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Microbial analysis of a composted product of marine animal resources and isolation of bacteria antagonistic to a plant pathogen from the compost

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A composting product of marine animal resources has been used as a fertilizer and a soil conditioner in Japan. This compost was produced by a repeated fed-batch fermentation system with three successive aerobic bioreactors. Composting temperature reached about 75°C without heating. The bacterial diversity in this compost was investigated by denaturing gradient gel electrophoresis (DGGE) and sequence determination of the V3 region in the 16S rRNA genes. The sequence analysis showed that a majority of retrieved sequences corresponded to those of *Bacillaceae*, and we frequently found sequences similar to the 16S rDNA sequences of *Bacillus thermocloacae* and *Bacillus thermoamylovorans*. In addition, a bacterium antagonistic to a *Fusarium* strain was isolated from the compost. The isolate (*Bacillus* sp. NP-1) produced an antifungal compound, iturin A. These results suggest that this compost serves as a valuable source of plant growth-promoting rhizobacteria including the antifungal bacteria.

Key Words——DGGE; iturin; plant growth-promoting rhizobacteria; 16S rRNA gene; soil fertilizer; thermophilic bacteria

Introduction

Composting is a biological process for conversion of degradable organic materials into stable and usable products such as fertilizers and soil conditioners (Keeling et al., 2003). A merchandized compost (produced by Miroku Co., Oita, Japan) is a degradative product of marine animal resources including small fishes, shrimps and crabs. This compost, referred to as MAR (marine animal resources) compost in this paper, has been used for more than 20 years in Japan. Application of MAR compost to soil resulted in an increase of crop yield, and suppression of plant diseases caused by several plant pathogens. From this observation, it has been postulated that plant growth-promoting rhizobacteria (PGPR) exist in the MAR compost. PGPR

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would increase plant growth via several different ways including growth inhibition of phytopathogenic microorganisms (Haas and Défago, 2005; Whipps, 2001). At present, studies of microbial communities in MAR compost are limited. Two thermophilic *Bacillus* spp. were isolated from MAR compost. These microorganisms grew on a colloidal-chitin plate, and their culture fluid contained several thermostable exo- and endochitinases (Sakai et al., 1994, 1998). However, no comprehensive analyses of bacterial diversity in MAR compost or isolation of antagonistic bacteria to plant pathogens have yet been conducted. The addition of an antifungal bacterium during the fermentation process can strengthen the activity of the compost for suppression of plant disease mediated by the plant pathogens as previously reported in the composting products of soybean curd residues (Mizumoto et al., 2006). Thus, characterization of PGPR in MAR compost would allow us to improve this compost which shows enhanced plant-growth promoting and antifungal activities.

The objectives of this research were to introduce a unique composting method which produces an interesting compost having two effects: promoting plant growth and suppressing plant pathogens. We further wished to analyze the bacterial community in this compost by using two complementary methods: polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) analysis of the V3 region of the 16S rRNA gene and determination of the nucleotide sequences found in the 16S rDNA library. Finally, we aimed to isolate microorganisms antagonistic to plant pathogens, to characterize the suppressive effect of the isolate and to identify antifungal compounds.

Materials and Methods

Composting. For production of MAR compost, a repeated fed-batch fermentation system was adopted. A process flow diagram is shown in Fig. 1. Composting was carried out using three bioreactors with the same volume (2 mϕ $\times$1.3 m height). Coffee-extracted residues were mixed with the marine animal resources, such as small fishes, shrimps, and crabs. Composting substrates equivalent to one-third volume of the bioreactor were loaded to the first bioreactor (F0) daily. Simultaneously, the fermented F0 products with the same volume as the input substrate were transferred to a second bioreactor (F1) to permit repeated feeding. Similarly, transfer of fermented F1 products to a third bioreactor (F2) was also carried out daily (Fig. 1). Mechanical stirring with an impeller was performed in each bioreactor to keep the reactor aerobic. The final withdrawn F2 products were dried by airing under the sun for 1 d, and then packaged. MAR compost, which consists of dried particulates of about 1–3 mm in size, was marketed as MIROKU or RAKUNOU-NO-TANE. For microbiological analyses, the MAR compost produced in April, May, June and November 2004, were subjected to PCR-DGGE analysis.

PCR-DGGE analysis. The diversity of bacterial communities in the MAR compost was analyzed by PCR-DGGE. Total DNA was isolated from about 0.1 g compost by using the ISOPLANT reagent (Nippon Gene,

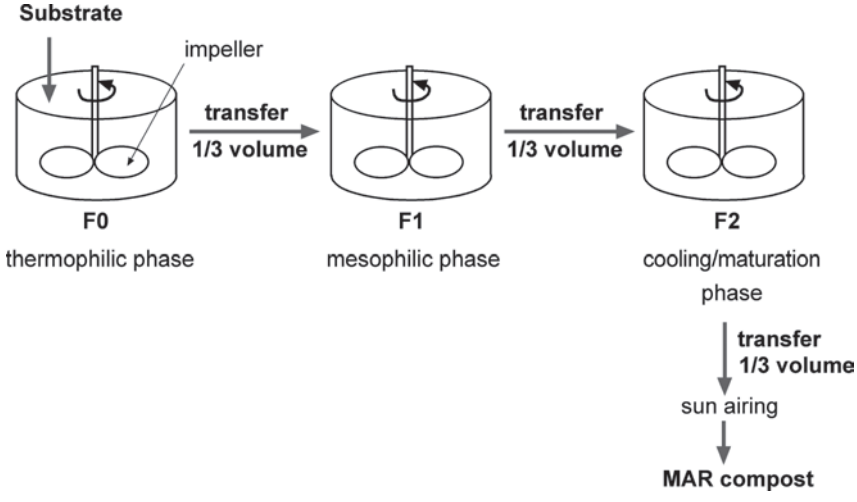

Fig. 1. Process flow diagram of production of MAR compost.
Substrate loading and transfer of fermented products were carried out every day.

Japan) according to the manufacturer's protocol. The variable region of bacterial 16S rDNA genes (338−534, *Escherichia coli* numbering system) (Brosius et al., 1978) was amplified by using following primers: Fs5; 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGGACTCCTACGGGAGGCAG CAG-3′, and Rs5; 5′-ATTACCGCGGCTGCTGG-3′ (Ovreas et al., 1997; Pedro et al., 2001). The resulting PCR fragments of about 240 bp in length were subjected to DGGE (Ishii et al., 2000).

DGGE was performed on a D-Gene apparatus (Bio-Rad, USA). PCR samples were applied onto 8% (w/v) polyacrylamide gels with a denaturing gradient ranging from 30% to 60%. One hundred percent of denaturant corresponded to 7 M urea and 40% (v/v) formamide. Samples were electrophoresed at a constant voltage of 45 V for 16 h. After electrophoresis, the gels were stained with SYBR Green I (TaKaRa, Japan) and photographed.

Sequence determination of the 16S rDNA fragments. The approximately 200-bp 16S rDNA fragments were amplified as mentioned above. The resulting fragments were blunt-ended with a BKL kit (TaKaRa) and cloned into the *Sma*I site of a plasmid vector, pUC119 (GenBank accession no. U07650) in accordance with the manufacturer's protocol. The cycle sequencing reaction was performed using a CEQ dye terminator cycle sequencing kit, and the resulting DNA ladder was detected on a Beckman CEQ2000 capillary sequencer. Alignments and database identification of the 16S rDNA sequences were carried out using BLASTn program. Operational taxonomic units (OTUs) were defined as clones that shared 97% or greater sequence similarity (McGarvey et al., 2004).

To elucidate the bacterial species in more detail, an approximately 570-bp-long variable region of bacterial 16S rDNA genes (341−907, *Escherichia coli* numbering system) was amplified by using following primers: ba341f (5′-CCTACGGGAGGCAGCAG-3′) and ba907r (5′-CCGTCAATTCMTTTRAGTT-3′). The resultant fragments were cloned and their sequences were determined as mentioned above. After the alignment of sequences was carried out, distances were determined with CLUSTAL W (Thompson et al., 1994) and the neighbor-joining trees were drawn with TREEVIEW (Page, 1996).

A nearly full-length 16S rDNA sequence of a bacterial strain showing the strongest inhibitory effect against the *Fusarium* mycelia growth was amplified by using following primers: 27f (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1525r (5′-AAAGGAGGTGATCCA GCC-3′). The resultant 1.5-kbp-long fragment was cloned and its sequence was determined as mentioned above.

Isolation of antagonistic bacterium. Fusarium sp. used in isolation of antagonistic bacterium was kindly provided by Dr. Shinoyama (Meisei University, Japan). To determine the fungal species, the 5.8S nuclear rDNA together with its flanking internal transcribed spacer (ITS) sequences were amplified by using the following primers: ITS5 (5′-GGAAGTAAAAGTCGTAA CAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG C-3′) (Turner et al., 1998). The resultant 691-bp-long fragment was cloned and its sequence was determined as mentioned above. This strain was isolated from mold on post-harvested banana peels, and was routinely maintained on Potato Dextrose Agar (PDA; Becton Dickinson, USA). The mycelia plug of *Fusarium* and a filter disc on which the granules of MAR compost were placed were incubated on PDA for 3−5 d at 25°C. Growth of bacteria was observed around the MAR compost and an inhibitory zone formed around the bacteria. Appropriate amounts of bacteria were suspended in 1 ml of sterilized water, and suspensions with different dilution ratios were spread on PDA and incubated at 25°C. Thirty nine colonies were isolated, and each was incubated with a mycelia plug of *Fusarium* on PDA for 3−5 d at 25°C. The bacterium which made the largest inhibitory zone was selected as the antagonist candidate and was named NP-1.

Antifungal sensitivity to pH and temperature of the strain NP-1 cell-free supernatant. Antifungal activity of cell-free supernatant was assayed as follows. Strain NP-1 was cultured in a 500-ml volume Erlenmeyer flask containing 100-ml no. 3 medium (3% polypeptone, 1% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$; Asaka and Shoda, 1996) in a rotary shaker at 26°C, 130 revolutions per min (rpm) for 3 d, and the cell-free supernatant was prepared with membrane filtration (0.2-μm-pore-size polytetrafluoroethylene membrane, Advantec, Japan). The spore suspension of *Fusarium* (approximately 2×10^5 spores per ml) was spread onto PDA plates and then a sterilized tube (5 mm in diameter × 7 mm in height) was placed on the plate. About 200-μl of cell-free supernatant sample was poured into the tube, and the diameter of the inhibition zone was measured after 3−5 d at 25°C incubation.

In the pH stability test, aliquots of the cell-free super-

natant were adjusted to pH 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0, respectively, and maintained for 20 h at 4°C. Antifungal activity was assayed after re-adjusting the supernatant to pH 7.4.

Test for temperature sensitivity of antifungal activity was performed by incubating aliquots of the cell-free supernatant at 40°, 60°, 80°, and 100°C, for 30 min, respectively, and autoclaving an additional aliquot for 15 min at 121°C. These samples were cooled to room temperature and then their remaining antifungal activity was assayed.

Detection of iturin A in the strain NP-1 cell-free supernatant. Strain NP-1 was cultured in a 300-ml volume Erlenmeyer flask containing 50-ml no. 3 medium in a rotary shaker at 26°C, 130 rpm, and the cell-free supernatant was prepared from the 3-d cultures. The supernatant was mixed with an equal volume of a solvent (acetonitrile−10 mM ammonium acetate (3:4 [v/v]), and then filtered through a 0.2-μm-pore-size polytetrafluoroethylene membrane (JP020, Advantec). The filtrate was injected into a high-performance liquid chromatography (HPLC: JASCO, 880-PU), equipped with a column (Chromolith Performance RP-18eb, 4.6 mm diameter×100 mm height, Merck, Germany), UV detector (JASCO, 870-UV) and column oven (JASCO, 865-CO). The mobile phase consisted of 10 mM ammonium acetate and acetonitrile (65:35 (v/v)). The flow rate was 2 ml/min and the injection loop was 20 μl. The oven temperature was 30°C, and the eluent was detected at 205 nm. The concentration of iturin A was determined by a calibration curve made of iturin A standard sample (Sigma-Aldrich Co.).

Nucleotide sequence accession numbers. The 15 most prevalent nucleotide sequences in the 200-bp 16S rDNA library have been deposited in the GenBank database under accession no. AB284992 to AB285006, those appearing in the phylogenetic tree were AB298559 to AB298585. The 16S rDNA sequence of *Bacillus* sp. NP-1 was deposited under no. AB379634.

Results

Composting process and physicochemical properties

In the production of MAR compost, a "loading and withdrawal" procedure was repeatedly carried out daily in each bioreactor (F0, F1 and F2). The typical time course of the temperature in each bioreactor during composting is shown in Fig. 2. The core temperatures of the F0 bioreactor decreased to 50°C when loading substrates, and then gradually increased with fermentation-associated self-heating (Fig. 2). After the temperature reached about 70°C, it was maintained constant for about 10 h, and then gradually decreased until the next loading of substrates. In the F1 and F2 bioreactors, the daily changes in temperature were essentially the same; namely, the temperature increased by 10−15°C after import of fermented products, and then stabilized for about 8 h followed by gradual decrease until the next import. The average temperature was around 55°C in the F1 and 50°C in the F2 bioreactor, respectively (Fig. 2). The pH value of the substrate was about pH 7.4, and increased slightly to pH 8.1 after fermentation in the F2 bioreactor. After airing in the sun for 1 d, the pH value of MAR compost was 7.8. Thus, pH remained constant during the composting process of MAR compost. The moisture content was high (about 65%) in the substrate but it was around 35% in the MAR compost.

Analysis of bacterial community in the MAR compost

The repeated fed-batch fermentation for production of MAR compost has been maintained for more than 10 years. The composition of bacterial species in MAR compost would be important for its use as a soil fertilizer. At first, the PCR-amplified 200-bp-long fragments for bacterial 16S rDNA sequences from the nucleic acid sample of MAR compost were subjected to PCR-DGGE analysis. The DGGE profiles of the MAR composts produced in April, May, June and November, 2004, were essentially the same (Fig. 3), indicating

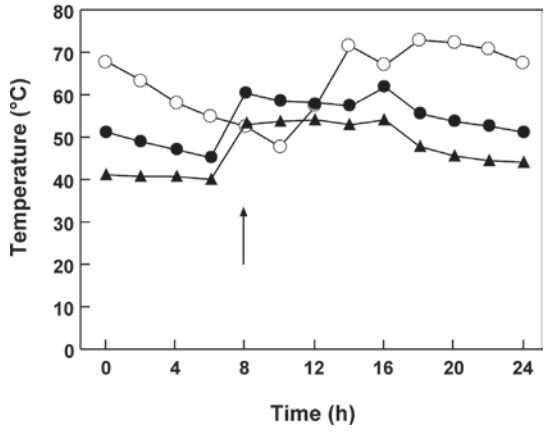

Fig. 2. Daily temperature changes in each bioreactor.

Temperatures of the bioreactor are shown by open circles (F0 bioreactor), closed circles (F1 bioreactor), and closed triangles (F2 bioreactor). The loading of substrates for each bioreactor was carried out at the time indicated by an arrow.

that a unified bacterial population was established by the thermophilic composting process.

The 200-bp-long 16S rDNA fragments were cloned to generate a 16S rDNA library. The resultant recombinant *E. coli* colonies were randomly selected, and their insert sequences were determined. The sequences comprised 5 phyla, with 63 OTUs and 258 clones (Fig. 4). Of a total of 258 bacterial 16S rDNA sequences retrieved, 246 sequences (95% of retrieved sequences) showed more than 97% identity to the sequences in GenBank. The other 13 sequences (5% of retrieved sequences) showed less than 97% identity to those in GenBank, indicating that they may be amplified from currently unknown bacterial species (Forney et al., 2004). The Firmicutes (low-G+C, Gram-positive bacteria) were most predominant phyla, representing 81% of total isolates with 32 OTUs and 209 clones. The phylum Actinobacteria (high-G+C, Gram-positive bacteria) represented 12% of the total clones (17 OTUs and 32 clones), followed by the Proteobacteria, representing 4% of the total clones (10 OTUs and 11 clones), the Chloroflexi represented 2% (2 OTUs and 5 clones), and the Bacterioidetes 1% (2 OTUs and 2 clones). Bacteria belonging to the *Bacillaceae* were predominant in this library. The 76 clones (30% of 258 retrieved sequences) had a DNA sequence closely related to a thermophilic bacterium *Bacillus thermocloacae* (termed 200-OTU1), and 49 clones (19%) were closely related to *Bacillus thermoamylovorans* (termed 200-OTU2). These OTUs were ranked according to their appearance frequencies in the library, and the 15 most prevalent OTUs have been deposited into the GenBank database (acc. no. AB284992 to AB285006). These 15 OTUs (termed 200-OTU1 to 200-OTU15) corresponded to 78% of retrieved sequences (201 of 258 sequences).

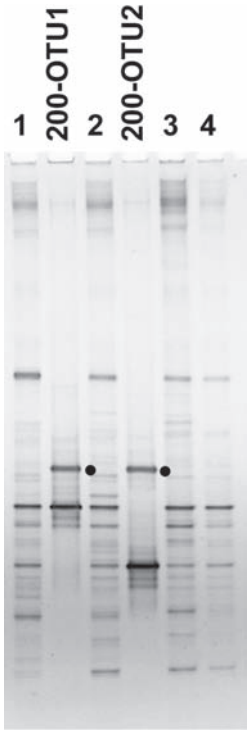

Fig. 3. DGGE profiles of the bacterial community in MAR compost that were produced in April (lane 1), May (lane 2), June (lane 3), and November (lane 4), 2004.

The DGGE profiles of the two most prevalent OTUs (200-OTU1 closely related to *B. thermocloacae* and 200-OTU2 related to *B. thermoamylovorans*) are also shown. These 200-bp-long 16S rDNA fragments were cloned into a plasmid, and the 16S rDNA fragments were directly amplified from the recombinant *E. coli* harboring the cloned sequences. Thus, the fingerprints of *E. coli* 16S rDNA fragments were also included in this DGGE profile and they are indicated by closed circles.

To identify the bacterial closest relatives in more detail, the 570-bp fragments amplified from the 16S rDNA gene were cloned. The 48 independent clones were subjected to sequence analysis, and 27 OTUs (termed 570-OTU1 to 570-OTU27) were generated (Fig. 5). Two major bacterial species found in the 200-bp-long 16S rDNA library, namely those related to *B. thermocloacae* and *B. thermoamylovorans*, were also found in the 570-bp-long 16S rDNA clone library. In the latter library, DNA sequences related to that of *B. thermoam-*

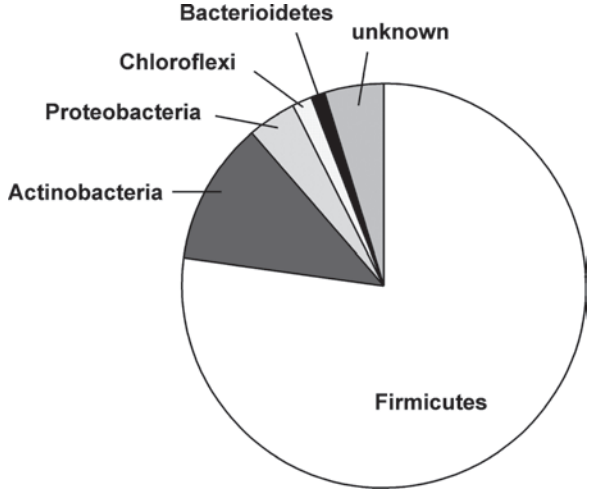

Fig. 4. Relative contribution of major bacterial divisions in the communities of MAR compost.

The proportion was calculated on the basis of relative abundance of each phylotype in the 258 cloned 200-bp-long 16S rDNA sequences.

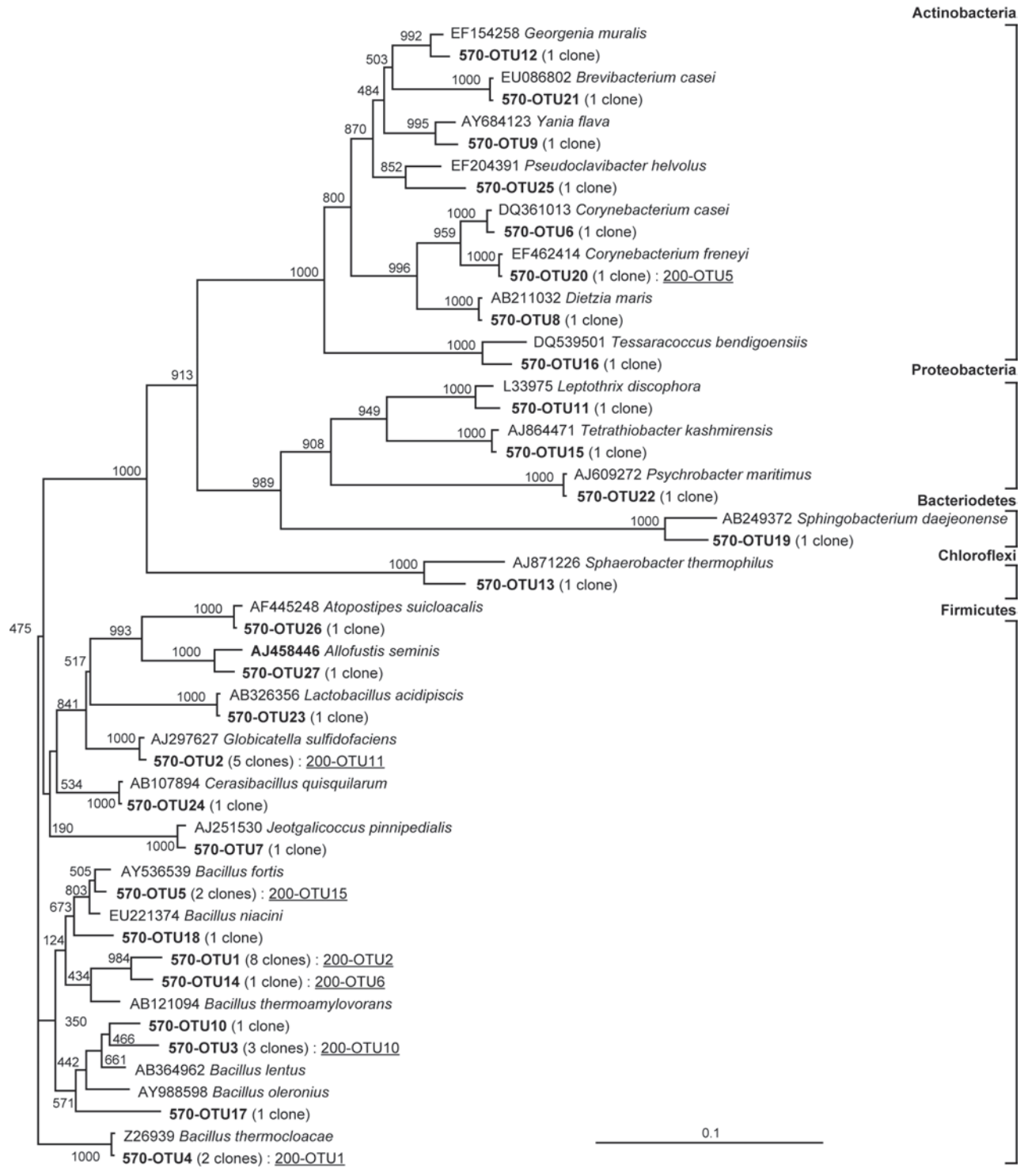

Fig. 5. The neighbor-joining tree of 16S-rDNA sequences isolated from the 570-bp-long library.

The 16S rDNA sequences that contained sequences identical with the 15 most prevalent OTUs obtained from the 200-bp-long library are given together with the corresponding number of 200-OTU (indicated by underlines). Accession numbers appear before the genus name. Scale is the substitutions per site. The number of clones in each OTU is indicated in parentheses. Numbers on the branches refer to bootstrap values for 1,000 times.

ylovorans (termed 570-OTU1) were most abundant (8 clones), and two clones harbored DNA sequences identical to that of *B. thermocloacae* (termed 570-OTU4). Although there are slight differences in the composition of bacterial species between the 200-bp-long and 570-bp-long 16S rDNA libraries, Firmicutes are obviously predominant bacteria in the MAR compost, and two thermophilic bacteria most related to *B. thermoamylovorans* and *B. thermocloacae* are present as representative bacteria in this compost.

Antifungal activity found in the MAR compost

It is unclear whether or not the representative thermophilic bacteria in MAR compost have an antagonistic activity against the phytopathogenic microorganisms under normal temperatures. We then investigated the in vitro antifungal activity in the MAR compost. A fungus was isolated from mold on post-harvested banana peels. The 691-bp fragment amplified from ITS and 5.8S rDNA gene of this fungus showed a 98% identity to the corresponding region of *Fusarium equiseti* (GenBank acc. no. EU326202). When this *Fusarium* sp. was cultured in the presence of MAR compost, inhibition of mycelium growth was observed around the compost-derived bacteria (Fig. 6a). A total 39 bacterial isolates were prepared from this antagonistic bacterial area, and their antifungal activities were assayed. One strain (named NP-1) displaying the strongest antagonistic activity to *Fusarium* sp. was further characterized (Fig. 6b). A 1.5-kb-long 16S rDNA fragment of this bacterium showed a 99.5% identity to *Bacillus subtilis* strain 168 (*Bacillus subtilis* Genome Database, http://bacillus.genome.jp/). On the basis of this result, strain NP-1 was classified as a *Bacillus* species.

Antifungal sensitivity to pH and temperature of the strain NP-1 cell-free supernatant

Antifungal sensitivity of the strain NP-1 cell-free supernatant to pH and temperature was determined. In Fig. 7, the diameter of the inhibition zone of the control sample was designated as 100%, and data for other samples are shown as a percentage of the control sample. The antifungal activity of *Bacillus* sp. NP-1 culture filtrates was highest at neutral pH, and was maintained at more than 80% of the antifungal activity of the control sample in the pH range from 2 to 10 (Fig. 7a). The antifungal metabolites of strain NP-1 were apparently heat stable. The inhibition zone by the autoclaved (121°C, 15 min) supernatant retained 80% of the corresponding zone by the control filtrate (Fig. 7b).

HPLC analysis of antifungal compounds produced by Bacillus sp. NP-1

As the antifungal compound produced by *Bacillus* sp. NP-1 was relatively heat stable and also stable in a broad pH range, this compound may be one of the cyclic lipopeptide iturin groups (Chitarra et al., 2003).

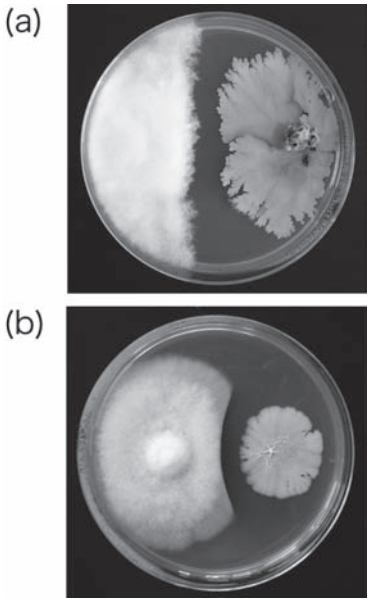

Fig. 6. Effects of MAR compost (a) and *Bacillus* sp. NP-1 (b) on *Fusarium* growth.

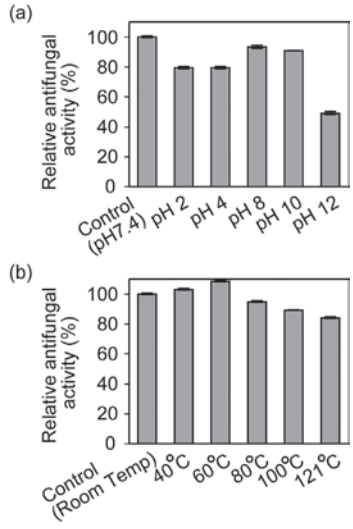

Fig. 7. Stability of antifungal metabolites secreted by *Bacillus* sp. NP-1 to pH (a) and heat (b).

The assay was performed using a *Fusarium* strain isolated from banana peels. Values are mean ± S.D. (n=3).

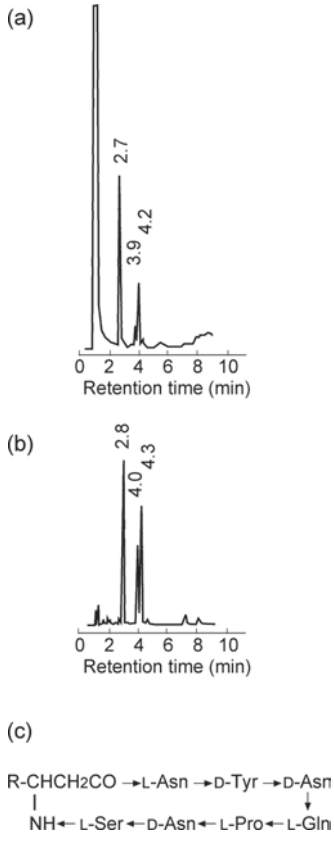

Fig. 8. Chromatograms of the cultural filtrate of *Bacillus* sp. NP-1 (a) and purified iturin A produced by *B. subtilis* RB-14 (b), and the structure of iturin A (c).

The numbers in chromatograms indicate the retention times of corresponding peaks for iturin A.

B. subtilis is well known to produce a number of antifungal compounds including iturins (Asaka and Shoda, 1996; Hiradate et al., 2002; Touré et al., 2004). The culture filtrate of strain NP-1 was subjected to HPLC analysis which had been developed for quantification of iturin A, a typical iturin group (Asaka and Shoda, 1996). Three major peaks were detected in the chromatogram of the culture filtrate of *Bacillus* sp. NP-1 (Fig. 8a) and their retention times were identical to the peaks observed in a chromatogram of purified iturin A which was produced by *B. subtilis* RB-14 (Asaka and Shoda, 1996) (Fig. 8b), indicating that strain NP-1 also secreted iturin A. The structure of iturin A is shown in Fig. 8c. Three peaks correspond to R=*n*-C_{14} (RT=2.7 and 2.8), R=*anteiso*-C_{15} (RT=3.9 and 4.0) and R= *iso*-C_{15} (RT=4.2 and 4.3). When strain NP-1 was inoculated into no. 3 medium, concentration of iturin A in the 3-d-cultured filtrate was about 92 mg/L.

Discussion

To characterize possible PGPR candidates, we first tried to isolate antagonistic bacteria from the MAR compost. MAR compost produced at different times constantly showed antifungal activity in vitro. For example, the composts produced in Oct. 2002, Sep. 2003, and April 2004 had antagonistic activity against *Fusarium* (unpublished results). However, such antagonistic bacterial species have not yet been identified. By screening bacteria antagonistic to *Fusarium*, strain NP-1 was isolated. The culture filtrates of strain NP-1 contained an antifungal compound, iturin A. Iturin A is a cyclic lipopeptide antibiotic containing four L-α-amino acids, three D-α-amino acids and one β-amino acid linked to a fatty acid side chain. Since the length of fatty acid chain varies from C-14 to C-17, five or more different homologs of iturin A exist (Touré et al., 2004). Iturins display strong antifungal activity and inhibit the growth of a wide range of plant pathogens including *Fusarium oxysporum* (Phae et al., 1990). We believe that iturin A is responsible for strain NP-1's antagonistic activity.

Several species of PGPR are commercially available, most of which belong to *Pseudomonas* and *Bacillus* spp. (Haas and Défago, 2005). The important features of PGPR are efficient colonization and persistence in treated soil or plant tissues (Cazorla et al., 2007). When soybean curd residue cultured with *Bacillus subtilis* RB14-CS was introduced into soil, iturin A in the fermented soybean curd residue prevented plant disease infection. However, the concentration of iturin A in soil declines rapidly. In this case, the introduced bacteria existed in soil mainly as spores, and not as vegetative cells (Mizumoto et al., 2006). At present, it remains to be determined whether strain NP-1 colonizes the soil or the plant roots. A biocontrol experiment using strain NP-1 and further determination of the active rhizobacteria derived from MAR compost is required.

The interesting properties of MAR compost originate from its substrates (fishes, shrimps and crabs) and its fermentation process. The main substrates for composting are ecological marine animals, and they are rapidly degraded in a repeated fed-batch fermentation system that consists of three successive aerobic bioreactors. After the F0 bioreactor is loaded with substrates, the temperature increases rapidly, reaches a maximum (70 to 80°C), and is maintained at this tem-

perature for about 10 h. In this thermophilic phase, the animal organic substrates are massively degraded. The bacterial species remaining active at temperatures above 60˚C were quite limited, one such thermophilic bacteria being *Bacillus* sp. (Strom, 1985a, b). Temperatures above 60˚C result in thermal inactivation of many bacteria, including mesophilic bacterial species (Fogarty and Tuovinen, 1991). Therefore, the thermophilic condition in the F0 bioreactor contributes to the establishment of the bacterial community characteristic of MAR compost even though natural variability in complex ecological substrates is expected (Fig. 3). Two bacterial species closest to *B. thermocloacae* and *B. thermoamylovorans* are the members of major bacterial inhabitants in the MAR compost, and they are present in every composting product tested (Fig. 3). In contrast, the 16S rDNA sequence of strain NP-1 was absent in the 258 sequences amplified directly from the MAR compost (Fig. 4), indicating that *Bacillus* sp. NP-1 is a minor bacterial inhabitant in the MAR compost. In this respect, we tested whether or not the major bacterial species in MAR compost can grow at room temperature. Most bacteria grown on the culture media (PDA and nutrient broth) were affiliated with bacterial species related to *Bacillus* spp., but their 16S rDNA sequences were absent in the 258 sequences amplified directly from the MAR compost (data not shown; the sequences have been deposited in GenBank database; acc. no. AB298586 to AB298591). Thus, bacterial species grown from the MAR compost should be strongly influenced by the culture conditions, and would not be proportional to the bacterial population in the MAR compost.

In most composting processes, the bacterial composition showed significant differences between the thermophilic and maturing stages. The level of Firmicutes was high in the thermophilic phase and then decreased greatly during the maturation phase (Ishii and Takii, 2003; Takaku et al., 2006). Unlike most other composting processes, the production of MAR compost via a repeated fed-batch fermentation system was complete within 3 d, which should allow Firmicutes (especially *Bacillaceae*) to still be the dominant bacteria in MAR compost. Since it is possible that the *Bacillaceae* bacteria competent to the introduced soil colonize the rhizosphere and act as PGPR, this unique bacterial community in MAR compost provides a valuable source of PGPR. Improved soil fertilizers and conditioners with enhanced plant growth promotion activity will be developed by identification of such valuable PGPR from the MAR compost.

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