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Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity

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ABSTRACT

Bacterial communities and chitinase gene diversity of vermicompost (VC) were investigated to clarify the influence of earthworms on the inhibition of plant pathogenic fungi in VC. The spore germination of *Fusarium moniliforme* was reduced in VC aqueous extracts prepared from paper sludge and dairy sludge (fresh sludge, FS). The bacterial communities were examined by culture-dependent and -independent analyses. Unique clones selected from 16S rRNA libraries of FS and VC on the basis of restriction fragment length polymorphism (RFLP) fell into the major lineages of the domain bacteria *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria* and *Firmicutes*. Among culture isolates, *Actinobacteria* dominated in VC, while almost equal numbers of *Actinobacteria* and *Proteobacteria* were present in FS. Analysis of chitinolytic isolates and chitinase gene diversity revealed that chitinolytic bacterial communities were enriched in VC. Populations of bacteria that inhibited plant fungal pathogens were higher in VC than in FS and particularly chitinolytic isolates were most active against the target fungi.

1. Introduction

The paper mill and dairy industries face considerable challenges particularly in the area of water availability, waste water discharge, chemical residues and solid waste disposal or utilisations. The main waste from the dairy industries is semi-solid of about 70% moisture content and contains protein, salts, fatty substances, lactose, and residues of additives. While, paper sludge is a complex material, typically composed of cellulose fibers, synthetic polymers, starch, clay and inorganic additives. In recent years, vermicomposting has received much attention as an efficient and low cost mean of composting organic waste such as paper-pulp and the sludge from paper mills and dairy plants (Banu et al., 2001). The end product of vermicomposting is rich in essential nutrients such as nitrogen, phosphorus, magnesium, zinc, calcium and contains abundant numbers and types of microorganisms (Banu et al., 2001; Elvira et al., 1998). Adding cast to soil improves soil structure, fertility, plant growth and suppresses diseases caused by soil-borne plant pathogens, increasing crop yield (Chaoui et al., 2002; Scheuerell et al., 2005; Singh et al., 2008). Vermicomposting decomposes organic materials through the joint action of earthworms and microorganisms that inhabit gut or composting substrates. The rapid transformation in physicochemical and biochemical properties makes vermicomposting suitable for management of industrial wastes (Garg et al., 2006; Vivas et al., 2009).

The microbial community in sludge is profoundly affected by the vermicomposting activity of various earthworm species. There tends to be an increase in bacterial population or activity of the cast after passage through the gut. The microbial flora of earthworm gut and cast are potentially active and can digest a wide range of organic materials and polysaccharides including cellulose, sugars, chitin, lignin, starch and polylactic acids (Aira et al., 2007; Vivas et al., 2009; Zhang et al., 2000).

Chitinases are digestive enzymes that hydrolyze the beta 1–4 glycosidic bonds of N-acetylglucosamine residues in chitin and are classified into glycosyl hydrolase subfamilies 18 and 19 on the basis of amino acid similarities within the catalytic domain (Henrissat and Bairoch, 1993). Chitinases are commonly found in many bacteria and fungi within soil which can degrade chitin, a component of fungal cell wall and a constituent of the exoskeletons of worms and arthropods (Hoster et al., 2005; Meanwell and Shama, 2008; Yu et al., 2008). Among the chitinolytic bacteria, several *Actinobacteria* and *Streptomyces* species are thought to degrade the chitinous cell wall of plant fungal pathogens through the production of chitinases and antibiotics (Kawase et al., 2006; Yu et al., 2008). Such chitinolytic bacteria are known to be involved in the suppression of plant fungal pathogens and are used

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for biocontrol of soil-borne fungal diseases (Hoster et al., 2005; Singh et al., 1999). From research and field trials around the world, evidence supports the hypothesis that soil-borne diseases are significantly suppressed by VC, with the suppression being frequently related to the level of bacterial community and activity in the vermicompost (Chaoui et al., 2002; Scheuerell et al., 2005). In this study, we observed that bacterial numbers were significantly increased in VC and that the aqueous extract of VC decreased the spore germination of *Fusarium moniliforme* as compared to FS. Presently, we assessed the influence of vermicomposting on the resident bacterial community of FS and chitinase gene diversity, which may be involved in the suppression of soil-borne plant pathogenic fungi, by using both culture-dependent and culture-independent methods. The recovered species were analyzed for their chitinase encoding gene profiles and screened for antifungal activity against several plant pathogenic fungi.

2. Methods

2.1. Sample collection and chemical analysis

FS and VC were collected from a commercial VC farm (Chung Weon Sang Sa, Masan, Korea). Paper sludge was mixed with dairy sludge to adjust the C:N ratio and FS was treated with earthworms (*Eisenia fetida*) in 10:1 ratio (w/w), respectively. The newly produced VC samples after 12–15 days composting were collected in the early February (winter) and June (summer) of 2006. The average temperature was 9 and 23 °C in the vermicompost reactor during February and June, respectively. The summer samples were immediately processed for cultivation and used for the rest of the experiments. The samples were stored at −70 °C for total DNA isolation and at 4 °C for chemical analysis. The pH was measured using digital pH meter (Schott, Germany) in a 1/10 (w/v) aqueous solution using deionized water. Total carbon (TC) was determined by the partial-oxidation method, total nitrogen (TN) was measured by the micro Kjeldahl method, and total phosphorus (TP) was analyzed colorimetrically with molybdenum in sulfuric acid (Bremner and Mulvaney, 1982). Exchangeable elements (K^+, Ca^{2+}, and Mg^{2+}) were determined after extracting the samples with ammonium acetate and analyzed using an Avanta atomic absorption spectrophotometer (GBC Scientific, Dandenong, Australia). To evaluate the results for field application, samples were collected from the commercial vermicomposting reactor producing fertilizer.

2.2. Cultivation experiments

Accurately weighed samples consisting of 1 g (fresh weight) from each sample were dispersed in 100 ml aliquots of sterile distilled water and were untreated or sonicated prior to serial dilution (10^{-3}–10^{-7}) and 100 μl of each dilution from each sample were spread on three replicate plates of each of the three solid media tested. The growth media used were nutrient broth (NB; 0.08 gm/L; Difco Laboratories), NB with 0.5% (w/v) swollen chitin derived from crab shell treated with H_3PO_4 and 0.1% Tryptic Soy broth (TSB; Difco Laboratories). All media were supplemented with 20% (w/v) of 0.2 μm filter sterilized sample aqueous extract and 1.5% (w/v) agar. The plates were incubated at 25 °C in the dark. After 2 weeks, colony forming units (cfu) were counted on nutrient agar media and cfu of the three replicates were used to estimate the population of bacteria. Swollen chitin media were used for analysis of chitinolytic bacteria. The highest dilutions showing growth in each medium were streaked onto solid media of the same composition for isolation of pure cultures.

2.3. 16S rRNA libraries

PCR reactions of the 16S rRNA gene were performed using the eubacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) from total genomic DNA. The modified PCR condition used on four replicates for each sample were: 94 °C for 3 min (1 cycle); 94 °C for 1 min, 55 °C for 45 s and 72 °C for 1.5 min (15 cycles); and 72 °C for 10 min (1 cycle) to minimize the accumulation of the known artifacts of *Taq* errors, chimeras and hetero duplex molecules. The four replicates of PCR amplifications were combined from each sample purified using a PCR purification kit (Qiagen, Valencia, CA) and resuspended in 40 μl of milliQ water. The resuspended PCR products were amplified again by five additional PCR cycles. The modified PCR products were ligated in the pCR2.1 vector of a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cloned insert DNAs were analyzed by RFLP using vector-specific primers (Jeon et al., 2003). Clones were grouped according to their RFLP patterns and representative clones containing distinct RFLP patterns were sequenced as described previously (Jeon et al., 2003).

2.4. Chitinase gene libraries

Chitinase clone libraries were constructed from community DNA extracted from FS and VC. Degenerate PCR primers targeted to a gene fragment from family 18 group A chitinases were used to amplify chitinase gene from the samples (Williamson et al., 2000). The thermocycling conditions consisted of 94 °C for 5 min, followed by 28 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 45 s with a final elongation step of 72 °C for 10 min. Reactions were performed in triplicate and subsequently pooled. Fragments (440 bp) were purified from 1.5 % low melting agarose gel and amplicons were ligated into a pCR2.1 vector. Cloned insert DNAs were analyzed by RFLP using vector-specific primers and sequenced as described previously (Jeon et al., 2003; Xiao et al., 2005). Family 18 chitinase genes from chitinolytic bacteria were amplified with the same set of primers and PCR conditions, grouped according to their RFLP pattern and sequenced as described for the chitinase gene libraries. The family 19 chitinase genes were amplified by using degenerate primers as described previously (Kawase et al., 2004).

2.5. Antifungal activity

The aqueous extract of each sample with a concentration of 50% (w/v) was filtered through Whatman filter paper no. 2, autoclave sterilized and sterilized by filtration through 0.2 μm pore size nitrocellulose membrane filter (Sartorious AG, Goettingen, Germany). Aliquots of 500 μl from FS, VC extracts and sterile tap water (control) were supplemented with 0.5% glucose and transferred to wells of a 24 well culture plate. Fungal spores from *F. moniliforme* were harvested by scratching mycelia mats with a rubber scraper and filtering the material through a double layer of cheese cloth. The filtered material was suspended in the each aliquot to give the final concentration of 4.15×10^6 ml^{-1} spores, as determined using a hematocytometer. The plate was incubated at 25 °C and the numbers of germinated spores were counted after 12 h of incubation (Bajpai et al., 2008).

Inhibitory activity of isolated strains on the growth of plant pathogenic fungi (*Rhizoctonia solani*, *Colletotrichum coccodes*, *Pythium ultimum*, *Phytophthora capsici* and *F. moniliforme*) was determined by confrontation bioassay using paper discs. Paper discs were impregnated with 20 μl of a bacterial suspension containing approximately 10^8 cfu ml^{-1} bacteria and were placed on potato dextrose agar opposite to the target fungi. The plates were