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NOTE

Diversity of Microorganisms in Decaying Maize Stalks Revealed by a Molecular Method

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Microbial diversity in decaying maize stalk was characterized by constructing and analyzing rRNA gene clone library. Total 47 OTUs were obtained from 82 bacterial clones, including Proteobacteria (64.6%), Actinobacteria (30.5%), Bacteroidetes (2.4%) and Firmicutes (2.4%). Most proteobacterial clones were members of *Rhizobium*, *Pseudomonas* and *Stenotrophomonas*. Eighty-four percent of Actinobacteria was related to *Microbacterium*. Only 14 OTUs were identified from 124 fungal clones, including Ascomycota (88%) and Basidiomycota (12%). Sixty percent of Ascomycota were members of *Eupenicillium* and *Paecilomyces* but all Basidiomycota were close to *Kurtzmanomyces nectairei*.

Keywords: microbial diversity, maize stalk, decomposition, rRNA clone library

How to manage effectively the postharvest residues has always been an important issue for sustainable development. In China, crop residue is numerically large, with a production of approximate 800 million tons per year. Traditionally, they are removed for fodder, cooking fuel, fencing, or are burned *in situ*. In harvest season, burning stalk residues *in situ* seriously decreases air visibility and disturbs traffic (Zhong *et al.*, 2003). These treatments also accelerate losses of soil organic matter, increasing CO_2 emissions and reducing soil microbial activity (Lal, 2004). In recent, return of residues into the field, such as leaving crop residues on the soil surface, incorporating them into the soil, has been popular in enhancing soil quality. However, these applications can create impediments to following cropping as incomplete microbial decomposition, and in some cases, they aggravate residue-born disease of crops (Bockus and Shroyer, 1998). Although the production of biofuel endows these materials with potential perspectives (Coombs *et al.*, 1987), the biotransformation is low effective due to the scarce knowledge of synergistic interactions among microbes (Lynd *et al.*, 2002). Therefore, understanding microbial community in stalk degradation process is essential for these biotechnological practices. However, previous studies have been focused on species with significant cellulose-degradation, such as white-rot and brown-rot fungi (Lynd *et al.*, 2002). In addition, most studies apply nutrient-rich media to isolate microbes. It is well-known that crop residues are biodegradation-resistant and most microorganisms in this ecosystem are assumed to be oligotrophic thus can not be cultured by traditional culture medium (Hu *et al.*, 1999).

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In this study, a molecular method was applied to characterize microbial species in decaying maize residues. Maize stalk (about 1 kg) was collected from field at Kunming, China, in January, 2005. Because of low temperature and moisture when we sampled, microbe is metabolically low. To observe active species for decomposition, we used soil block culture to stimulate their growth till significant decay occurred (Jasalavich *et al.*, 2000). Decay stalks were then grounded into fine powder with liquid nitrogen and total microbial DNA was extracted by the DNeasy Plant Mini kit (Qiagen). rRNA genes were amplified with universal bacterial primers F984 and R1401 (Nubel *et al.*, 1996) and with fungal primers SSU-0817 and SSU-1196 (Borneman and Hartin, 2000). The purified PCR products were ligated into the pGM18-T vector (Takara, China) and transformed into competent cells (*Escherichia coli* HB101) to construct rRNA gene clone library. Correct inserted DNA fragment obtained from transformant was digested separately with 1.0 U of *Rsa*I, *Bsu*RI, and *Hin*fI (Bio Basic Inc.) (Filion *et al.*, 2004). Clone with unique restriction fragment length pattern (RFLP) was considered as an operational taxonomic unit (OTU). Subsequently each representative clone of each OTU was commercially sequenced. DNA sequences were edited and deleted primer using DNAstar package. Chimeras were checked and excluded from further analysis using the CHIMERA_CHECK program (http://rdp.cme.msu.edu/html/). The closest relatives were downloaded from the RDP database for bacteria and NCBI for fungi. Sequence similarity of bacteria was represented by S_ab value, which was defined as the number of (unique) oligomers shared between our sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences (http://rdp8.cme.msu.edu/docs/seq_match_doc.html). All sequences were aligned

using CLUSTAL X1.83 and neighbor-joining tree was constructed using Phylip 3.65 (Felsenstein, 1989). Species richness was determined by the Shannon-Weaver diversity index (H') (Hill *et al.*, 2003). These sequences were deposited in GenBank with accession numbers from DQ881468 to DQ881515 for bacterial clones and from DQ881454 to DQ881467 for fungal clones.

In laboratory, soil block culture simulated a warm and moist season when stalks were rapidly decomposed in nature. After two months' cultivation, half of maize stalk biomass (51%) was lost. A variety of microbes was detected in this decomposition ecosystem. Total 14 OTUs were obtained

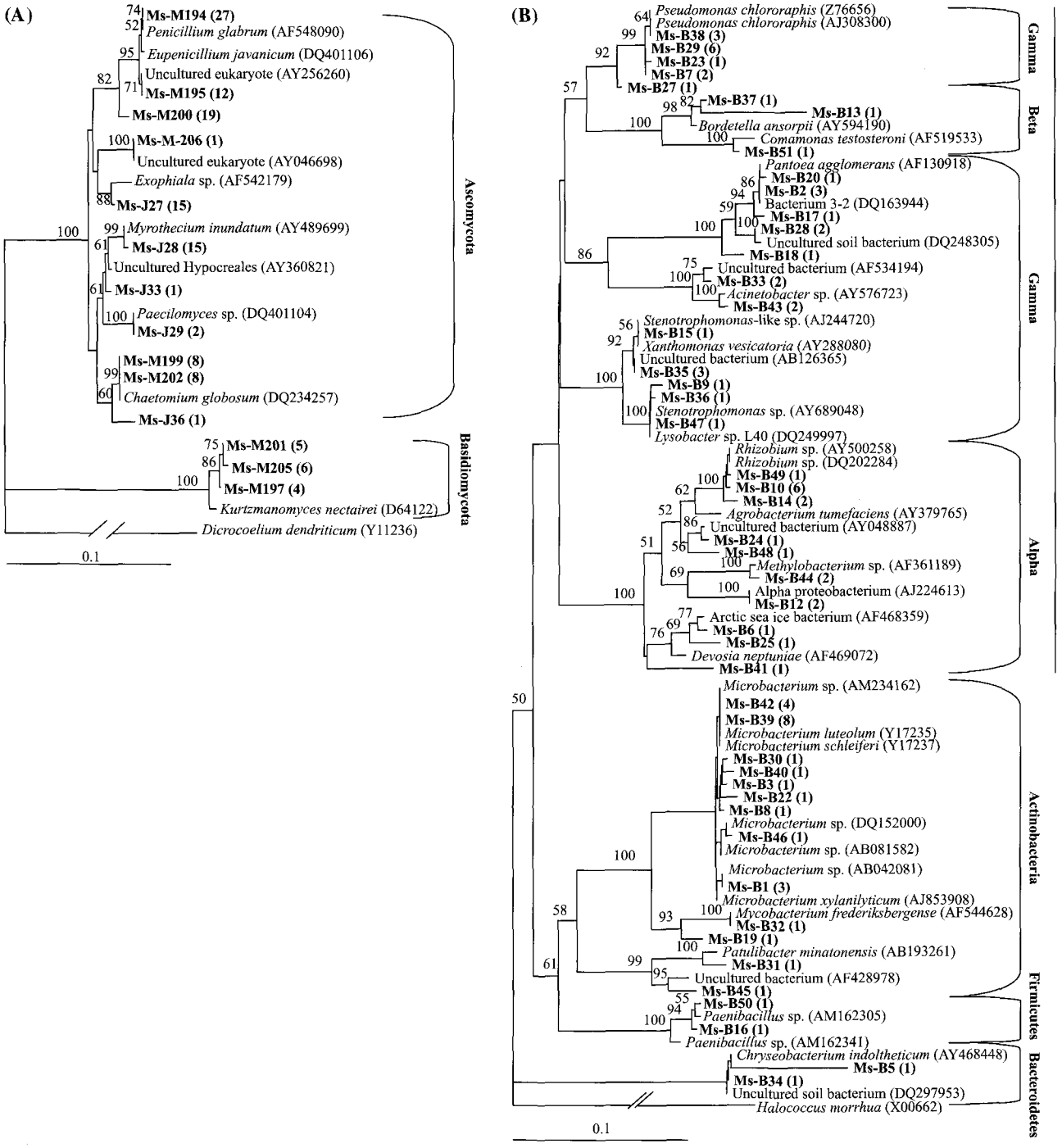

Fig. 1. The phylogenetic relationships of fungal (A) and bacterial (B) OTU sequences to the most closely related sequences obtained from GenBank and RDP database. Clone number of each OTU was indicated in the following parenthesis. The numbers at the nodes are the occurrence percentage with 1,000 bootstrap resamplings (values below 50% are not shown). Trematodes *Dicrocoelium dendriticum* (Y11236) is used as an outgroup in fungal phylogenetic tree, whereas an archaebacteria *Halococcus morrhua* (X00662) is used as an outgroup in bacterial phylogenetic tree. The bar represents 0.1 substitution per site.

from 124 fungal clones (no chimera was found). These OTUs were grouped into Ascomycota and Basidiomycota (Fig. 1A). Ascomycota contained 11 OTUs (88% of total fungal clones). Some OTUs were related to the previously reported cellulose-degrading species. For example, three OTUs, Ms -M194, -M195, and -M200 (involving 58 clones) were clustered with a cellulose-producing species *Eupenicillium javanicum* (Tanaka *et al.*, 1981). Three OTUs (Ms-J36, -M199, and -M202) were closely related to *Chaetomium globosum* (sequence similarities of 98-100%), one of the most widespread cellulose- and xylan-degrading moulds (Gandhi *et al.*, 1993). OTU Ms-J28 was close to *Myrothecium inundatum* (a sequence similarity of 99%). Although many species of this genus can degrade cellulose (Ahrazem *et al.*, 2000), no report is found to describe this ability of *M. inundatum*. OTU Ms-J29 was related to a common lignin-degrading fungus *Paecilomyces* (a sequence similarity of 100%). No clone was found to affiliate with well-confirmed cellulose-degrading *Aspergillus* and *Trichoderma* (Lynd *et al.*, 2002). Possibly these species played a minor role in the degradation of maize stalks. Notably, all Basidiomycota (three OTUs, 15 clones) were closely related to *Kurtzmanomyces nectairei* (a sequence similarity of 98%), a species rarely mentioned in literature describing cellulose-decomposing microbes.

Compared to fungal role in degrading cellulosic materials, bacteria are greatly ignored (Lynd *et al.*, 2002). However, 47 OTUs were obtained from 82 clones (8 chimeras were excluded from analysis). Bacteria showed a more diversified distribution in decaying maize stalk than fungi (H'=3.59 vs 2.28). These sequences fell into Proteobacteria (64.6%), Bacteroidetes (30.5%), Firmicutes (2.4%), and Actinobacteria (2.4%) (Fig. 1B). Proteobacteria included α-, β-, and γ-subdivision. Half of α-proteobacterial clones were members of genus *Rhizobium* and other nitrogen-fixing bacteria such as *Devosia neptuniae*, agreeing with previous viewpoint (Spano *et al.*, 1982), that is, nitrogen-fixing bacteria are a common component of the microflora active in cellulose decay. Subdivision β only contained 3 OTUs, including 2 *Bordetella* and 1 *Comamonas* species. γ-Proteobactria consisted of 17 OTUs (32 clones), related to *Pseudomonas, Pantoea, Acinetobacter,* and *Stenotrophomonas*. Thirteen clones were affiliated with *Pseudomonas chlororaphis* (S_{ab} values ranged from 0.924 to 0.982). To our knowledge, no cellulolytic ability is reported to this species. However, it is a common biocontrol agent for fungal pathogen (Tombolini *et al.*, 1999). Their roles in maize decay system still needed to be characterized.

The majority of Actinobacteria (84% of 25 clones) was related to one of aerobic cellulolytic bacteria *Microbacterium* (S_{ab} values ranged from 0.962 to 1.000) (Lynd *et al.*, 2002). OTU Ms-B32 was identical to a polycyclic aromatic hydrocarbon (PAH)-degrading species *Mycobacterium frederiksbergense* (S_{ab}=1.000). Additionally, there were only four clones related to division Firmicutes and Bacteroidetes. Notably, many bacteria common in anaerobic degradation process, such as *Clostridium, Cellulomonas* (Hethener *et al.*, 1992; Lynd *et al.*, 2002), are not observed in present study. Possible reason is that the aerobic soil block culture hinders their growth or a significantly different bacterial community maybe carries out the decomposition of these maize stalks.

Because soil block culture completely hampered species succession between stalk and natural soil microbial communities, current sequences only represented those microbes colonized on stalks when we sampled and grown up under culture condition. In addition, due to low resolution, RFLP analysis of 400 bp-length fragment of rRNA gene also tended to underestimate microbial diversity in decaying maize stalk. Therefore, the actual microbes involving into stalk decay in nature may be more complex than present data. However, in the first time we applied molecular method to reveal the diversified microbial species in this ecosystem. Beside some cellulose-degrading species, this snapshot of microbial community also verified the existence of many species without any described cellulose-degrading activity, such as *Kurtzmanomyces nectairei.* Moreover, 2 fungal and 7 bacterial OTUs were grouped only with the uncultured clones (Fig. 1). Our data are valuable either for understanding the interaction between fungal and bacterial species, and between cellulolytic and non-cellulolytic species, during the process of maize stalk decay or for searching novel microbes decomposing maize residues by specific isolation strategy.

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