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Molecular characterization of fungal community dynamics in the initial stages of composting

Ann M. Hansgate [a], Patrick D. Schloss [b,d], Anthony G. Hay [c], Larry P. Walker [b,*]

[a] *School of Chemical and Biomolecular Engineering, Olin Hall, Cornell University, Ithaca, NY 14853, USA*
[b] *Department of Biological and Environmental Engineering, Riley Robb Hall, Cornell University, Ithaca, NY 14853, USA*
[c] *Department of Microbiology, Riley Robb Hall, Cornell University, Ithaca, NY 14853, USA*
[d] *Department of Plant Pathology, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706, USA*

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Abstract

Composting relies on a complex network of bacteria and fungi to process crude organic material. Although it is known that these organisms drive dynamic changes in temperature and pH, little is known about the temporal dynamics of fungal populations during the rise to thermophilic conditions. This study employed F-ARISA (fungal-automated rRNA intergenic spacer analysis) and 18S rRNA gene cloning and sequencing to examine changes in community structure during this period. Sequencing of the 18S rRNA portion of cloned F-ARISA products revealed the presence of four distinct fungal genera including *Backusella* sp., Mucoraceae, *Geotrichum* sp. and the yeast *Pichia* sp. Based on the presence and absence of these ARISA operational taxonomic units (A-OTUs), we observed a shift in fungal community structure between 48 and 60 h. This change in community structure preceded a rise in pH and coincided with an increase in temperature. Clone libraries constructed using fungi-specific 18S rRNA primers contained sequences similar to several other fungal genera including *Penicillium* sp., *Aspergillus* sp., *Hamigera* sp., *Neurospora* sp. and the yeast *Candida* sp. While the fungal species richness was relatively low at any time point, the community structure was dynamic and paralleled changes in bacterial community structure.

Keywords: ARISA; Compost; Fungi; 18S rRNA

1. Introduction

Composting is a dynamic process of organic solids degradation carried out by a complex microbial community. The metabolic activities of the microorganisms that populate compost piles drive dramatic changes in the physical and chemical structure of the pile that, in turn, drive changes in the microbial community structure [1]. There has been much attention focused on measuring and modeling changes in the physical and chemical state variables of the compost environment [1–6] and recent focus on bacterial community dynamics in compost [7–16], but the ecology of fungi has largely been ignored [12].

Previous analyses of a variety of composting environments have focused on the dynamic bacterial community using 16S rRNA gene cloning [7,8,10], fingerprinting [9,11–14] and hybridization blotting [17]. In our earlier work [14], we employed bacteria-based automated rRNA intergenic spacer analysis (ARISA) for community composition analysis and showed that shifts in the bacterial community composition followed pH shifts in the early stages of composting. Subsequently, the dominant

* Corresponding author. Tel.: +1 607 255 2478; fax: +1 607 255 4080.
E-mail address: lpw1@cornell.edu (L.P. Walker).

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bacterial populations in the same samples were quantified using DNA hybridization probing. Surprisingly up to 65% of the rRNA genes that annealed to the universal rRNA gene probe did not anneal to a bacteria-specific 16S rRNA gene probe during the rise to thermophilic conditions [17]. Since no PCR product was obtained using archea-specific primers or plant-specific primers [8,18], it was hypothesized that the difference in the signal from the universal and bacterial probes represented fungal 18S rRNA genes.

Recently, Ranjard et al. [19] extended the use of ARISA to fungal communities (F-ARISA) by designing primers that allow differentiation between fungal populations by determining the length heterogeneity of the region between the 18S and 28S rRNA genes, which includes the 5.8S rRNA gene as well as two internal transcribed spacer regions (ITS). As the ecological and metabolic role of fungi in composting has rarely been examined, our goal was to begin to evaluate the hypothesis that fungi are present and active members of the composting microbial community. In this study, we therefore sought to identify and track fungal groups and to relate these changes to the temperature and pH of the organic matrix during the first 96 h of composting in a model bench-scale reactor system.

2. Materials and methods

2.1. Sample preparation and DNA purification

Compost reactors with a substrate consisting of equal dry weights of Big Red Food (Pro-Pet, Syracuse, NY) pellets and maple wood chips (Costal Lumber, Cayuta, NY), adjusted to a final moisture content of 55% (wet basis) were operated as described previously without inoculation [14]. Dog food has been used by our research group as a model substrate because it has the same fat, fiber, and protein content as food waste, and is available in a consistent, commercial formulation [5,14,20–22]. Monitoring both temperature and pH throughout the process, three replicate reactors were run simultaneously for 12, 24, 36, 48, 60, 72, 84, and 96 h [14]. In addition, we repeated each time point grouping of three replicate reactors three times. Therefore, there were nine reactors operated for each time point in addition to initial samples taken at the beginning of each trial, giving 96 total samples.

Reactors were sacrificed after running the duration specified for each group of three reactors, then DNA was extracted from the initial material using a bead-beating extraction method with polyethylene glycol precipitation and glass milk purification (Express Matrix, Bio101, Inc., Vista, CA) [14]. The DNA preparations used in this analysis were the same as those used previously [14].

2.2. F-ARISA

In a 10 μl PCR reaction, 1 μl template DNA was added to the reaction mixture containing 500 nM universal 16S/18S rRNA forward primer 1406f (TGYACACACCGCCCGT) [23] labeled with TET, 500 nM 28S rRNA reverse primer 3126r (ATATGCTTAAGTTCAGCGGGT) [19], 2.5 mM $MgCl_2$, and 1× *Taq* PCR Master Mix (Qiagen, Inc., Valencia, CA) containing 0.5 U of *Taq* DNA Polymerase, 1× PCR buffer, and 200 μM of each dNTP. PCR was initiated by 2 min denaturing at 94 °C, then cycled 34 times through 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. The final annealing step was 10 min at 72 °C [24]. The region amplified by these primers includes the last 120 bp of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, and ITS2, with the 3126r primer matching the 5′ end of the 28S rRNA gene. The length of F-ARISA fragments was expected to range between 500 and 1200 bp [19].

The PCR products were purified (Express Matrix, Bio101, Inc., Vista, CA) and the resulting F-ARISA fragment lengths in each sample were analyzed using an ABI377 sequencer (Perkin–Elmer Inc., Foster City, CA). GeneScan (Perkin–Elmer, Inc., Boston, MA) was used to analyze each lane and assign fragment sizes based a rhodamine labeled size standard (MapMarker 1000, Bioventures, Murfreesboro, TN).

2.3. F-ARISA analysis

Any peak that had signal intensity greater than 5% of the highest peak and was between 350 and 1000 bp long was defined as an ARISA operational taxonomic unit (A-OTU). Asymmetric binary coefficients a, b, and c were used in the following equation to calculate the Jaccard distance coefficient for each pair of time points [25]:

$$D(i,j) = 1 - \frac{a}{a+b+c}, \quad (1)$$

where $D(i,j)$ is the Jaccard distance between the ith and jth sample; a, the number of fragments contained in both samples; b, the number of fragments contained in sample i, but not in sample j; c, the number of fragments contained in sample j, but not in sample i.

The Jaccard distance matrix was interpreted with a dendogram generated by Tree View (version 1.6.5) using the unweighted pair-group method by the arithmetic averages (UPGMA) algorithm contained within PHYLIP (http://evolution.genetics.washington.edu.phylip.html).

2.4. A-OTU clone library construction

Samples with high intensity bands of the most common A-OTUs were chosen to create an F-ARISA clone library. PCR was used to amplify a larger portion of the

18S rRNA in addition to the ITS1, 5.8S rRNA gene, ITS2 and part of the 28S rRNA gene region using the 18S1196f (GGAAACTCACCAGGTCCAGA) and 3126r primer pair. The PCR products were purified (Express Matrix, Bio101, Inc., Vista, CA), ligated according to manufacturers specifications into the pGEM-T EZ vector (Promega, Madison, WI), and transformed into *Escherichia coli* JM109. Each insert was re-amplified and double digested using *Hae*III and *Dpn*II restriction enzymes to identify unique clones. The exact length of the intergenic region for each clone was determined using F-ARISA as described above. The 18S rRNA gene fragment contained within the cloned PCR products that had ARISA fragments that were the same size as the previously identified A-OTUs were sequenced at the Cornell University Bioresource Center using primer 18S1196F. If more than one clone was identified with either the same restriction digest pattern or ARISA band, then at least two clones were sequenced. The phylogenetic origin of each clone was determined by comparing the DNA sequence to GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. 18S rRNA gene clone library construction

Two samples from reactors run for 0, 24, 48, 72, and 96 h were chosen at random as a template for the amplification of fungal 18S rRNA gene fragments using the 18S rRNA fungi-specific primers 18S817F (TTAGCATGGAATAATRRAATAGGA) and 18S1536R (ATTGCAATGCYCTATCCCCA) [24]. Unique cloned 18S rRNA gene fragments were identified after analysis of restriction endonuclease digestions of the PCR amplified inserts using *Hae*III, *Dpn*II and *Rae*I. Unique inserts were then sequenced as described above with the 18S817F primer.

3. Results

3.1. F-ARISA results

The average sample had 1.8 F-ARISA peaks and overall, there were 27 different A-OTUs and each sample contained at least one A-OTU. Table 1 summarizes the occurrence of the eight most common A-OTUs during the first 96 h. We screened 90 clones using restriction digests and ARISA and although all of the most common A-OTUs were targeted for cloning and sequencing, we were only able to clone and sequence four of the eight most common A-OTU fragments (Table 1).

A dendrogram generated from the A-OTU data (Fig. 1) shows that a similar community structure was found during the first 48 h and that the community structure in samples obtained from 60 to 96 h were most similar to each other. The difference in the community structure appears to be the result of populations similar to *Backusella* sp. (A-OTU of 769 bp) and *Amylomyces* sp. or *Rhizopus* sp. (A-OTU of 760 bp) being eliminated from the substrate and due the emergence of three populations with F-ARISA fragment sizes of 540, 552, and 621 bp. The clone with an F-ARISA fragment size of 621 bp was most similar to a *Pichia* sp. (99%). This change in community structure coincided with a rise in pH and temperature (Fig. 2). Both *Amylomyces* sp. or *Rhizopus* sp., which are in the Mucoraceae family, matched the 760 bp fragment.

3.2. Cloning and sequencing fungal DNA

As we were only able to identify four of the A-OTUs, we constructed 18S rRNA gene libraries as an alternative method for accessing the fungal diversity in the compost. Based on RFLP analysis of the 18S rRNA gene clones obtained from the four samples, we selected 26 of 80 clones for sequencing. All of the cloned sequences were at least 99% similar at the nucleotide level to previously sequenced 18S rRNA gene fragments (Table 2). Two of the most commonly found 18S rRNA gene fragments in the four clone libraries were similar to *Aspergillus* sp. and *Backusella* sp. Although there was little similarity in the collection of 18S rRNA sequences observed using the two cloning methods (ARISA vs 18S rRNA gene fragments), we found fragments similar to *Backusella* sp. with both approaches. No clones were obtained from the 96 h sample.

Table 1
Frequency of A-OTUs during the first 96 h of composting and the phylogenetic identity of four A-OTUs

A-OTU size (bp)	Closest match	Accession number	Time (h)								
			0	12	24	36	48	60	72	84	96
470			3	4	0	0	0	0	0	0	0
490			14	9	6	9	7	4	6	8	0
502	*Geotrichum* sp.	AB000652	1	0	0	6	4	5	5	0	0
540			6	0	0	0	0	3	3	1	9
552			2	0	0	0	0	3	1	0	2
621	*Pichia* sp.	X96461	1	0	0	0	0	1	0	0	5
760	Mucoraceae gen.	AY054696	0	0	1	2	3	0	0	0	0
769	*Backusella* sp.	AF157122	0	2	4	5	5	0	0	0	0

The frequency represents data pooled from 24 samples for 0 h and from nine samples for the other time points. Phylogenetic identity was obtained by BLAST and all database matches were greater than 99% for A-OTU sizes larger than 562 bp.

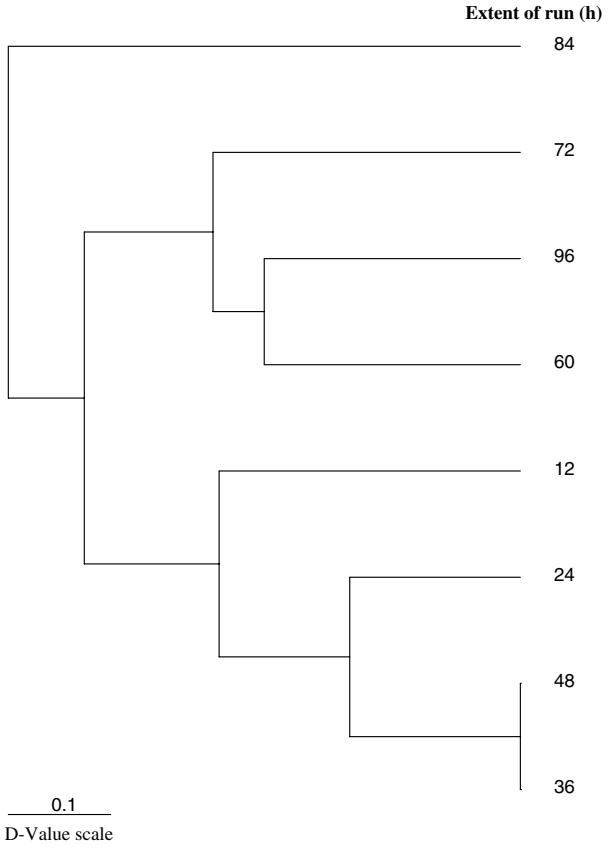

Fig. 1. Dendrogram of fungal community composition at 12 h intervals during the first 96 h of composting. Distances are calculated using the Jaccard distance matrix as described in Section 2.

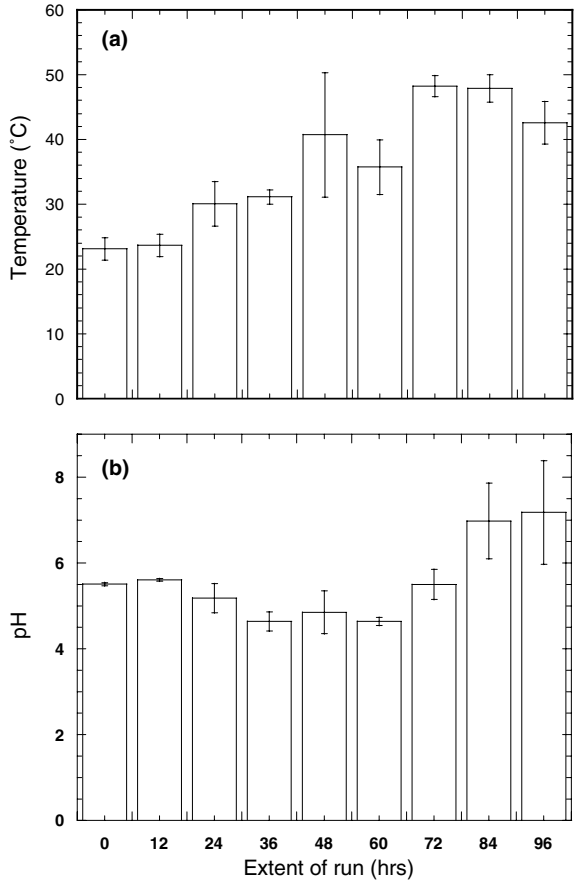

Fig. 2. Mean temperature and pH data collected from nine reactors at each time point. Error bars represent the standard deviation.

Table 2
Frequency and phylogenetic identity of 18S rRNA sequences obtained from libraries constructed at 0, 24, 48, 72, and 96 h

Time (h)	Number of clones	Closest BLAST match	Accession number
0	4	*Penicillium* sp.	AF218786
	1	*Candida* sp.	AB018151
	1	*Galactomyces* sp.	AB000646
	1	*Williopsis* sp.	Y12111
24	3	*Backusella* sp.	AF157122
	1	*Mucor* sp.	AF113430
48	3	*Aspergillus* sp.	M55626
	2	*Hamigera* sp.	AB003948
	1	*Backusella* sp.	AF157122
72	4	*Aspergillus* sp.	AB008397
	3	*Backusella* sp.	AF157122
	2	*Neurospora* sp.	X04971

Phylogenetic identity determined by BLAST of 724 bp against the GenBank database. All matches were greater than 99%.

4. Discussion

Fungi are major players in the degradation of biomass, yet their presence and participation during the initial phases of composting has been poorly characterized.

Our results show that the species and strain level richness reflected in the fungal A-OTUs was small when compared to the A-OTUs for bacteria [14]. This result was observed previously by Ranjard et al. [19] who found that fingerprinting techniques yield much less complex profiles when using primers specific for eukaryotes rather than bacteria.

Previous work using the same DNA template showed that *Weissella* sp., *Lactobacillus* sp., *Lactococcus* sp., and *Pediococcus* sp., members of the lactic acid bacteria, were present during the initial stages of our compost and might have been suspected to have played a role in the pH drop [14]. However, hybridization probing revealed that their abundance was very low until 72 h, well after the pH dropped. The presence of fungal lactic acid producers such as *Rhizopus* sp. or *Amylomyces* sp. is consistent with Schloss et al.'s [17] hypothesis that fungi contributed to the production of acidity and thereby changed the environment. The clear shift in community structure, which included the appearance of *Pichia* sp. and the disappearance of *Backusella*, *Amylomyces* or *Rhizopus* sp., coincided with a change in pH.

Although one previous study used molecular methods to characterize the fungal community of compost [12], they employed a much less frequent sampling regime to broadly characterize several different phases of composting. Of the putative fungal OTUs examined, they only identified two *Candida* species after compost had reached thermophilic conditions [12]. In contrast, the present work focuses more specifically on a single phase, the rise to thermophilly, and demonstrates that 13 different fungal genera (Tables 1 and 2) were detected during this dynamic period.

Although it is difficult to discern whether the fungal populations we have identified were active or if we merely sampled genomic DNA from resting cells, the dynamic temporal shifts for each F-ARISA fragment indicate that there was obvious turnover in the DNA. This suggests that the populations represented by these sequences were actively growing rather than vegetative. If the sequences were of vegetative populations, those detected early on should likely have been detected throughout the life of the experiment. This however, was not the case. In our previous analysis using 16S rRNA genes we reached a similar conclusion when we observed dynamic changes in the presence of gene fragments belonging to spore-forming bacteria [14]. These results demonstrate that the bacterial and fungal community structure in compost is dynamic. Despite these obvious shifts, it remains unclear as to which factors were causative and which were merely the result of community changes. It is possible that shifts in community composition were initiated due to changes in substrate availability, temperature, or pH. They may also have been affected by more specific microbe–microbe interactions.

The combination of F-ARISA and cloning was an effective way to quickly gather a large amount of information about the fungal community and the changes that occurred throughout composting. PCR and cloning biases however have been well documented [26,27] and for this reason further experimentation must be conducted before any quantitative assessments of community structure can be made. One possible outcome of PCR bias may have been our identification of *Backusella* sp. at 72 h with the 18S rRNA fragment primer set, but not with the F-ARISA primer set. Others have shown that the successful amplification of some small subunit rRNA genes can be influenced by the abundance and type of other rRNA genes present in the template [26]. It is therefore possible that the PCR competitiveness of the *Backusella* template became limiting as the total fungal community template changed. There is also a risk of poor specificity in our 18S rRNA gene primers. Ranjard et al. [19] found homologous regions in the BLAST database for some non-fungal eukaryotes.

Another limitation of the 18S rRNA gene cloning methodology employed here is the limited species-level sequence differences in the 18S rRNA gene. In several cases cloned sequences differed by only 1 or 2 base pairs from multiple different published sequences representing different genera, making more accurate identification impossible. Table 1 demonstrates this problem, with the sequence obtained for the 760 bp A-OTU identically matching species from two different genera preventing us from typing this A-OTU beyond the family level.

The relatively small number of bands observed in each sample, although they may have accurately reflected limited species richness, increase the chance that the relatedness profile presented in Fig. 1 could have been unduly influenced by idiosyncratic analytical anomalies. Although none were detected, these points highlight the need to complement fingerprint-based methods with more exhaustive cloning and sequencing strategies and the use of multiple PCR primer sets.

Although the experimental conditions employed here did not permit the compost to attain a thermophilic state as is achieved using large reactors, composting operations are diverse with no single method or set of conditions being universally applied [1]. Thus rather that trying to mimic a specific set of field conditions, we have pursued a line of research using a variety of laboratory scale reactors with a consistent substrate in an effort to model the core of the compost process. Given the high level of variability [20,28] between even model systems, much remains to be learned about the effect of reactor design, substrate, and various operational schemes. However, until we can identify the sources of variability in simple systems such as the one used in this study, it seems unlikely that analyses of more complex systems will be feasible.

Our study has begun to address the hypothesis that fungal populations play an intricate role in the early stages of composting. The results of this study suggest that a modest variety of fungi were present over the course of the first 96 h of composting. This qualitative assessment of fungal population dynamics is an important first step that may be useful in designing probe-based strategies for future quantitative studies. Such information will give a more complete picture of microbial succession in compost.

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