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# Identification of fungi associated with municipal compost using DNA-based techniques

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ABSTRACT

Fungi are important in terrestrial decay processes. However, fungi associated with organic decay during composting are still not well known. In this study culture-independent methods were used to identify fungi associated with composting organic municipal wastes to gain a better understanding of the diversity of fungi associated with this process. Fungal communities from 0, 210, and 410 day-old compost samples were assessed with DNA fingerprinting using denaturing gradient gel electrophoresis (DGGE) and by the analysis of DNA sequences from rDNA clone libraries. From 207 rDNA sequences, 82 fungal OTU's were detected. A disproportionate number of yeast sequences were detected in Day 0 clone libraries, including the human pathogens *Candida tropicalis* and *Candida krusei* (Saccharomycetales). Basidiomycetes accounted for over half of the clones from the Day 210 sample. Clones of *Cercophora* and *Neurospora* species accounted for most of the fungal clones of the Day 410 sample. No Zygomycetes or *Aspergillus* species were detected in this study. These findings call for a reassessment of long held views about the organisms involved in the composting of organic municipal wastes.

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## 1. Introduction

Composting organic wastes is an important pathway for carbon flow and cycling of nutrients, both in industrial and developing countries. Our knowledge of the microbiology of this process is limited, particularly concerning the fungi. Culture-based studies have yielded important, yet limited, information on fungal diversity in compost. The use of molecular tools is expected to increase awareness of the specific fungi involved in the composting process, due to the increased sensitivity of molecular methods over culture-based approaches for detecting microbial diversity. Knowledge of the fungi involved in composting could be valuable to microbial engineering efforts aimed at managing and improving the efficiency of composting and biomass-conversion processes.

Modern large-scale composting is accomplished by grinding organic matter into smaller particles, which are conveyed into long rows (windrows). The composting process is characterized by four phases: (1) the initial mesophilic phase (10–42 °C) during which the temperature rapidly rises, (2) the thermophilic phase (45–70 °C) distinguished by prolonged high temperatures, (3) the middle mesophilic phase (65–50 °C) during which temperatures decrease, and (4) the finishing phase (50–23 °C) during which the organic matter and biological heat production stabilize (Miller, 1996; Ryckeboer et al., 2003a,b). Although composting is considered to be an aerobic microbial process (Isikhuemhen et al., 1996), the high biological oxygen demand may lead to anaerobic fermentation. Forced aeration may be used to prevent anaerobic conditions and to accelerate the composting cycle (Fasidi et al., 1996; Kulcu and Yaldiz, 2004).

Although fungi are known to dominate terrestrial decomposition processes, their importance in composting is considered to be minor in comparison to that of bacteria (Miller, 1996). This is because self-heating that occurs during composting may reach temperatures too extreme for survival of eukaryotic organisms (>65 °C) and sometimes high enough to induce spontaneous combustion (Waksman et al., 1939; Cooney and Emerson, 1964; Peters et al., 2000; Ryckeboer et al., 2003a,b). Fungi have been cultured from all four phases of the compost cycle, but appear most prevalent during the initial and middle mesophilic phases (De Bertoldi et al., 1983; Ryckeboer et al., 2003a,b). The most dominant fungi cultured from the mesophilic phases are species in the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Emericella*, *Fusarium*, *Geotrichum*, *Mortierella*, *Mucor*, *Pencillium*, *Pseudallescheria*, *Scopulariopsis* and *Trichoderma*. *Absidia*, *Aspergillus*, *Chaetomium*, *Coprinus*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizomucor*, *Scytalidium* and *Thermomyces* are thermotolerant genera that have been isolated at higher temperatures (Waksman et al., 1939; Ghazifard et al., 2001; Vijay et al., 2002; Ryckeboer et al., 2003a,b; Anastasi et al., 2005). Pathogenic fungi recovered from

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composts include: *Aspergillus* sp., *Phialophora richardsii*, *Geotrichum candidum*, *Trichophyton*, *Epidermophyton*, *Candida albicans*, *C. krusei*, *C. tropicalis*, *C. guillermondii*, *Cryptococcus neoformans*, and *Trichosporon* sp., however these fungi are typically associated with composts amended with sewage sludge additions (Gaby, 1975; Burge and Miller, 1980; De Bertoldi et al., 1983; Dumontet et al., 2001).

Most information concerning the biology of composting is based on direct observation and culture studies. Culture-based studies may be biased against more fastidious or unculturable organisms. Culture-independent DNA-based methods offer an alternative approach to studying microbial diversity, and are usually more sensitive in detecting species than culturing. DNA sequence and fingerprinting approaches have already been used successfully to profile and characterize fungal communities in soils (O'Brien et al., 2005; Perez-Piqueres et al., 2006; Anderson et al., 2008; Bates and Garcia-Pichel, 2009). This pilot study was conducted to determine the feasibility of using DNA sequencing and fingerprinting to identify fungi associated with compost produced by the local municipality.

## 2. Methods

### 2.1. Study site

Compost samples were taken from three windrows at the City of Durham Composting Facility, North Carolina, USA during June 2004. This facility does not use forced aeration or irrigation, and consequently, a complete composting cycle may last for over 16 months. The source material of the sampled compost consisted of an unspecified mix of the following substrates that were collected from city residences and shredded: grass clippings, tree branches/stumps, and wood pallets, although small amounts of plastic and metal inevitably ended up in the mix. No wastewater or biosolid sludge was added to the composting mix. Windrows were moved and mixed about every 6 months with heavy equipment, and large uncomposted debris was separated out during these times. Sampling dates and times were coordinated with windrow mixing dates to ensure field samples were well homogenized.

### 2.2. Sampling

Compost windrows were sampled during spring and included; a mix of freshly shredded substrates (Day 0) that had accumulated over a few months, material that had composted 7 months and that was being mixed and separated for the first time (Day 210), and finished compost that was being mixed and separated for a third time (Day 410). For each time period sampled, approximately 0.5 $m^3$ of homogenized compost was collected from the conveyer belt after mixing. Compost windrow temperatures were measured in the field by inserting a thermometer 0.3 m into each newly formed compost windrow and taking a measurement after 15 min. Samples were immediately brought back to the laboratory for processing and were thoroughly mixed and shaken. Sub-samples were then taken to calculate field moisture, pH, and carbon and nitrogen concentrations.

Sub-samples for carbon and nitrogen analysis were freeze-dried before being ground to a fine dust in a Wiley mill. Carbon and nitrogen concentrations were analyzed on a Carlo Erba Elemental Analyzer (Milan, Italy). Equal volumes of compost sample and deionized water were stirred into a slurry in a 250 ml beaker, and after 30 min pH measurements were taken with an Orion 310 pH electrode. Percent moisture was calculated by the difference in mass between samples at field moisture and after being dried in an oven for three days at 65 °C.

Compost samples used for DNA analyses first were sieved (2 mm) and then were frozen in liquid nitrogen and ground to a dust with a sterile mortar and pestle. Total DNA was extracted from a 0.5 g subsample of this material with the MoBio Laboratories Ultraclean Soil DNA extraction kit and according to manufacturer's instructions (MoBio, Solana Beach, CA). In order to minimize DNA extraction biases, two DNA extractions were performed for each of the three samples. Purified DNA samples were eluted in 50 μl of sterile double distilled water and were stored at −20 °C until further analysis.

### 2.3. Molecular methods

Fungal DNA fingerprinting through Denaturing Gel Gradient Electrophoresis (DGGE) as outlined by Janse et al. (2004) was used to assess the complexity of eukaryotic community assemblages in the compost samples. Total DNA extracted from compost samples was PCR amplified with the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), the former of which was modified with a 40-bp GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) to stop DNA migration during the gel electrophoresis (White et al., 1990; Muyzer et al., 1993). The ITS ribosomal DNA gene region was chosen because it shows high variation between most species of fungi in both length and sequence and is commonly used in molecular fungal ecology (Smith et al., 2007; Peay et al., 2008). The PCR protocol included a final extension at 72 °C PCR for 30 min to reduce PCR artifacts (Janse et al., 2004). The urea denaturant gradient of the gel increased from 0% to 60%.

Although the ITS region is valuable for identifying species and operational taxonomic units (OTUs), the ribosomal large subunit (LSU) is more value for community phylogenetic approaches because it can be aligned across the fungal kingdom. Therefore, for clone libraries, the 5.8S ribosomal gene and both the internal transcribed spacer region 2 (ITS2) and a portion of the LSU were amplified from compost DNA extracts using universal primer set 5.8SR and LR5 (Vilgalys and Hester, 1990). Two PCR reactions were made for each DNA extract to minimize PCR biases. The PCR protocol began with an initial denaturation at 94 °C for 3 min, followed by 20 cycles at 94 °C with a 2 min extension time, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Twenty cycles were used rather than 35 in order to reduce PCR bias against rare or long DNA templates. Each 25 μl PCR reaction consisted of 0.2 mM each dNTPs, PCR buffer including 1.5 mM of MgCl, 25 μg BSA, 0.5 μM each 5.8SR and LR5 primers, 0.75 U *Taq* DNA polymerase, and 10 μl DNA extract.

PCR products were cloned into *Escherichia coli* plasmids with the Topo-TA 5-min PCR cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. One clone library was made from each PCR reaction, resulting in a total of 12 clone libraries. Maximum recommended incubation times were used for each step, and 2 μl of PCR product was used in each reaction. Inserts from white colonies were amplified by adding whole cells directly to PCR reactions using the primer set M13 F and M13 R (Invitrogen, Carlsbad, CA). A "colony PCR" protocol beginning with an initial denaturation of 94 °C for 10 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min was used to amplify cloned regions. Each 25 μl PCR reaction consisted of 0.2 mM of each dNTPs, PCR buffer including 1.5 mM of MgCl, 10 μg BSA, 0.5 μM forward primer M13 F, 0.5 μM reverse primer M13 R, 0.75 U *Taq* DNA polymerase, and 15 μl water. PCR products containing the insert were purified by affinity chromatography with QIAquick spin columns (Qiagen, Valencia, CA). Cycle sequencing was performed with Big Dye chemistry version 3.1 (Applied Biosystems, Foster City, CA)

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