1

Examination and Comparison of Microbial Diversity in Field-Scale Sewage Sludge Composters

Akihiro Ohnishi[1], Akihiro Nagano[2],
Naoshi Fujimoto[1] and Masaharu Suzuki[1]
[1]*Department of Fermentation Science, Faculty of Applied Bio-Science, Tokyo University of Agriculture*
[2]*Technical Environmental Systems Division, Sanki Engineering Co. Ltd. Japan*

1. Introduction

While the earth's biodiversity has been studied in detail, to date, microbes have been completely omitted from meta-analytical studies of biodiversity data sets (Balmford & Bond, 2005; Díaz et al., 2006). In fact, biodiversity data sets are far from being considered a comprehensive global resource (Collen et al., 2008). Since the origin of prokaryotes on Earth over 3.5 billion years ago, the extent of evolutionary diversification within this group has been truly immense (DeLong & Pace, 2001; Payne et al., 2009). Microbial communities play important biological roles, such as the global cyclical change of materials in various environments (Díaz et al., 2006). As a result, existing biodiversity includes a vast and largely undiscovered diversity of microbes, which are probably very important for the sustainability of ecosystems (Swift et al., 2004). Hence, detailed investigation to characterize the global biodiversity of microbes is a very important task.

Microbes have always formed a major component of global biodiversity, either as producers (e.g., phototrophic blue-green algae) or decomposers (e.g., heterotrophic bacteria) (Naeem et al., 2000). Furthermore, in the future, they may serve as producers of useful alternative energy sources (Ohnishi et al., 2010). For example, phototrophic microorganisms use the energy from light for the production of biomass, which is an energy source stored in all living organisms. In fact, microbial decomposers are used in industry to convert microbial biomass and organic waste materials, such as domestic garbage, into biofuels, such as methane, ethanol, and hydrogen (Swift et al., 2004; Kayhanian et al., 2007). Microbes are also used for bioremediation, which is the cleanup of pollution caused by human activities (Jørgensen et al., 2000). In this process, various microbes have been isolated from nature, which are capable of degrading spilled oil, solvents, and other environmentally toxic pollutants. Furthermore, the breadth of microbial diversity on Earth provides genetic resources that offer solutions for environmental and energy issues, and research in this area is currently expanding. Considering the serious environmental and energy issues that humans face today, a better understanding of the ecophysiology of environmental microbes is warranted to address problems such as resource depletion and environmental pollution.

These problems could be resolved by converting anthropogenic waste into renewable resources, such as clean biofuels or fertilizers (Pimentel et al., 1994). As microbial ecologists, we are interested in understanding the mechanisms underlying the existence of an individual microbe, its involvement in a microbial community, and its special abilities.

Because microbes are invisible to the naked eyes, morphological analysis is difficult (Gest, 2003). Therefore, it is necessary to study microbial diversity from a variety of perspectives, including the physiological, genetic, and phylogenetic characteristics of species, as well as other taxonomic levels. One of the primary tasks for studying the naturally occurring microbial diversity is to perform accurate macroscopic analysis of the variety, population, and/or activity of microorganisms present in a specific habitat. In the past, the lack of appropriate methodologies has hindered this task, and thereby affected the progress in studying microbial diversity (Torsvik et al., 1998). Traditionally, microbiologists have used culture-dependent approaches for the detection and isolation of environmental microbes, and the methods currently in use are based on those developed in the late 19th century (Okabe et al., 2009). These culture-dependent approaches present one of the most serious limitations to studying microbes, as they are essentially very effort intensive and slow down data assimilation (Moter & G bel, 2000). Therefore, concerted efforts are required to develop novel techniques for elucidating the taxonomic positions and activities of as-yet unknown microbes, which might contribute towards enhancing our understanding of the microbial world.

Recently, problems related to culture-dependent approaches have been resolved through the application of methods from the discipline of molecular biology, such as culture-independent approaches (Amann et al., 1990; Muyzer, 1999). Compared to culture-dependent approaches, culture-independent approaches provide a broader view of the microbial population and/or its activity, without the necessity of isolating and culturing individual organisms (Hugenholtz et al., 1998; Ranjard et al., 2000). Thus, these approaches, such as fluorescence *in situ* hybridization (FISH) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), generate accurate results in a short time. As a result, the molecular method has been used to analyze the microbial diversity of a wide range of environments, which has generated many beneficial findings. Examples include solid waste composters (Nakasaki et al., 2009), wastewater treatment plants (Wagner et al., 2002), agricultural soils (Ranjard et al., 2000), and natural rivers (Brummer et al., 2000). Furthermore, culture-independent approaches have been used to identify many novel bacterial and archaeal lineages from different environments (Oren, 2004). As a consequence, studies using these approaches have shown that the microbial world is genetically and functionally more complex and diverse than previously predicted from culture-dependent studies.

However, culture-independent approaches also have certain disadvantages, as the strains in a sample cannot be distinguished and the unique properties of a particular strain cannot be identified (Rapp & Giovannoni, 2003). While correct data about the microbial ecology is generated using a culture-independent approach, the characteristics and activities of microbial strains cannot be studied without isolation. Therefore, it would be very difficult to use these culture-independent approaches to conduct a detailed study of an individual strain for the development of an applied technology. In other words, despite the widespread use of culture-independent approaches, cultural isolation will continue to be an important but necessary method to generate new technologies.

Our research group previously used the culture-dependent approach to provide important information about unknown microbial diversity and potential available resources in a field-scale model of a sewage sludge composter that was set up in Sapporo, Japan (Ohnishi et al., 2011). During composting, microorganisms decompose solid wastes, such as urban wastes, sewage sludge, and food garbage. This process is the first step by which organic matter is recycled and absorbed into plants or other autotrophs. However, the mechanisms underlying these activities remain unclear. In this study, we investigated the microbial diversity of culturable bacteria obtained from a field-scale sewage sludge composter that was set up in Tendo, Japan. PCR amplification of 16S rRNA gene from 32 isolates was performed using universal primers, followed by gene sequencing. The gene sequences were compared with the sequences available in the GenBank databases to identify closely related sequences. The closely related sequences were aligned to construct a phylogenetic tree for these bacteria. Then, these sequences were deposited in GenBank under different accession numbers. In addition, the detailed data sets, including ribo-patterns and carbon source utilization, were compared with those obtained in a previous study by the same research group.

2. Materials and methods

2.1 Sample collection

Samples were obtained from a field scale composter (Tendo Compost Plant; 38° 21′ N, 140° 37′ E) that was used for the treatment of sewage sludge (10 t/d) from Tendo City. This composter, which had aeration holes at the bottom, was operated as a silo-type. The composted material was sewage sludge mixed with return compost and sawdust, with a final water concentration of approximately 60%. Primary decomposition was completed after 14 d (the highest temperature was 75 °C), and secondary decomposition was completed after 60 d (at a temperature of approximately 40 °C). The sample (approximately 5 kg) was taken from the surface of the compost (at a depth of 30 cm) after the completion of primary decomposition. The sample was packed in ice for transportation to the laboratory, and maintained at 4 °C until the initiation of the experiment.

2.2 Plate counts and strain isolation

Mesophilic and thermophilic bacterial counts were performed following aerobic bacterial culture on nutrient agar plates (1.0% meat extract, 1.0% polypeptone, 0.5% NaCl, and 1.5% agar (pH 7.0)). In brief, 10 g of compost sample and 90 ml distilled water were placed in a shaking flask (volume, 500 ml). The solution was homogenized by shaking at 230 rpm for 20 min. The homogenate was then serially diluted, and 100-µl aliquots of 10^1 to 10^9 sample dilutions were plated. Incubation was performed at 37 °C or 50 °C for mesophilic bacteria and at 60 °C for thermophilic bacteria. After 7 d incubation at these temperatures, colonies on plates containing 30–300 colonies were counted. A number of colonies were randomly selected from plates that had been inoculated with the highest dilution, and in which the colonies were well separated. These colonies were then purified by repeated dilution plating. The isolates were stored at 4 °C with continuous subculturing, and stocks were deep frozen at -80 °C.

2.3 16S rRNA gene sequence determination

DNA extraction from each isolate was performed using the bead beating method (Ohnishi et al., 2010). After cells were grown for 24 h, they were suspended in 1 ml sterile distilled water

by using a sterile swab, and centrifuged for 5 min (12,000 g, 4 °C). The cell pellet (1–3 mg) was then resuspended with 1 ml of extraction reagent (0.1 M NaCl, 0.5 M Tris-HCl, and 0.5% SDS (pH 8.0)). After vortexing, the suspension was transferred into a 2-ml screw-capped tube containing 0.3 g of zirconium beads (diameter, 0.1 mm), and the cells were crushed by 10 cycles (3000 rpm, 30 s) of bead beating at 30-s intervals by using an MB-200 Multi-beads shocker (Yasui Kikai). The beads and cell debris were removed by centrifugation at 20,000 *g* for 5 min. The crude DNA was purified by phenol-chloroform extraction followed by ethanol precipitation, and dissolved in TE buffer (pH 8.0). The DNA extract was then subjected to PCR amplification.

Gene fragments that were specific to the 16S rRNA-coding regions of the isolates were amplified by PCR using the 2 primers, 20F (5´-AGTTTGATCATGGCTCA-3´, positions 10–26) and 1540R (5´-AAGGAGGTGATCCAACCGCA-3´, positions 1521–1541) (*Escherichia coli* numbering system (Brosius et al., 1978)), following the method of Yanagi and Yamasato (1993). PCR amplification was carried out in a PTC200 thermal cycler (MJ Research) using reagents from a Taq PCR kit (Takara). The amplified 16S rRNA gene was directly sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems). The following 5 primers were used: 20F, 1540R, 350F (5´-CCTACGGGAGGCAGCAGT-3´, positions 341–358), 800F (5´-GTAGTCCACGCCGTAAACGA-3´, positions 803–819), and 900R (5´-CGGCCGTACTCCCCAGGCGG-3´, positions 879–898) (Ohnishi et al., 2011).

2.4 Phylogenetic analysis

Multiple alignment was performed using Clustal X (version 1.8 (Thompson et al., 1997)). Phylogenetic distances (Knuc) for the aligned sequences were calculated using the 2-parameter method of Brummer (Kimura, 1980). The neighbor-joining method (Saitou and Nei, 1987) was used for the construction of a phylogenetic tree. The topology of the phylogenetic tree was evaluated by bootstrapping with 1000 replications (Felsenstein, 1989).

2.5 Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been submitted to the DDBJ, GenBank, and EMBL databases under the following accession numbers: AB210952–AB210984 (see Table 1).

2.6 Riboprinting

Ribotyping was performed using the DuPont (Wilmington, DE) Qualicon RiboPrinter (Bruce, 1996). Some single colonies from a 24-h culture on agar plates were suspended in sample buffer, and heated at 80 °C for 15 min. After the addition of a lytic enzyme, the samples were transferred to the RiboPrinter. Further analysis, including the use of the *Eco*RI enzyme, was carried out automatically. The ribotypes were aligned according to the position of a molecular size standard, and compared with patterns stored in the library. The ribotyping profiles were transferred and analyzed with the FPQuest software (Bio-Rad Laboratories), using the Pearson correlation and default settings for optimization (2.0%) and position tolerance (1.00%) for genetic similarity. The dendrogram was generated by the unweighted pair-group method, using arithmetic averages (UPGMA) to determine profile relatedness.

2.7 Carbon source utilization

The carbon source utilizing-ability of the isolates was investigated with a Biolog GP2 microplate (Biolog Inc.). This standard 96-well microplate contained a dried film of 95 different sole carbon sources, which are mainly carbohydrates, but also other carbons such as amino and carboxylic acids, and 1 negative control. Each well contained a redox dye (tetrazolium violet) for the colorimetric determination of respiration, due to oxidization of the carbon source by cells. This system is generally used to test the ability for sole carbon source utilization of microorganisms. Basically, when a cell metabolizes a carbon source (by chemical oxidation), a redox dye (such as a tetrazolium salt) is irreversibly reduced to a purple formazan, which is then assayed colorimetrically (Bochner & Savageau, 1977).

One colony was selected from the pre-culture by using a sterile cotton swab, subcultured on BUG medium (Biolog universal growth medium), and then incubated overnight. Cells were harvested using sterile cotton swabs, and then suspended in 20 ml of GN/GP-IF inoculating fluid.

The cell density was adjusted to 20% of transmittance with a 2% range, which was assessed using a photometer model according to the range specified by the manufacturer. Thioglycolate was added to the suspension at a final concentration of 5 mM, to inhibit the production of a bacterial capsule. An aliquot of 150 µl of the suspension was immediately dispensed into each well of the GP2 microplate by using a multichannel pipette. The microplate was then incubated for 16–24 h and analyzed using an automated Biolog microplate reader at 2 different wavelengths (590 nm and 750 nm). Reactions were interpreted as positive or negative by the Biolog MicroLog 3 software, version 4.20 (Biolog Inc.).

3. Results

3.1 Culturable count

Bacteria that were grown on media under mesophilic (37 or 50 °C) or thermophilic conditions (60 °C) in Tendo Compost were counted. The number of mesophilic bacteria that grew at 37 and 50 °C was 7.0 and 5.3 × 10^9 CFU g^{-1} dry matter, respectively, while that of thermophilic bacteria was 9.1 × 10^8 CFU g^{-1} dry matter.

3.2 Phylogenetic analysis

Thirty-two isolates from Tendo Compost, comprising 15, 9, and 8 isolates grown at 37, 50, and 60 °C, respectively, were randomly selected and purified. Comparative 16S rRNA gene sequencing analysis of the isolates was completed based on gene fragments approximately 1,500 bp in size. Table 1 and Fig. 1 show the results of phylogenetic analysis of the 16S rRNA gene sequencing for the isolates from Tendo Compost, along with 18 related prokaryote species.

Using 97% 16S rRNA gene-sequence similarity as the definition of a species (Stackebrandt & Goebel, 1994), the remaining isolates appeared to represent new species. Among the isolates, 4 could be identified only to the genus level due to their low sequence similarity. Of the unknown species, 3 groups (designated as NoID D, E, and F) belonged to the phylum Firmicutes, and 1 group (designated as NoID G) to the phylum Actinobacteria. “NoID” indicates that a taxon could not be identified to the species level based on 16S rRNA gene sequence similarity. From the isolates of the NoID groups, the 16S rRNA gene sequence similarity values for known species ranged between 93.0 and 96.7%.

A phylogenetic relationship of isolates from Tendo Compost and Sapporo Compost was evaluated. The phylogenetic trees of every phylum are shown in Fig. 1. Of the 32 isolates from Tendo Compost, 14 were classified to the genus level and 18 to the species level, including 4 unknown taxa. Of the 49 isolates from Sapporo Compost, 13 were classified to the genus level and 16 to the species level, including 5 unknown taxa. In addition, from the strains isolated from Tendo Compost and Sapporo Compost, a very high correlation was shown for the 5 common phylogenetic groups, *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, No ID D group, and No ID E group.

3.3 Characterization by automated ribotyping

Isolated strains from Tendo Compost and Sapporo Compost were analyzed using ribotyping. *Eco*RI digestion was incomplete for some groups, mainly the Actinobacteria and No ID groups (without No ID C and D group). As the riboprint identification database provided by the manufacturer was not complete enough to affiliate patterns to taxon names, clustering was executed based on the similarity in band profile by FPQuest. The riboprint patterns and a dendrogram of 62 isolates are shown in Fig. 2. Clustering analysis of the ribotype patterns led to the separation of strains isolated from Tendo Compost and Sapporo Compost into clusters that corresponded with the phylogenetic group obtained from the 16S rRNA gene analysis, in most cases.

3.4 Carbon source utilization of common phylogenetic groups

Twenty isolates belonging to 5 common phylogenetic groups that were extracted from Tendo Compost and Sapporo Compost were tested for 95 carbon sources. Based on the Biolog GP2 microplate results, all strains of the same common phylogenetic group showed high similarity in their carbon sources utilization pattern; specifically, *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, No ID D group, and No ID E group (Table 2).

Seven of the ten utilized sole carbon sources from isolates belonging to *A. aneurinilyticus* were correlated for Tendo Compost versus Sapporo Compost (Table 2). Likewise, 23 of the 29 isolates of *B. subtilis* were correlated between the 2 compost types. For *T. fusca*, 15 of the 26 isolates were correlated. For the "No ID D" group, 17 of the 25 isolates were correlated, and for the "No ID E" group, 1 of the 7 isolates was correlated.

4. Discussion

In this study, the bacteria obtained from Tendo Compost were analyzed using the cultivation method at various temperatures, the results of which clearly showed the diversity of bacteria that could potentially be cultivated. The results indicate that various types of mesophilic and thermophilic bacteria were present in the compost at a density of around 10^9 CFU g^{-1} dry matter. Since this population size of the isolates is higher than that recorded in standard commercial composts (10^3 to 10^7 CFU g^{-1} matter), the detected isolates seem to comprise a very active group in the Tendo composting process (Pedro et al., 1999; Vaz-Moreira et al., 2008). In addition, the phylogenetic diversity of culturable bacteria was also very high. Of the 32 isolates obtained from Tendo Compost, a total of 19 species (including 4 unknown taxa) belonging to 16 genera were detected (Table 1). Hence, there was a very high diversity of bacteria at the phylogenetic level that actively contributed to the primary role of organic matter decomposition in the field-scale composting process.

Strain No.	Related species	Similarity	Accession Number	Isolation temperature
	Actinobacteria			
SSCT48	*Arthrobacter mysorens* (AJ617482)	99.7%	AB210984	37 °C
SSCT78	*Cellulosimicrobium cellulans* (X79456)	98.7%	AB210961	50 °C
SSCT54	*Cellulosimicrobium cellulans* (X79456)	99.5%	AB210980	37 °C
SSCT73	*Cellulosimicrobium cellulans* (X79456)	99.6%	AB210965	50 °C
SSCT58	*Microbacterium esteraromaticum* (AB099658)	99.1%	AB210977	37 °C
SSCT49	*Rhodococcus rhodochrous* (X79288)	99.7%	AB210983	37 °C
SSCT69	*Rhodococcus rhodochrous* (X79288)	99.8%	AB210967	50 °C
SSCT55	*Sanguibacter keddieii* (X79450)	96.7%	AB210980	37 °C
SSCT81	*Thermomonospora fusca* (AF002264)	99.9%	AB210960	50 °C
	Firmicutes			
SSCT74	*Aneurinibacillus aneurinilyticus* (AB101592)	99.9%	AB210964	50 °C
SSCT75	*Bacillus foraminis* (AJ717382)	96.7%	AB210963	50 °C
SSCT76	*Bacillus novalis* (AJ542512)	95.6%	AB210962	50 °C
SSCT84-2	*Bacillus pocheonensis* (AB245377)	93.0%	AB210954	60 °C
SSCT51	*Bacillus subtilis* (Z99104)	99.9%	AB210982	37 °C
SSCT68	*Bacillus subtilis* (Z99104)	99.9%	AB210968	50 °C
SSCT72	*Brevibacillus panacihumi* (EU383032)	99.4%	AB210966	50 °C
SSCT85	*Geobacillus thermodenitrificans* (AB190135)	99.8%	AB210952	60 °C
SSCT83	*Geobacillus thermodenitrificans* (AY608963)	99.3%	AB210956	60 °C
SSCT82-1	*Geobacillus toebii* (AY608982)	98.8%	AB210959	60 °C
SSCT82-2	*Geobacillus toebii* (AY608982)	99.6%	AB210958	60 °C
SSCT82-3	*Geobacillus toebii* (AY608982)	99.0%	AB210957	60 °C
SSCT84-1	*Geobacillus toebii* (AY608982)	99.6%	AB210955	60 °C
SSCT84-3	*Geobacillus toebii* (AY608982)	98.7%	AB210953	60 °C
	Proteobacteria			
SSCT56	*Citrobacter freundii* (AF025365)	99.5%	AB210978	37 °C
SSCT63	*Morganella morganii* (AY464464)	99.5%	AB210972	37 °C
SSCT65	*Pantoea agglomerans* (AY335552)	99.0%	AB210971	37 °C
SSCT59	*Pantoea agglomerans* (AY335552)	99.3%	AB210976	37 °C
SSCT62	*Pantoea agglomerans* (AY335552)	99.3%	AB210973	37 °C
SSCT66	*Pantoea agglomerans* (AY335552)	99.3%	AB210970	37 °C
SSCT67	*Pantoea agglomerans* (AY335552)	99.1%	AB210969	37 °C
SSCT60	*Stenotrophomonas maltophilia* (AJ131116)	98.3%	AB210975	37 °C
SSCT61	*Stenotrophomonas maltophilia* (AJ131116)	98.8%	AB210974	37 °C

Table 1. Sequence similarities of the isolated strains.

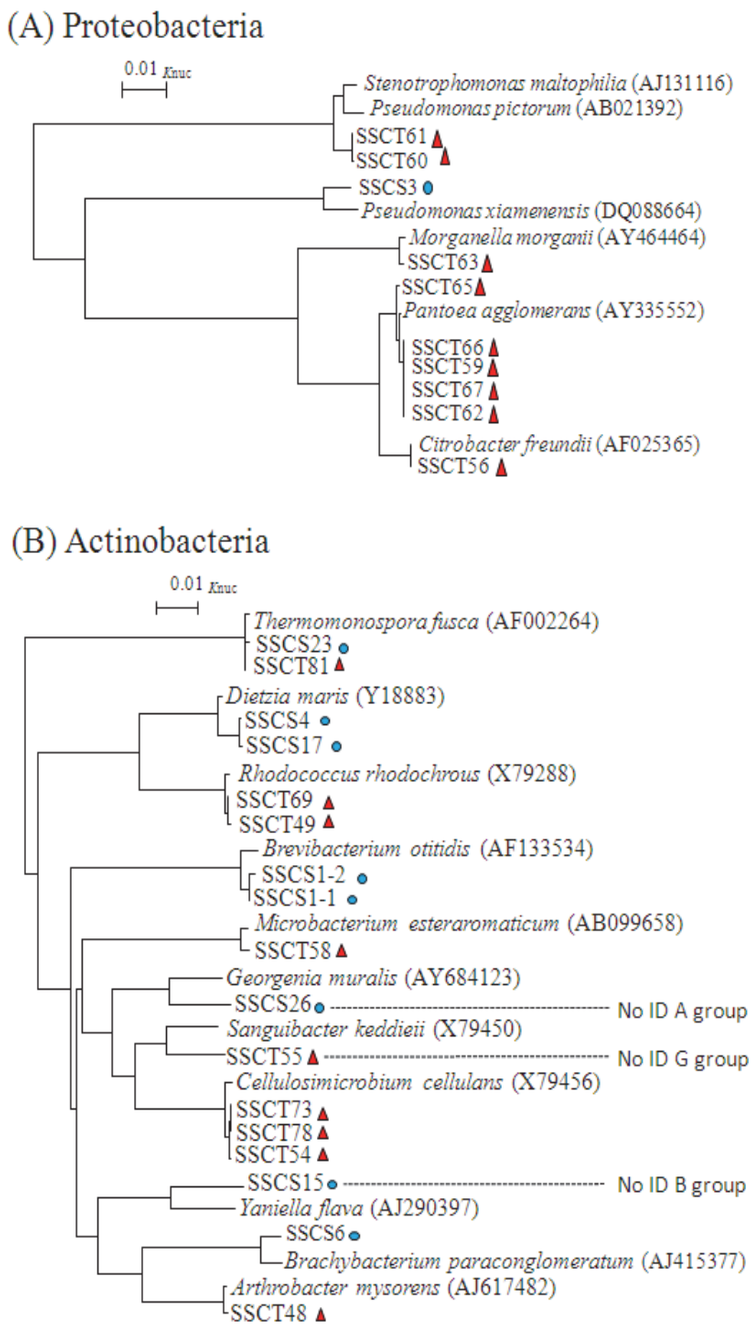

Fig. 1. Continued

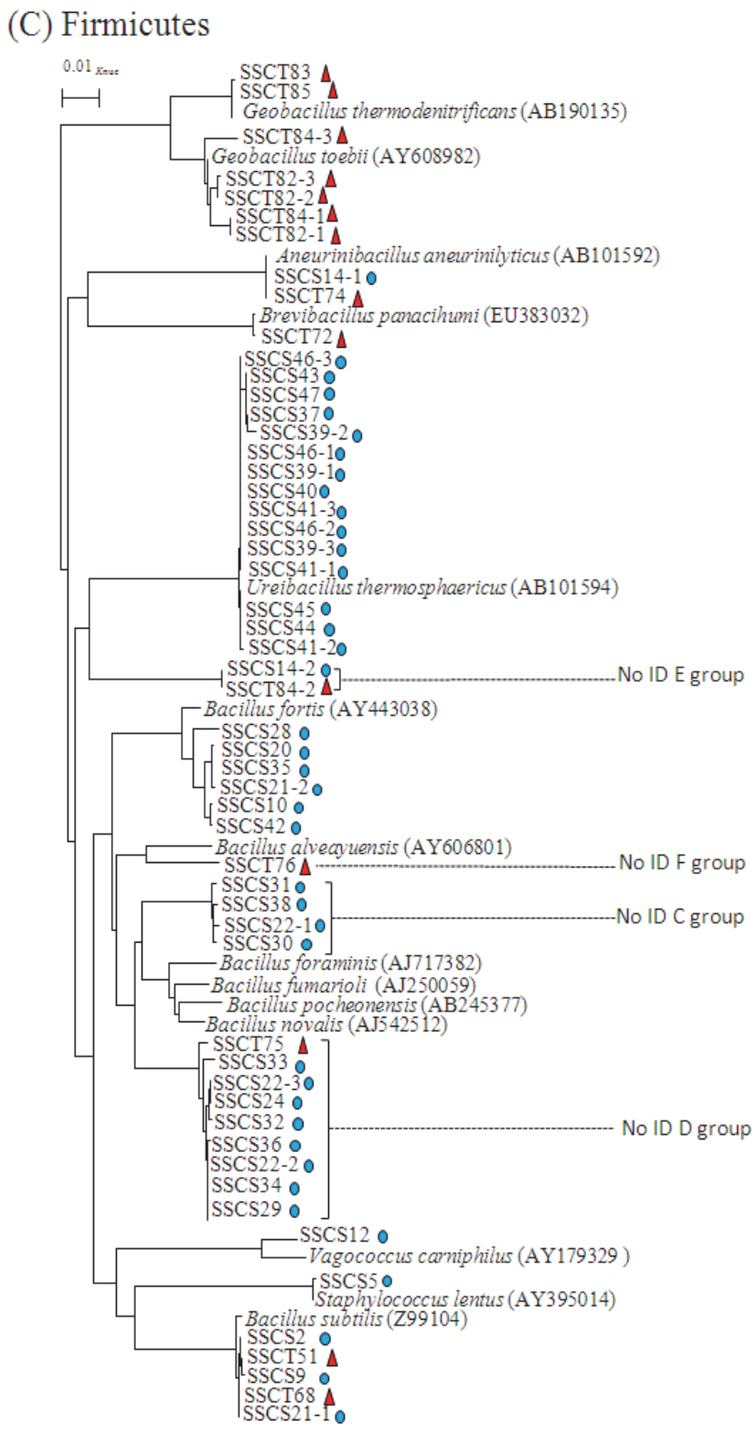

Fig. 1. Phylogenetic relationship between the isolates and other related bacteria based on 16S rRNA gene sequences. (A) Phylum Proteobacteria. (B) Phylum Actinobacteria. (C) Phylum Firmicutes. The phylogenetic tree, which was constructed using the neighbor-joining method, is based on the comparison of approximately 1,400 nucleotides of the 16S rRNA gene. Symbols are isolated: ▲, from Tendo Compost; ●, from Sapporo Compost in previous study.

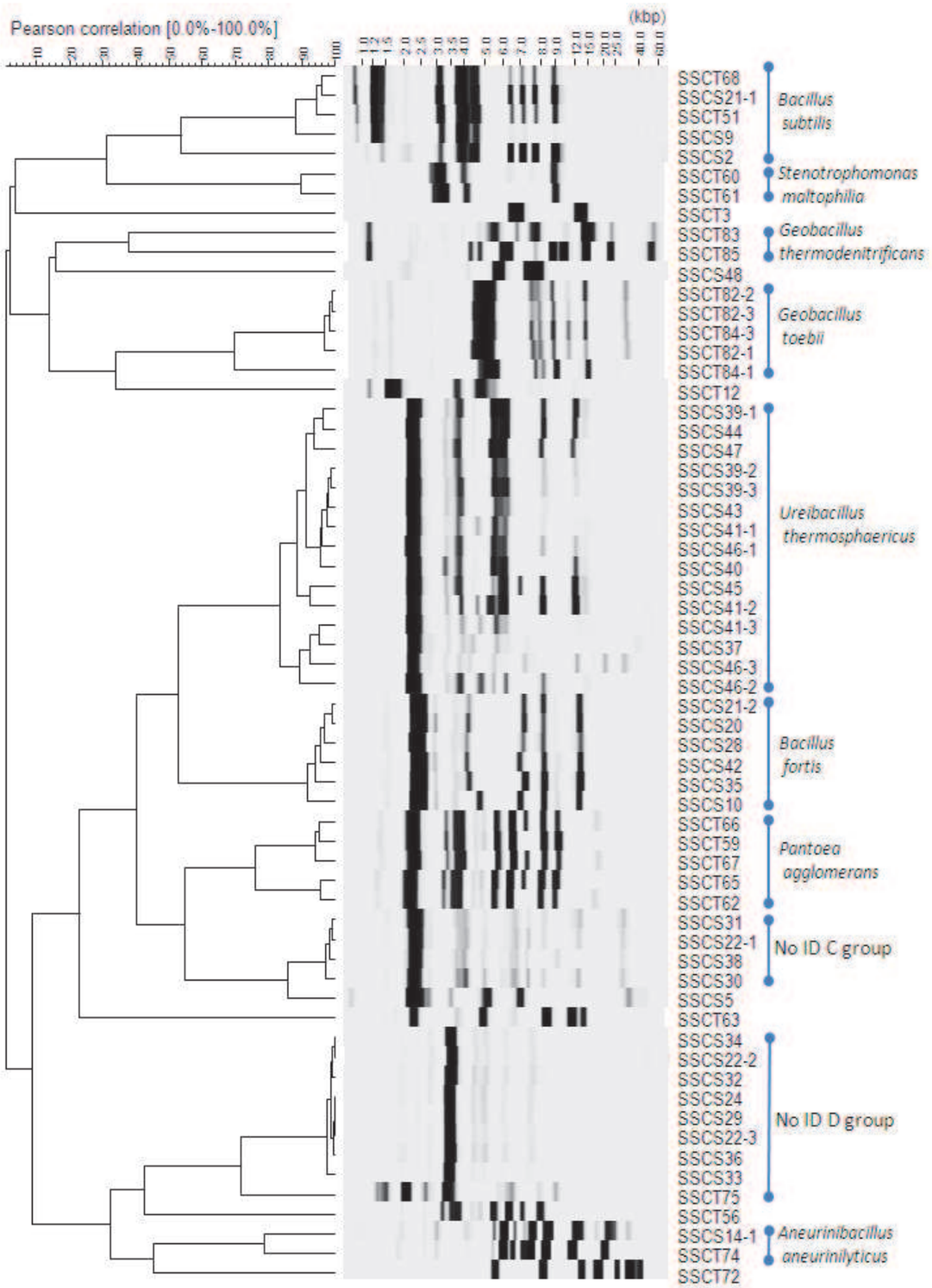

Fig. 2. Riboprint pattern obtained from isolates that were derived from Tendo Compost and Sapporo Compost. Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UPGMA) based on the Pearson correlation coefficient.

Species identification	*Aneurinibacillus aneurinilyticus*		*Bacillus subtilis*		*Thermomonospora fusca*		No ID D group		No ID E group	
	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS	SSCT	SSCS
Dextrin			+	+	+	+			+	
Glycogen	+									
Mannan	+									
Tween 40	+	+	50	33						+
Tween 80	+	+								+
N-Acetyl-D-Glucosamine			50							
N-Acetyl-β-D-Mannosamine			+	33						
L-Arabinose					+	+				
D-Cellobiose			50	67						
D-Fructose			+	33	+	+	+			
D-Galactose			50	67						
Gentiobiose				33						
α-D-Glucose			+	+	+	+				
Maltose			+	67	+	+				
Maltotriose			+	67	+	+				
D-Mannitol						+				
D-Mannose			+	33						
3-Methyl Glucose			+	67						
α-Methyl-D-Glucoside			+	33						
β-Methyl-D-Glucoside			+	67						
Palatinose			+	67						
D-Psicose			+	+			+			
D-Ribose	+	+		+	+	+	+		+	
Sedoheptulosan			+	33						
D-Sorbitol			+	67						
Sucrose			+	+						
D-Trehalose			+	+						
Turanose			+	+						
D-Xylose						+				
Acetic Acid					+	+				
α- Hydroxybutyric Acid					+					
β- Hydroxybutyric Acid							+	88		
α- Ketoglutaric Acid	+	+				+				
α- Ketovaleric Acid	+	+			+	+	+	63	+	+
L-Malic Acid			+							
Pyruvatic Acid Methyl Ester	+	+	+	+	+		+	+		
Succinic Acid Mono-methyl Ester					+		+	63	+	
Pyruvic Acid	+	+	+		+	+	+	75	+	
Succinamic Acid	+									
Succinic Acid								75		
L-Alaninamide							+	+		
D-Alanine					+		+			
L-Alanine					+		+	38		
L-Alanyl-Glycine							+	75		
L-Glutamic Acid							+	75		
Glycyl- L-Glutamic Acid							+	75		
L-Serine							+	75		
2,3-Butanediol			+	+		+				
Glycerol			+	+		+	+	75		
Adenosine					+	+	+	75		
2'-Deoxy Adenosine					+	+	+	75		
Inosine			+		+	+	+	75		
Thymidine					+	+	+	75		
Uridine			50		+	+	+	75		
Thymidine-5'-Monophosphate						+				

+, positive reaction (100%); 1–99, percent positive reaction. SSCT, Isolates from Tendo Compost; SSCS, Isolates from Sapporo Compost. In addition to the above data, none of these isolates were able to use α–Cyclodextrin, β–Cyclodextrin, Inulin, Amygdalin, D–Arabitol, Arbutin, L–Fucose, D–Galacturonic Acid, D–Gluconic Acid, m–Inositol, α–D–Lactose, Lactulose , D–Melezitose, α–Methyl–D– Galactoside, β–Methyl–D–Glucoside, α–Methyl–D–Mannoside, D–Raffinose, L–Rhamnose, Salicin, Stachyose, D–Tagatose, Xylitol, γ– Hydroxybutyric Acid, p–Hydroxy–Phenylacetic Acid, Lactamide, D–Lactic Acid Methyl Ester, L–Lactic Acid, D–Malic Acid, Propionic Acid, N–Acetyl–L–Glutamic Acid, L–Asparagine, Putrescine, Adenosine–5'–Monophosphate, Uridine–5'–Monophosphate, D–Fructose–6–Phosphate, α–D–Glucose–1–Phosphate, D–Glucose–6–Phosphate, D–L–α–Glycerol Phosphate.

Table 2. Percentile positive results of the isolated strains using traditional biochemical tests and in BiOLOG.

However, differences in the diversity of culturable bacteria at each growth temperature were clearly demonstrated by 16S rRNA gene sequence determination. The temperature (from below 40 °C to over 60 °C) and nutritional status during the composting process was usually subject to dynamic changes. For example, the 9 isolates belonging to the Proteobacteria phylum were isolated only from mesophilic conditions at 37 °C; the 9 isolates belonging to the Actinobacteria phylum were isolated from mesophilic conditions at 37 and 50 °C; the 14 isolates belonging to the Firmicutes phylum were isolated from mesophilic and thermophilic conditions at 60 °C. Based on these observations, it appeared that in Tendo Compost, Proteobacteria and Actinobacteria are mesophiles that actively participate at temperatures below 37 °C and 50 °C, respectively. In addition, since all isolates under the thermophilic condition (60 °C) belonged to the *Geobacillus* and *Bacillus* genera, thermophiles that actively contribute at temperatures over 60 °C appear to belong to Firmicutes. Hence, the main phylogenetic groups of bacteria that actively contribute to the composting process under each temperature condition vary according to the phylum level. The same tendency was observed for Sapporo Compost (Ohnishi et al., 2011).

The commonality in culturable bacteria between Tendo Compost and Sapporo Compost was demonstrated by 16S rRNA gene sequence determination, Ribotyping, and the Biolog system (Figs. 1 and 2, and Table 2). For example, 32 isolates from Tendo Compost were classified into 16 genera comprising 19 species, including 4 unknown taxa. In comparison, 49 isolates from Sapporo Compost were classified into 13 genera comprising 16 species, including 5 unknown taxa. Five common phylogenetic groups based on 16S rRNA gene sequence were determined as *A. aneurinilyticus*, *B. subtilis*, *T. fusca*, "No ID D" group, and "No ID E" group (Fig. 1). These results show that approximately 17% of phylogenetic groups that were detected were common. Furthermore, Ribotyping showed that there was high similarity at the species level of isolates belonging to the 3 common phylogenetic groups (*A. aneurinilyticus*, *T. fusca* and "No ID D"group). In addition, carbon source utilization analysis showed that common phylogenetic groups had similar carbon source utilization abilities. Of additional significance, it was found that common phylogenetic groups existed between the 2 sewage sludge composters, despite their being located approximately 500 km apart.

The similarity in the role of isolated common phylogenetic groups between Tendo Compost and Sapporo Compost can be evaluated on the basis of carbon source utilization, in parallel to the results of past studies. For example, *A. aneurinilyticus* mainly utilized carbohydrates, polymers, and carboxylic acids, while *T.* fusca utilized a wide range of carbon sources, such as carbohydrates, polymers, carboxylic acids, and nucleosides. Furthermore, *A. aneurinilyticus* and *T. fusca* may be involved in the degradation of lignin when conditions are mesophilic during the composting process, since the bacteria of these taxa are known to degrade lignin (Chandra et al., 2007; Crawford & Crawford, 1976). Most strains of *B. subtilis* were able to utilize 23 sole carbon sources, which were mainly carbohydrates, polymers, carboxylic acids, and alcohols. The hydrolysis of starch and casein has also been observed for members of this group (Ohnishi et al., 2011). In addition, *B. subtilis* may inhibit fungal growth when compost is applied to agricultural land (Phae et al., 1990). The "NoID D" group can also hydrolyze casein (Ohnishi et al., 2011), and utilize 17 sole carbon sources, which primarily include carboxylic acid, amino acid, and nucleoside. However, this group shows weak ability to utilize carbohydrate. In addition, the "NoID D" group may be involved in the degradation of proteins when conditions are mesophilic during the

composting process (Ohnishi et al., 2011). While there is noticeable bias for carbon source utilization in 1 common phylogenetic group, this role seems to compensate for the decomposition of complicated organic matter, which occurs during the process of composting. The bias of characteristics within common phylogenetic groups seems to lead to the complementary decomposition of complex organic matter in the composting process. The "No ID E" group utilizes only a few sole carbon sources. The ability of this group to utilize a variety of sole carbon sources is very limited during the composting process. Hence, it would be of interest to identify the role of the "NoID E" group in the composting process.

5. Conclusions

The current study clarified that the diversity of cultivable bacteria is extremely high, including undiscovered phylogenetic groups that were found in Tendo Compost and their commonality to Sapporo Compost. This study found differences in the temperature required for growth in the phylogenetic groups of bacteria that were isolated. In other words, because the environmental conditions (i.e., temperature and nutritional status) of the composting process are subject to dynamic changes, the microflora that actively participates in the composting process is very protean. The growth temperature was different for the phylogenetic groups of bacteria of each phylum that were isolated from Tendo Compost. For isolates grown at 37 °C, the primary phylum was Proteobacteria; Actinobacteria also formed a large proportion of the phyla, and Firmicutes to some extent. In comparison, isolates grown at 50 °C primarily comprised Actinobacteria and Firmicutes, while those at 60 °C comprised only Firmicutes. In general, the nutritional requirement of each phylum was different, (Fierer et al. 2007), and a wide range of temperatures were recorded during the composting process (i.e., from below 40 °C to over 60 °C) within a 1-day period. Therefore, because each phylum plays different roles in decomposting, in parallel to regularly fluctuating temperatures, the early decomposition of complex organic matter may be achieved. This may be the factor that composting goes very early. Furthermore, we identified several phylogenetic groups that showed a strong correlation to a composting system that was constructed over 500 km away. These similarities may indicate that specific phylogenetic groups play a very important role in the field-scale composting process of sewage sludge.

6. Acknowledgements

We thank Yasuko Ohba, Megumi Kikuchi, Yuko Kitayama, Mitsuru Fukasawa, Taro Jingu, Masako Hisatomi, Noriko Makino, and Xiao-yu Huang (Faculty of Applied Bio-Science, Tokyo University of Agriculture) for their technical assistance with the sequence analysis.

7. References

Amann, R., Krumholz, L. & Stahl, D. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology, *Journal of Bacteriology* 172: 762.

Balmford, A. & Bond, W. (2005). Trends in the state of nature and their implications for human well]being, *Ecology Letters* 8: 1218–1234.

Bochner, B. & Savageau, M.A. (1977). Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms, *Applied and Environmental Microbiology* 33: 434.

Brosius, J., Palmer, M.L., Kennedy, P.J. & Noller, H.F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli, *Proceedings of the National Academy of Sciences of the United States of America* 75: 4801.

Bruce, J. (1996). Automated system rapidly identifies and characterizes microorganisms in food, *Food Technology* 50: 77–81.

Brummer, I., Fehr, W. & Wagner-Dobler, I. (2000). Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle, *Applied and Environmental Microbiology* 66: 3078.

Chandra, R., Raj, A., Purohit, H. & Kapley, A. (2007). Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste, *Chemosphere* 67: 839–846.

Collen, B., Ram, M., Zamin, T. & McRae, L. (2008). The tropical biodiversity data gap: addressing disparity in global monitoring, *Tropical Conservation Science* 1: 75–88.

Crawford, D.L. & Crawford, R.L. (1976). Microbial degradation of lignocellulose: the lignin component, *Applied and Environmental Microbiology* 31: 714.

Díaz, S., Fargione, J., Chapin, F.S. & Tilman, D. (2006). Biodiversity loss threatens human well-being, *PLoS Biology* 4: 1300–1305.

DeLong, E.F. & Pace, N.R. (2001). Environmental diversity of bacteria and archaea, *Systematic Biology* 50: 470.

Felsenstein, J. (1989). PHYLIP-phylogeny inference package (version 3.2), *Cladistics* 5: 164–166.

Fierer, N., Bradford, M.A. & Jackson, R.B. (2007) . Toward an ecological classification of soil bacteria. *Ecology* 88: 1354-1364

Gest, H. (2003). *Microbes: an invisible universe*, Amer Society for Microbiology.

Hugenholtz, P., Goebel, B.M. & Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* 180: 4765.

Jørgensen, K., Puustinen, J. & Suortti, A.M. (2000). Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. *Environmental Pollution* 107: 245–254.

Kayhanian, M., Tchobanoglous, G. & Brown, R. (2007). Biomass Conversion Processes For Energy Recovery. Handbook of Energy Efficiency and Renewable Energy.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *Journal of Molecular Evolution* 16: 111–120.

Moter, A. & G bel, U.B. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms, *Journal of Microbiological Methods* 41: 85–112.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems, *Current Opinion in Microbiology* 2: 317–322.

Naeem, S., Hahn, D., & Schuurman, G. (2000). Producer-decomposer co-dependency influences biodiversity effects, *Nature* 403: 762–764.

Nakasaki, K., Tran, L.T.H., Idemoto, Y., Abe, M. & Rollon, A.P. (2009). Comparison of organic matter degradation and microbial community during thermophilic

composting of two different types of anaerobic sludge. *Bioresource Technology* 100: 676–682.

Ohnishi, A., Bando, Y., Fujimoto, N. & Suzuki, M. (2010). Development of a simple bio-hydrogen production system through dark fermentation by using unique microflora, *International Journal of Hydrogen Energy* 35: 8544–8553.

Ohnishi, A., Nagano, A., Fujimoto, N. & Suzuki, M. (2011). Phylogenetic and physiological characterization of mesophilic and thermophilic bacteria from a sewage sludge composting process in Sapporo, Japan, *World Journal of Microbiology and Biotechnology*: 333–340.

Okabe, S., Oshiki, M., Kamagata, Y., Yamaguchi, N., Toyofuku, M., Yawata, Y., et al. (2010). A great leap forward in microbial ecology, *Microbes and Environments* 25: 230–240.

Oren, A. (2004). Prokaryote diversity and taxonomy: current status and future challenges, *Philosophical Transactions B* 359: 623–638.

Payne, J.L., Boyer, A.G., Brown, J.H., Finnegan, S., Kowalewski, M., Krause, R.A., et al. (2009). Two-phase increase in the maximum size of life over 3.5 billion years reflects biological innovation and environmental opportunity, *Proceedings of the National Academy of Sciences* 106: 24.

Pedro, M.S., Hayashi, N.R., Mukai, T., Ishii, M., Yokota, A. & Igarashi, Y. (1999). Physiological and chemotaxonomical studies on microflora within a composter operated at high temperature. *Journal of Bioscience and Bioengineering* 88: 92–97.

Phae, C.G., Sasaki, M., Shoda, M. & Kubota, H. (1990). Characteristics of *Bacillus subtilis* isolated from composts suppressing phytopathogenic microorganism, *Soil Science and Plant Nutrition* 36: 575-586.

Pimentel, D., Rodrigues, G., Wang, T., Abrams, R., Goldberg, K., Staecker, H., et al. (1994). Renewable energy: economic and environmental issues, *BioScience* 44: 536–547.

Ranjard, L., Poly, F. & Nazaret, S. (2000). Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment, *Research in Microbiology* 151: 167–177.

Rapp, M.S. & Giovannoni, S.J. (2003). The uncultured microbial majority, *Microbiology* 57: 369.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Molecular Biology and Evolution* 4: 406.

Stackebrandt, E. & Goebel, BM. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology, I*nternational Journal of Systematic and Evolutionary Microbiology* 44: 846-849.

Swift, M.J., Izac, A. & van Noordwijk, M. (2004). Biodiversity and ecosystem services in agricultural landscapes--are we asking the right questions? *Agriculture, Ecosystems & Environment* 104: 113–134.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. & Higgins, D. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Research* 25: 4876.

Vaz-Moreira, I., Silva, M.E., Manaia, C.M. & Nunes, O.C. (2008). Diversity of bacterial isolates from commercial and homemade composts, *Microbial Ecology* 55: 714–722.

Torsvik, V., Daae, F.L. & Sandaa, R.A. (1998). Novel techniques for analysing microbial diversity in natural and perturbed environments, *Journal of Biotechnology* 64: 53–62.

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N. & Daims, H. (2002). Microbial community composition and function in wastewater treatment plants, *Antonie Van Leeuwenhoek* 81: 665–680.

Yanagi, M. & Yamasato, K. (1993). Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiology Letters* 107: 115–120.

Changing Diversity in Changing Environment
Edited by PhD. Oscar Grillo

ISBN 978-953-307-796-3
Hard cover, 392 pages
Publisher InTech
Published online 14, November, 2011
Published in print edition November, 2011

As everybody knows, the dynamic interactions between biotic and abiotic factors, as well as the anthropic ones, considerably affect global climate changes and consequently biology, ecology and distribution of life forms of our planet. These important natural events affect all ecosystems, causing important changes on biodiversity. Systematic and phylogenetic studies, biogeographic distribution analysis and evaluations of diversity richness are focal topics of this book written by international experts, some even considering economical effects and future perspectives on the managing and conservation plans.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Akihiro Ohnishi, Akihiro Nagano, Naoshi Fujimoto and Masaharu Suzuki (2011). Examination and Comparison of Microbial Diversity in Field-Scale Sewage Sludge Composters, Changing Diversity in Changing Environment, PhD. Oscar Grillo (Ed.), ISBN: 978-953-307-796-3, InTech, Available from: http://www.intechopen.com/books/changing-diversity-in-changing-environment/examination-and-comparison-of-microbial-diversity-in-field-scale-sewage-sludge-composters

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen