%) and *Streptomyces* sp. (15%). *Proteobacteria* and *Firmicutes* sp. were also detected. Aerobic bacterial populations in the vermicompost reached levels greater than 10^7 CFU/g vermicompost. The level of bacteria diversity and large aerobic bacterial populations in the compost supported the hypothesis that microorganisms in vermicompost may be able to outcompete and reduce soil-borne pathogen populations, making vermicompost a viable disease-control method for the agriculture industry.

Keywords Vermicompost • Cow manure • Bacterial community composition • Soil-borne pathogens

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Introduction

Soil fumigation is a common practice in agriculture and is recommended to reduce a variety of soil-borne pathogens (Yakabe et al. 2010). By sterilizing soil before crops are planted, weeds, pests, and other harmful organisms are eradicated (Ibekwe et al. 2001). However, beneficial organisms are eliminated as well, and the soil microbial community has difficulty recovering after treatment with fumigants (Ibekwe et al. 2001). Some of the eliminated organisms are capable of protecting plants against other pathogens, therefore making plants grown in fumigated soil susceptible to a host of diseases. Compost has been used as a method of returning soil health and sustainability by providing a microbial community that may resist such diseases (Contreras-Ramos et al. 2005).

Standard compost is generated through the decomposition of solid animal or plant waste. Vermicompost is generated with the assistance of the earthworm digestive system. This process creates a soil product with a rich bacterial community (Domínguez et al. 2010), which may be able to outcompete and eliminate other bacteria and pathogens that are harmful to key crops (Contreras-Ramos et al. 2005).

Lab studies recently determined that vermicompost suppresses *Agrobacterium tumefaciens* (Strauss et al. *in press*), which is able to colonize and persist in soil after fumigation (Yakabe at al. 2010). *A. tumefaciens* is the cause of crown gall, which inhibits and limits the productivity of

walnuts and other agriculturally important hosts (Yakabe et al. 2010). Vermicompost application may protect orchards from diseases such as crown gall by using naturally occurring soil bacteria rather than chemical methods.

The objective of this study was to gain a better understanding of bacterial communities inhabiting vermicompost made from cow manure (Terra Vesco, Sonoma, CA, USA) by isolating and identifying individual bacteria species from the compost. Vermicompost samples were diluted, and bacteria strains were isolated, grown, and analyzed using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP). The culture-based community composition of vermicompost was determined by calculating the percentage of each species identified.

Materials and Methods

Sample Collection and Serial Dilution

Tryptic soy broth (TSB), 10% TSB, and Reasoner's 2A (R2A) media were made using Milli-Q water. Cyclohexamide (100µg/mL) made with dimethyl sulfoxide (DMSO) was added to each media type to reduce fungal growth. Serial dilutions (1:10) were made from a suspension of vermicompost (Terra Vesco, Sonoma, CA, USA) and sterile water. 100µL from 10^{-3} to10^{-5} dilutions were plated onto TSB, 10% TSB, and R2A media. Serial dilutions were performed for three separate vermicompost samples. Serial dilutions were also performed for a vermicompost sample that was heated in an oven at 85˚C for two 24-hour periods. The heat-treated sample was diluted with the same procedure as the non-treated vermicompost samples. The serial dilution plates were incubated at 28˚C for 48 hours. After incubation, the bacteria colonies were counted under a light magnifier. CFU/mL based on bacteria counts were converted to CFU/g for analysis.

Pure Colony Isolation

Single colonies were randomly selected from the serial dilution plates for isolation and each colony was re-streaked onto TSB, 10% TSB, and R2A media plates. The plates were incubated at 28˚C for 24 hours, and plates with pure colonies were stored in a refrigerator at 4˚C. Plates with colonies that were not pure were streaked again until all plates had only pure colonies.

PCR Amplification, Sequencing, and Analysis

The bacterial 16S rDNA was amplified using the polymerase chain reaction (PCR) with universal eubacterial primers 338F (Lane 1991) and 518R (Muyzer et al. 1995) using a colony PCR technique. Colonies from TSB and 10% TSB plates were picked and transferred into nuclease-free water (Promega, Madison, WI, USA) to make the DNA templates. The templates were heat-treated in the thermocycler at 95˚C for 5 minutes to lyse the cell for PCR and then stored on ice. The PCR mixture consisted of 2X GoTaq master mix (Promega, Madison, WI, USA) and 100µM of each primer. 1µL of DNA template was used for each PCR tube, and 1µL of *A. tumefaciens* pure culture and 1µL of nuclease-free water were used for the positive and negative control tubes, respectively. PCR was performed with 30 cycles. The PCR products were resolved on a 1X tris-acetate-EDTA (TAE) gel with Gel Red (Phenix Research, Candler, NC, USA) and 1 kb ladder (TrackIt, Invitrogen, Carlsbad, CA, USA).

The PCR products were purified using the Qiagen (Germantown, MD, USA) PCR Purification Kit. Manufacturer's protocol was followed. The DNA was eluted with 30µL of sterile water and quantified for sequencing using Nanodrop (Thermo Scientific, Wilmington, DE, USA). The purified and quantified PCR products were then sent to the University of California, Davis DNA Sequencing Lab. The resulting DNA sequences were checked for sequence quality with the Sequencher 5.1 software (Gene Codes Co., Ann Arbor, MI, USA) and then identified using NCBI BLAST and RDP. Alignment Name, Max ID, and Accession Number, and taxonomical information were recorded.

The experiment was conducted three times.

Statistics

Means and standard errors of the three trials were calculated. ANOVA was used to compare the means of different treatments and media types.

Results

Confirmation of Microbial Presence in Vermicompost

Comparison of the heat-treated and non heat-treated samples showed that microbial presence was significantly greater (Fig. 1, $P = 0.0085$) in non-treated vermicompost than in heat-treated vermicompost. The non-treated samples contained approximately 10,000 times more colonies per gram.

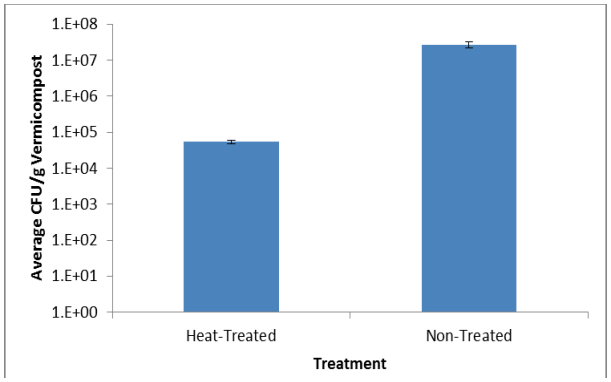

Fig. 1 Comparison of heat-treated and non-treated samples based on logarithmic scale confirms microbial presence in vermicompost

Bacterial Populations of Non-treated Vermicompost and Media Comparisons

Serial dilutions and isolation of non-treated vermicompost produced a range of bacteria species. During incubation of pure colonies, some bacteria only grew on one of the media types examined. There were no colonies on seven of the TSB plates while 10% TSB and R2A each had two plates with no growth. There tended to be more growth on 10% TSB than on TSB and R2A (Fig. 2), but ANOVA analysis showed that these differences were not significant (Fig. 2, $P = 0.13$).

Media comparisons between the three repetitions produced consistent data. 10% TSB showed the largest populations in all of the repetitions, followed by R2A then TSB (Fig. 2).

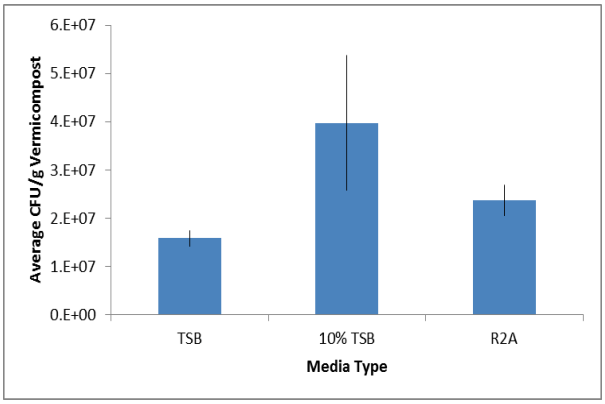

Fig. 2 Media comparison based on the combination of all three repetitions. 10% TSB consistently showed most growth, followed by R2A then TSB. Standard errors are shown.

Sequence Results

The 16S rDNA genes in the bacterial isolates were sequenced and assigned to phylogenetic groups by comparing NCBI BLAST and RDP results (Table 1). *Actinobacteria* was the most abundant phylum, followed by *Proteobacteria* then *Firmicutes* (Fig. 3). All of the *Actinobacteria* isolates grouped within the order *Actinomycetales* and genera *Arthrobacter*, *Streptomyces*, *Microbium*, and *Rhodococcus*. The majority of *Actinobacteria* isolates belonged to *Arthrobacter* (68%) and *Streptomyces* (29%). *Arthrobacter* and *Streptomyces* were also the most abundant genera across all three vermicompost samples, with 58% and 15% of the total analyzed population, respectively.

Proteobacteria showed the greatest phylogenetic diversity, with orders *Burkholderiales*, *Pseudomonadales*, *Rhizobiales*, and *Xanthomonadales* (Fig. 4). Each order grouped into only one genus; the *Burkholderiales* isolate was associated with the genus *Cupriavidus*, the *Pseudeomonadales* isolates were associated with the genus *Pseudomonas*, the *Rhizobiales* isolates were associated with the genus *Rhizobium*, and the *Xanthomonadales* isolate was associated with the genus *Rhodanobacter*.

All of the *Firmicutes* isolates grouped within the order *Bacillales*.

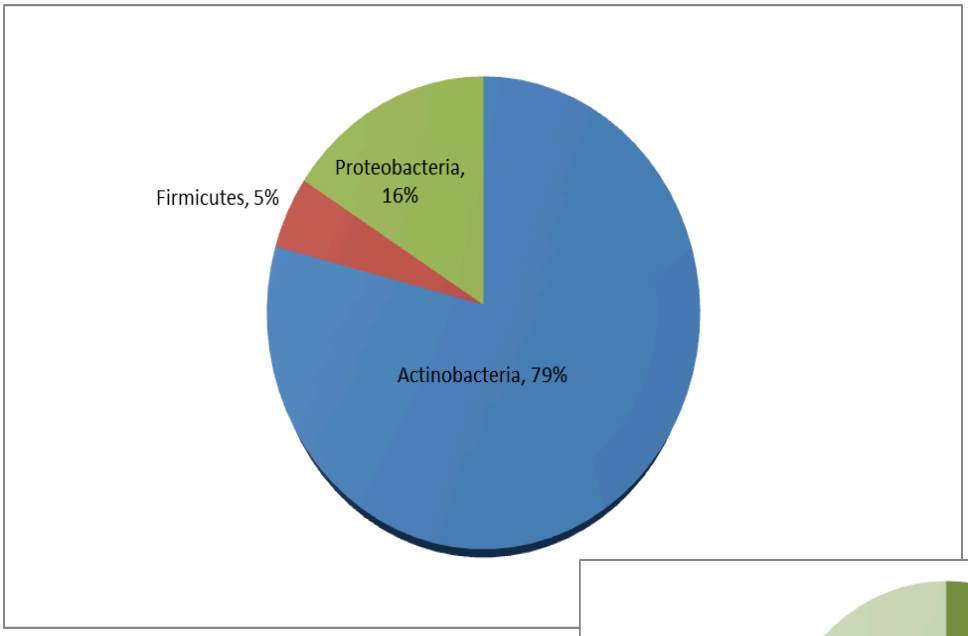

Fig. 3 Percentages of identified bacteria phylum.

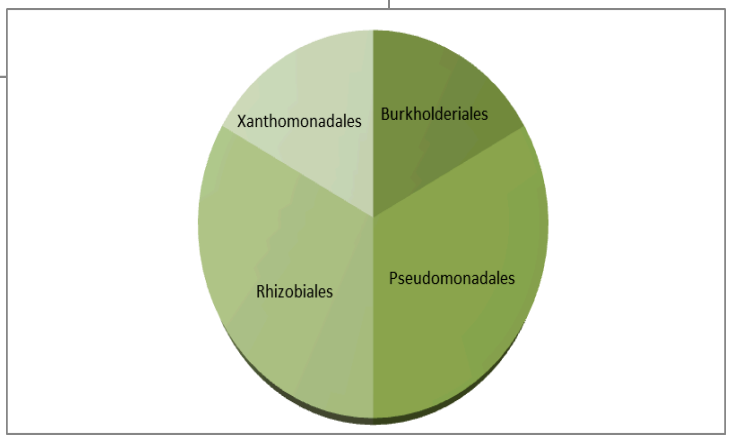

Fig. 4 Four orders identified from the *Proteobacteria* phylum and their relative presence in vermicompost.

Isolate Name	Phylum	Order	Genus	BLAST Max Iden
C	Actinobacteria	Actinomycetales	Arthrobacter	100%
E	Actinobacteria	Actinomycetales	Arthrobacter	100%
F	Actinobacteria	Actinomycetales	Arthrobacter	100%
G	Actinobacteria	Actinomycetales	Arthrobacter	100%
H	Actinobacteria	Actinomycetales	Arthrobacter	99%
R	Actinobacteria	Actinomycetales	Arthrobacter	100%
S	Actinobacteria	Actinomycetales	Arthrobacter	100%
T	Actinobacteria	Actinomycetales	Arthrobacter	100%
U	Actinobacteria	Actinomycetales	Arthrobacter	100%
V	Actinobacteria	Actinomycetales	Arthrobacter	100%
W	Actinobacteria	Actinomycetales	Arthrobacter	100%
X	Actinobacteria	Actinomycetales	Arthrobacter	100%
AA	Actinobacteria	Actinomycetales	Arthrobacter	99%
BB	Actinobacteria	Actinomycetales	Arthrobacter	99%
CC	Actinobacteria	Actinomycetales	Arthrobacter	99%
DD	Actinobacteria	Actinomycetales	Arthrobacter	99%
EE	Actinobacteria	Actinomycetales	Arthrobacter	100%
HH	Actinobacteria	Actinomycetales	Arthrobacter	100%
II	Actinobacteria	Actinomycetales	Arthrobacter	100%
JJ	Actinobacteria	Actinomycetales	Arthrobacter	100%
KK	Actinobacteria	Actinomycetales	Arthrobacter	100%
NN	Actinobacteria	Actinomycetales	Microbacterium	100%
OO	Actinobacteria	Actinomycetales	Microbacterium	100%
LL	Actinobacteria	Actinomycetales	Rhodococcus	100%
MM	Actinobacteria	Actinomycetales	Rhodococcus	100%
A	Actinobacteria	Actinomycetales	Streptomyces	100%
B	Actinobacteria	Actinomycetales	Streptomyces	100%
M	Actinobacteria	Actinomycetales	Streptomyces	98%
N	Actinobacteria	Actinomycetales	Streptomyces	99%
O	Actinobacteria	Actinomycetales	Streptomyces	100%
P	Actinobacteria	Actinomycetales	Streptomyces	100%
D	Firmicutes	Bacillales	Bacillus	99%
FF	Firmicutes	Bacillales	Brevibacillus	100%
I	Proteobacteria	Burkholderiales	Cupriavidus	99%
Y	Proteobacteria	Pseudomonadales	Pseudomonas	100%
Z	Proteobacteria	Pseudomonadales	Pseudomonas	100%
K	Proteobacteria	Rhizobiales	Rhizobium	100%
L	Proteobacteria	Rhizobiales	Rhizobium	98%
Q	Proteobacteria	Xanthomonadales	Rhodanobacter	100%

Table 1 Sequences obtained by analysis of 16S rDNA from 39 bacteria isolates. Phylum and order from RDP listed with genus determined by analysis of both NCBI BLAST and RDP results. Max Identification from BLAST also listed.

Discussion

The analysis of 16S rDNA sequences from the vermicompost samples revealed that a majority of bacteria were *Actinobacteria*, a major phylogenetic group of soil bacteria (Agrios 2005). In a study of vermicompost microbial community composition using both culture-dependent and culture-independent methods, *Actinobacteria*, *Proteobacteria*, and *Firmicutes* were also identified, with *Actinobacteria* dominating culture isolates (Yasir et al. 2009). The *Actinobacteria* found in our vermicompost are also consistent with the Yasir et al. (2009) study, which found suborders *Micrococcineae* and *Streptomycinea*, which correspond with genera *Arthrobacter* and *Streptomyces*, respectively.

In another study that used similar methods to analyze 16S rDNA from isolates, 72% of isolates belonged to the genus *Bacillus* (Mocali et al. 2006). Although *Bacillus* only represented 3% of the bacterial community from our study, it was still identified. The Mocali et al. (2006) study also found genera *Arthrobacter* and *Pseudomonas*, which were present in this study as well.

In comparison to investigations that studied vermicompost from fruit and vegetable wastes, there was a different microbial community composition in the cow manure vermicompost used in our study. Mocali et al. (2006) identified *Sporosarcina*, *Staphylococcus*, *Alcaligenes*, and *Brachybacterium*, none of which were identified in our vermicompost. The Fernández-Gómez et al. (2012) study, which used culture-independent techniques rather than culture-dependent techniques, identified 34 different genera from four vermicompost samples, in contrast to the 10 genera identified in this study. This is most likely because vermicompost from different wastes that are processed by different earthworms produce vermicompost with varying communities (Aira et al. 2005). Another possibility is that culture-dependent and culture-independent techniques produce different results. Because each method may only be able to identify certain bacteria species, major strains could be missed unless both methods are used.

The bacterial counts of non heat-treated vermicompost samples were approximately 10,000 times more than the counts of heat-treated vermicompost, suggesting that the vermicompost has an active microbial population. The consistency between the three repetitions based on bacterial count comparison between media types also suggests uniformity in bacteria composition between the samples.

Because the pure colony isolates from R2A plates also grew on 10% TSB media, R2A plates were excluded from identification. The relative similarity between bacteria growth on 10% TSB and R2A is likely because 10% TSB and R2A are both nutrient-poor media while TSB is a nutrient-rich media. Different bacteria are able to survive on each type of media depending on the amount and variety of nutrients provided. Despite the similarity in nutrient level between 10% TSB and R2A, it is evident that bacteria grew on each media type differently (Fig. 2), so some strains may have been missed.

In conclusion, sequences identified in cow manure vermicompost using culture-based methods were similar to the sequences identified in other studies using similar methods (Yasir et al. 2009, Mocali et al. 2006). However, the bacteria composition was different from those obtained by culture-independent methods (Fernández et al. 2012). The application of individual bacteria strains to *A. tumefaciens* and/or further analysis of the effects of each strain are required to identify the function of individual bacteria species in vermicompost.

Using vermicompost in agriculture may provide a method to reduce soil-borne pathogens and improve the productivity of the agricultural industry.

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