Vol. 13(7), pp. 134-144, 21 February, 2019
DOI: 10.5897/AJMR2018.9022
Article Number: 3BAD8DB60269
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and identification of bacteria from high-temperature compost at temperatures exceeding 90°C

Kikue Hirota[1], Chihaya Miura[1,2], Nobuhito Motomura[3], Hidetoshi Matsuyama[4] and Isao Yumoto[1,2]*

[1]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan.
[2]Laboratory of Molecular and Environmental Microbiology, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan.
[3]Chitose Recycling Factory, Chuuou Chitose 066-0007, Japan.
[4]Department of Bioscience and Technology, School of Biological Science and Engineering, Tokai University, Minamisawa, Minami-ku, Sapporo 005-8601, Japan.

Received 21 November, 2018; Accepted 15 January, 2019

Conventional composts exhibit temperatures ranging from 50 to 80°C during organic waste degradation by microorganisms. In high-temperature compost, temperatures can reach ≥90°C with appropriate bottom aeration. To elucidate specific characteristics of the bacterial activity in high-temperature compost and to regenerate a high-temperature compost from isolates, bacterial isolation and characterization were performed. Although the isolated taxa varied depending on sample and temperature, the use of gellan gum medium and cultivation at 60°C led to high diversity among the isolated taxa. In addition, combining the use of the compost extract with water-solvent medium led to the isolation of more diverse species. Based on 16S rRNA gene sequencing, the isolates shared ≥99% similarity with *Geobacillus thermodenitrificans*, *Ureibacillus* spp. (*Ureibacillus suwonensis*, *Ureibacillus themosphaericus*) and *Aeribacillus pallidus*, and these isolates were isolated from both steady-state and newly prepared small-scale composts. Thus, these taxa were considered to be frequently observed regardless of the composting process. Although the frequency of isolation of mesophilic bacteria from this high-temperature compost was lower than that from ordinary composts, these bacteria have been isolated from ordinary composts and there was a discrepancy between the *in situ* compost temperature (≥90°C) and their maximum growth temperature (≤70°C).

Key words: High-temperature compost, *Geobacillus thermodenitrificans*, *Ureibacillus suwonensis*, *Ureibacillus thermosphaericus*, *Aeribacillus pallidus.*

INTRODUCTION

Composting is an environmentally friendly process for degradation of organic waste. Organic waste is broken down concomitant with successive changes in the microbiota at various stages of composting. Wastes that have been completely broken down are then used to fertilize soil for plant growth. During ordinary composting, the temperature increases to approximately 50–80°C, at which microbial activity increases (Ryckeboer et al.,

*Corresponding author. E-mail: i.yumoto@aist.go.jp. Tel: +81-11-857-8925.

2003; Kumar, 2011). The heat produced is convenient for fertilization of soil for agricultural purposes because this heat is detrimental to harmful microorganisms and weed seeds. When composts are subjected to appropriate aeration, the temperature can exceed 90°C. Although there have been many studies on the constituent microorganisms in ordinary composts (Strom, 1985; Dees and Ghiorse, 2001; Ryckeboer et al., 2003; Wang et al., 2007; Kumar 2011; Antunes et al., 2016; Cerda et al., 2017), there have been only a few studies on microorganisms in high-temperature composts (≥90°C) (Oshima and Moriya, 2008; Yoshii et al., 2013; Tashiro et al., 2016). Although several examples of high-temperature composts are available, the operational procedures and the types of organic wastes applied might differ. Therefore, a different microbiota may exist in each type of high-temperature compost.

The ratio of microorganisms that can be isolated to preexisting background microorganisms in compost samples is low similar to that in ordinary environmental samples (Amann et al., 1995; Rappé and Giovannoni, 2003). Composting environments are specific environments that fluctuate over relatively large temperature ranges. In addition, many possible microbes can be present in these environments, depending on maintenance procedures and added substrates. Therefore, isolation of microorganisms is of great interest for identification of novel genetic resources. Several microorganisms associated with novel genera have been isolated (Ohno et al., 2000; Hatayama et al., 2005, 2006; Yabe et al., 2010, 2011a, b; Moriya et al., 2011; Wu et al., 2014; Sakai et al., 2015; Siddiqi et al., 2016). Microorganisms that belong to new genera can potentially be isolated from composts by testing new samples and developing new media.

Isolates from high-temperature compost have not been well explored. Application of isolates from compost will allow rapid production of high-temperature compost, which will increase the efficiency of waste decomposition. In this study, to understand the type of bacteria exist in high-temperature compost and to regenerate high-temperature compost by utilizing these isolates in a future study, this study aimed to isolate bacteria from the following high-temperature composts (≥90°C): an already running steady-state compost in several temperature states and a newly prepared small-scale compost without added substrate. Attempts were made to isolate the associated bacteria using various media, and then examined the taxonomic positions of these isolates. Finally, the isolates were compared with identified clones using a culture-independent method.

MATERIALS AND METHODS

Compost samples

Compost samples were obtained from the Chitose Recycling Factory (42° 51' 35" N, 141° 42' 31" E) in Chitose, Hokkaido. Various solid wastes or effluents from raw garbage, the food industry and fisheries were added to a pail (approximately 10 × 10 × 3 m, width × length × height) with this compost to degrade the organic compounds and evaporate the water in the waste. Samples from a steady-state compost, which was maintained for 10 years at different temperatures (90-100°C), were obtained at different sampling times (Table 1). In addition to the steady-state compost, samples were obtained from a newly prepared compost (approximately 1 × 1.5 × 1.5 m, width × length × height), which was not affected by loaded wastes. The compost was prepared by adding only water, no substrate, to the original material containing a composted tree-sourced material mixture (mostly root and bark), and this mixture was used as a sample for bacterial isolation.

Bacterial isolation and cultivation

Compost extract was used as an ingredient in the medium to accelerate colony formation by bacterial strains that are difficult to grow in ordinary media. The compost extract was prepared by mixing 100 g of the obtained compost with 1 L of distilled water and autoclaving at 121°C for 20 min. Then, the supernatant was obtained for use. The medium listed in Table 1 for bacterial isolation was used for the corresponding sample described in Table 2. The compost aliquot was first diluted ten-fold (w/w) with sterilized physiological saline. The liquid was serially diluted up to 10^5-fold, and the 10^3–10^5-fold dilutions were used to inoculate the medium, followed by incubation under aerobic conditions at 50–70°C for 2 days. We isolated colonies that were dependent on the variation and re-isolated these colonies at least three times from each medium or culture procedure. The composting process involves degradation introduced wastes. Therefore, it was important to examine whether the isolates could decompose substrates. Hydrolysis of starch (amylase) and casein (protease) and catalase activity were examined according to the methods described in Cowan and Steel's manual (Barrow and Feltham, 1993). Hydrolysis of xylan and cellulose was determined using 1% of each substrate according to the method described by Teather and Wood (1982).

DNA extraction, PCR and clone library construction

Colonies of the isolates from the compost samples were grown on the corresponding agar plates as described in Table 1, and DNA was extracted using the InstaGene matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. For the culture-independent approach based on a 16S rRNA gene library, DNA was directly extracted from an aliquot of the compost sample using ISOIL (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. PCR was performed to identify isolates obtained via the culture-dependent approach and to construct the 16S rRNA gene library in the culture-independent approach using the DNA extracted from the isolates and from the compost samples, respectively. The universal primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1514R (5'-AAGGAGGTGATCCAGCC-3') were used for PCR (Paster et al., 2002). The solution (100 μL) for the PCR consisted of 10 μL of 10 × PCR buffer, 8 μL of 2.5 mM dNTP mix, 100 ng of isolated DNA, 5 U of Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) and 20 pmol of each primer. The PCR was performed under the following conditions: 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1.5 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The PCR products from the isolates were directly used for sequence determination. The PCR product for the 16S rRNA clone library (culture-independent analysis) was cloned in *Escherichia coli* DH5α with the pT7Blue-2 vector system (Novagen, Madison, WI, USA) according

Table 1. Different media used in this study to isolate bacteria from elevated-temperature compost.

Medium name	Medium composition
SMA*	5 g casein peptone, 2.5 g yeast extract, 1 g glucose, 15 g agar in 1 L distilled water (pH 7)
PYG	5 g casein peptone, 3 g yeast extract, 1 g K_2HPO_4, 30 g gellan gum in 1 L distilled water (pH 8)
A-1	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L compost extract (pH 8)
A-2	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L distilled water (pH 8)
B-1	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L distilled water (pH 7.5)
B-2	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L compost extract (pH 7.5)

*Standard method agar.

Table 2. Elevated-temperature compost samples used for isolation and clone analysis.

Sample name	Temperature of compost (°C)	Steady state or early phase	Method	Medium used	Culture temperature (°C) and pH
CS1	96	Steady state	Isolation	SMA	50, pH 7
CS2	100	Steady state	Isolation	SMA	50, pH 7
CS3	90	Steady state	Isolation, clone analysis	PYG	60, 70, pH 8
CS4	90	Steady state	Isolation	A-1, A-2	60, pH 8
ChS	90	Early phase	Isolation	B-1, B-2	60, pH 7.5

to the manufacturer's instructions. Approximately 30 randomly selected clones were examined for the correct insert size by vector-targeted PCR followed by gel electrophoresis. Approximately 400–600-bp partial sequences from the amplified approximately 1,500-bp full-length sequence were analyzed as described below.

DNA sequencing and sequence assignment

The DNA sequence was determined by the dideoxy chain termination method using a BigDye Terminator Cycle Sequence Kit (Applied Biosystem, Foster City, CA, USA) and an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). The sequence assignments were determined by a BLAST search (Altschul et al., 1990). The sequences reported in this study were deposited in the DDBJ database under DDBJ/EMBL/GenBank accession numbers LC315695–LC315768 for the isolates and LC315983–LC316008 for the culture-independent clone libraries.

Phylogenetic analysis

Phylogenetic analysis was performed using the determined 16S rRNA gene sequence of the family Bacillaceae. The sequences were aligned, and the consensus sequence was determined using CLUSTAL W (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbor-joining method, and the distances between sequences (K_{unc} value) were calculated using Kimura's two-parameter model (Kimura, 1980; Saitou and Nei, 1987) in MEGA 7 (Kumar et al., 2016). The confidence values for the branches of the phylogenetic tree were determined by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings.

RESULTS

Isolation of bacteria

To understand the species of bacteria that can be isolated from high-temperature (≥90°C) compost, 80 strains were isolated from 5 samples that exhibited different temperatures (90–100°C). In addition, although four samples were obtained from the steady-state compost (including 2 composts at temperatures higher than 95°C), one sample was obtained from newly prepared small-scale compost without added substrate for microbial growth (Table 2). However, several types of media were used for bacterial isolation from each sample. Notably, these media used agar or gellan gum for solidification and included or lacked compost extract (Table 1). Although the *in situ* temperature was ≥90°C, no growth was observed at temperatures greater than 80°C for any of the samples used in this study in either solid or liquid medium. Although the medium composition and temperature for isolation were different for each sample, the differences in the isolated bacteria were dependent on the sample. This result suggests that the microbiota of high-temperature compost varies depending on the *in situ* temperature, sampling location or waste degradation phase.

The most diverse species of bacteria were isolated from the CS3 sample using PYG (peptone, yeast extract and gellan gum) medium (incubated at 60°C), which contained gellan gum (Table 1) because of the characteristics of the sample and the use of gellan gum. Only *Thermus thermophilus* or *T. thermophilus*-related strains were isolated when PYG medium was used for the CS3 sample when the incubation temperature of the culture was 70°C (Table 3). Based on a comparison of results for media A-1 with A-2 and B-1 with B-2, the combined use of the compost extract with water-solvent medium has led to the isolation of highly diverse species.

Table 3. Summary of the isolates recovered using the different media. Bacterial identification was based on ≥99% similarity in the 16S rRNA gene sequence.

Medium/Sample names Sample/Culture temp. (°C)	SMA/CS1 96/50	SMA/CS2 100/50	PYG*/CS3 90/60	PYG*/CS3 90/70	A-1**/CS4 90/60	A-2/CS4 90/60	B-1/ChS 90/60	B-2**/ChS 90/60	Total number of isolates[¶]
Bacillus hisashii	3								3 (1)
Bacillus thermoamylovorans	1								1 (1)
Bacillus subtilis	1								1 (1)
Bacillus thermoamylovorans	1								1 (1)
Bacillus thermocloaceae			1		4				5 (2)
Bacillus kokeshiformis			1						1 (1)
Bacillus termolactis			1						1 (1)
Bacillus borborid								1	1 (1)
Ureibacillus suwonensis		6						1	7 (2)
Ureibacillus thermosphaericus			3					1	4 (2)
Ureibacillus terrenus			11						11 (1)
Geobacillus thermodenitrificans			4			7	6		17 (3)
Aeribacillus pallidus						1	1	1	3 (2)
Noviobacillus thermophilus					2				2 (1)
Thermobifida fusca			2						2 (1)
Thermus thermophilus				5					5 (1)
No. of strains exhibiting ≤98% similarity to reported species	1	1[§]	1[§]	5	1[¶]				

*Only this medium used gellan gum for solidification. ** Compost extract-containing media. [¶]Numbers in the brackets indicate detected sample counts.
[§]This was due to the sequence quality. DNA extraction for 16S rRNA gene sequence analysis was performed by using the stain grown in the corresponding isolation medium (Table 2).
[¶]This strain was CS4-7 (LC315748) exhibited 91% similarity with *Melghiribacillus thermophilus* (NR_134761) (data not shown).

Ureibacillus spp. were isolated from medium that contained compost extract, while *Geobacillus thermodenitrificans* was not isolated from this medium (Table 3). Although most of these isolates were not very distantly taxonomically related to reported species, one strain, namely, CS4-7, was very different from a reported species (*Melghiribacillus thermohalophilus*, 91%). This strain was isolated from A-1 medium, which contained compost extract.

Although *Ureibacillus terrenus* and *T. thermophilus* were isolated from only one sample (CS3), based on the identification of isolates by 16S rRNA gene sequencing, strains that exhibited higher than 99% similarly with *G. thermodenitrificans*, *Ureibacillus suwonensis*, *Ureibacillus thermosphaericus*, *U. terrenus*, *Bacillus thermocloaceae*, *Aeribacillus pallidus* and *T. thermophilus* were frequently isolated when the incubation temperature of the medium was 50–70°C (Table 3). Although high-temperature compost was used in the present study as the source of these isolates, the isolated species are commonly observed in ordinary composts. In addition, the isolates did not exhibit growth at temperatures greater than 80°C (data not shown), and there have been no reports of the corresponding identified species growing at temperatures greater than 80°C (Table 4), which indicated a discrepancy between the isolated taxa and the *in situ* temperatures of the sources. However, this study indicates that the frequency of isolation of mesophilic microorganisms was lower in high-temperature compost (only two strains, identified as *Bacillus subtilis* (>99% similarity) and *Sphingomonas melonis* (98% similarity) isolated from CS1 sample) than in moderate-temperature compost (Dees and Ghiorse, 2001; Li et al., 2014; López-González et al., 2015).

Table 4. Growth temperature ranges and substrate utilization of the isolates from the CS3 sample.

Strain	Closely associated phylogenetic relative	Growth temperature range (°C)*	Cellulase	Xylanase	Protease (casein)	Amylase	Catalase
CS3-1	*Bacillus thermocloaceae* (NR_036986)	55-65	ND	ND	–	ND	ND
CS3-2	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-3	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-4	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-5	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-6	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-7	*Ureibacillus terrenus* (NR_025394)	42-65		–	+	–	+
CS3-8	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-9	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-10	*Thermobifida fusca* (NR_14411)	35-60	+	+	+	+	+
CS3-11	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-12	*Bacillus kokeshiformis* (NR_133	35-61 (50)	–	–	ND	+	+
CS3-13	*Thermobifida fusca* (NR_14411)	35-60	+	+	+	+	+
CS3-14	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-15	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-16	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-17	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-18	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-19	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-20	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-21	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-22	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-23	*Bacillus thermolactis* (NR_115226)	40-60	–	–	–	+	+
CS3-24	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	ND	ND	ND

All isolates exhibited 99% similarity with closely associated phylogenetic relatives. *Growth temperature ranges were cited from Zarilla and Rerry (1987); Zhang et al. (1998); Fortina et al. (2001); Kim et al. (2006); Cihan et al. (2011); Coorevits et al. (2011); Wang et al. (2013); Poudel et al. (2014); and Yang et al. (2015). The numbers in brackets are the optimum growth temperatures.

It is expected that many strains can produce macromolecule degradation-related enzymes because various wastes were frequently added to this compost. Although eight strains exhibited amylase activity among the twenty-four strains, only three strains exhibited protease activity. *Thermobifida fusca* strains, belonging to the phylum Actinobacteria, were isolated from the CS3 sample. Only these two strains produced multiple enzymes that degraded various macromolecules (Table 4).

The temperature of the compost exceeded 100°C when the CS2 sample was obtained. Although the heat was apparently produced as a result of vigorous microbial activity, the diversity of the isolates decreased at this temperature (Table 3). The colony morphology on the plate inoculated with a sample from the compost with a temperature greater than 100°C was homogenous (data not shown). Seven colonies were picked from the plate, and the 16S rRNA gene sequences

of the isolates were determined. According to the results, although one strain exhibited 98% similarity to *U. suwonensis* due to the low quality of the sequence, 6 isolates were very similar (≥99% similarity) to *U. suwonensis* (Table 3).

There is a possibility that the generation of high temperatures (≥90°C) is dependent on the mass of the compost and the types of waste added. Therefore, we tried to prepare small-scale compost by adding only water without adding waste. In addition, to understand the types of bacteria that can be isolated from the early phase of high-temperature compost, bacterial isolation was performed with two kinds of medium one of which contained compost extract. As in the steady-state compost, *G. thermodenitrificans*-related strains (≥99% similarity) were most frequently isolated from this sample (6 strains; Table 3). In addition to these species, *A. pallidus*-related strains (≥99% similarity) were isolated from both types of medium (with and without compost extract; Table 3). Isolates sharing ≥99% similarity with *G. thermodenitrificans*, *Ureibacillus* spp. (*U. suwonensis*, *U. themosphaericus*) and *A. pallidus* were isolated from both steady-state and newly prepared small-scale composts. Therefore, these taxa were considered to be frequently observed isolates regardless of the composting process. The introduction of a mixture of these species at the start of composting may accelerate the initiation of high-temperature composts in the absence of materials such as compost seed, from which the expected microbiota originates.

To understand the phylogenetic relationships among the isolates, a neighbor-joining phylogenetic tree was constructed (Figure 1). According to the tree, the predominant isolates throughout the samples, namely, the *G. thermodenitrificans*-related strains, were quite similar phylogenetically and had slightly different phylogenetic positions from the type strain of *G. thermodenitrificans*. In addition, the *U. suwonensis* and *U. thermosphaericus*-related strains exhibited slightly different phylogenetic positions from the type strains of each species belonging to this genus. In the clade containing *Bacillus kokeshiiformis*, various species exhibited similar phylogenetic positions (Coorevits et al., 2011). Therefore, to assess the novelty of the strains CS3-12 and CS3-23, additional detailed experiments, such as DNA-DNA hybridization, are necessary. Other isolates located at almost the same phylogenetic positions corresponded to the most closely related taxa.

Culture-independent analysis

The most diverse species of bacteria were isolated from the CS3 sample using PYG, which contained gellan gum. The culture-independent approach (clone analysis) was also applied to the CS3 sample (Table 5). Although many diverse bacterial strains were isolated from the samples, there was a difference in the bacterial diversity of the isolates between the culture-dependent and culture-independent approaches. More than 42% of the isolated clones shared less than 95% similarity with reported gene sequences. The results described above suggest that most of the bacteria in the high-temperature compost could not be easily isolated by ordinary bacterial isolation methods. The predominant group was the family Bacillaceae. Based on classification at the genus level, the genera *Bacillus*, *Ureibacillus* and *Paenibacillus* were major constituents. Although strains sharing 95-96% similarity with *Paenibacillus sabinae* were frequently detected in the culture-independent analysis, this taxon was not isolated. Although strains sharing greater than 99% similarity with *T. fusca*, which exhibited multiple enzyme activities (Table 4), were isolated in the medium containing gellan gum, this taxon was not detected by the culture-independent approach. Although the compost was aerated, obligate anaerobic bacterial clones belonging to the family Thermoanaerobacteraceae were detected. This finding indicates that although the compost was aerated, there were niches where obligate anaerobes could thrive in this compost.

DISCUSSION

To understand the differences between the bacteria isolated from high-temperature compost and ordinary compost, both culture-dependent and culture-independent approaches were used to study high-temperature compost. We examined the probability of isolating bacteria from high-temperature compost using different samples and media. However, there was a discrepancy between the *in situ* compost temperature (≥90°C) and the maximum growth temperature (<80°C) ranges in the medium used to grow the isolates. This discrepancy could be a result of the temperature inside the compost being higher than out sites (that is, at the surface), with most of the microbial activity occurring in these low-temperature niches. Our isolates, which were buried inside the compost, were not able to propagate at temperatures higher than 80°C, in the laboratory experiment. These isolates are thought to have remained alive *in situ* because they were isolated from the environment. However, it is not known whether these isolates were metabolically active *in situ*. On the other hand, according to the culture-independent analysis, there may exist unculturable microorganisms that can grow at temperatures greater than 80°C under specific culture conditions that were not tested in this study, such as strictly anaerobic, microaerobic or low-nutrient conditions (Janssen and Yates, 2002).

Although isolates from high-temperature compost (≥90°C) were not able to grow at temperatures higher than 90°C, the frequency of isolation of mesophilic bacteria was lower than that from ordinary temperature compost (50 to 80°C) (Dees and Ghiorse, 2001; Li et al., 2014; López-González et al., 2015). However, no isolate

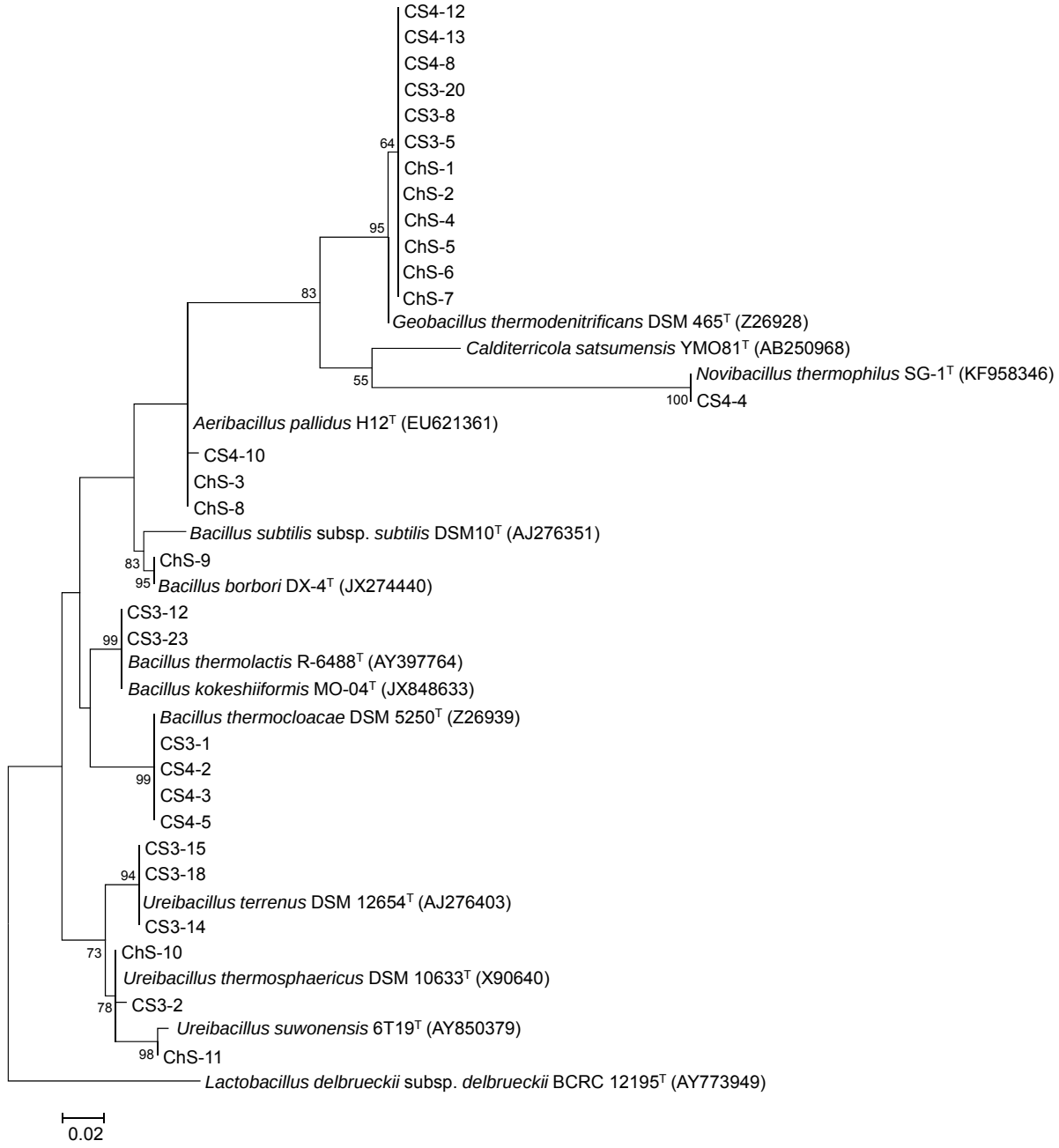

Figure 1. Phylogenetic tree showing the positions of isolates from three different samples of high-temperature compost. The strain name represents the sample name listed in Table 2. Bootstrap percentages (based on 1000 replicates) >50% are shown at the nodes. *Lactobacillus delbrueckii* subsp. *delbrueckii* BCRC 12195^T was used as an outgroup. Scale bar=0.01 substitution per nucleotide position.

could grow at temperatures higher than 90°C. This finding indicates that selective pressure against mesophilic bacteria in high-temperature compost was higher than that in ordinary compost. Although there are two previous examples of isolates of new species of bacteria that can grow at 80°C (Moriya et al., 2011), the isolation of bacteria that can grow at temperatures higher than 90°C from high-temperature compost has not been previously reported (Oshima and Moriya, 2008; Yoshii et al., 2013). We believe there was little possibility of isolating microorganisms that could grow at temperatures higher than 80°C from the compost used in the present study. Aliquots of the compost samples were used to inoculate broth medium, and microbial growth was never

Table 5. Sequence analysis of 16S rDNA obtained from clone library constracted from the samples from 90°C compost.

Clone	Length (bp)	Accessing no.	Closely identified phylogenetic relative	Similarity (%)	Clone	Length (bp)	Accessing no.	Closely identified phylogenetic relative	Similarity (%)
CC-1	438	LC315983	*Ureibacillus thermosphaericus* (NR_040961)	98	CC-14	590	LC315996	*Bacillus thermotolerans* NR_118456	99
CC-2	535	LC315984	*Ureibacillus thermosphaericus* (NR_040961)	97	CC-15	496	LC315997	*Anoxybacillus calidus* NR_125532	92
CC-3	547	LC315985	*Bacillus infernus* (NR_027227)	93	CC-16	593	LC315998	*Bacillus acidicola* NR_041942	94
CC-4	549	LC315986	*Bacillus massiliogorillae* (NR_133029)	93	CC-17	556	LC315999	*Bacillus thermocloacae* (NR_036986)	99
CC-5	466	LC315987	*Geobacillus icigianus* (NR_134736)	90	CC-18	427	LC316000	*Fervidicola ferrireducens* (NR_044504)	94
CC-6	556	LC315988	*Bacillus thermocloacae* (NR_036986)	98	CC-19	418	LC316001	*Paenibacillus sabinae* (NR_121732)	96
CC-7	553	LC315989	*Ureibacillus thermosphaericus* (NR_040961)	95	CC-20	430	LC316002	*Caldilinea aerophila* (NR_074397)	88
CC-8	561	LC315990	*Geobacillus thermodenitrificans* (NR_043021)	100	CC-21	403	LC316003	*Paenibacillus sabinae* (NR_121732)	96
CC-9	560	LC315991	*Paenibacillus sabinae* (NR_122066)	96	CC-22	528	LC316004	*Melghiribacillus thermohalophilus* (NR_134761)	87
CC-10	575	LC315992	*Bacillus thermocloacae* (NR_036986)	99	CC-23	459	LC316005	*Thermus thermophilus* (NR_037066)	98
CC-11	564	LC315993	*Bacillus thermocloacae* (NR_036986)	99	CC-24	565	LC316006	*Bacillus subterraneus* (NR_104749)	94
CC-12	570	LC315994	*Ureibacillus thermosphaericus* (NR_040961)	98	CC-25	580	LC316007	*Bacillus thermocloacae* (NR_036986)	90
CC-13	574	LC315995	*Thermacetogenium phaeum* (NR_074723)	92	CC-26	580	LC316008	*Paenibacillus sabinae* (NR_122066)	95

observed at 80°C (data not shown). This finding suggested that the *in situ* compost environment has not been adequately reproduced in the laboratory. Although we isolated as many as 80 colonies of bacteria from the compost using various conditions, none of the isolates could grow at 80°C. Therefore, the existence of many types of bacteria that can grow at temperatures higher than 80°C under laboratory culture conditions is highly unlikely. Bacteria that exhibited growth at sites with low localized temperatures (e.g., at the surface) were occasionally introduced into the interior of the compost and we isolated surviving microorganisms that were not able to grow at temperatures higher than 80°C.

Another possibility is that there is a difference in moisture content between the *in situ* compost and the recovery medium in the laboratory. The relatively higher moisture content in the media used in the laboratory may have hindered bacterial growth. Although some thermophiles exhibit metabolic activity at temperatures higher than 90°C, these organisms can be expected to exhibit growth at lower temperatures. Under such extreme conditions, there is a gap between the temperatures suitable for metabolism and those suitable for propagation. For example, it has been reported that *Amphibacillus iburiensis* can survive at pH values higher than 10.5 and exhibits indigo-reducing activity at those pH values (Hirota et al., 2013). However, bacterial propagation is observed at relatively low pH values under laboratory conditions. Although there was apparent metabolic activity in the form of acid production by the inoculated cells in broth adjusted to a pH value greater than pH 10, actual growth was initiated at pH 8.0–9.1, with optimum growth observed at pH 8.9–9.1 (30°C) (Hirota et al., 2013). Thus, we hypothesize that there are bacterial niches that exhibit temperatures lower than that inside the compost and that the temperature range in which bacteria are able to perform metabolic activity is different from the range in which bacterial propagation can occur.

We attempted to isolate as many colonies as possible from various compost samples that exhibited different temperatures (90-100°C). However, in many cases, no colony diversity was observed when an ordinary agar plate was used for isolation using composts with >90°C temperatures. When gellan gum was used and in sample CS3 (90°C), relatively diverse micro-organisms were isolated. The superiority of gellan gum over agar has been reported previously due to the prevention of H_2O_2 production by this material (Tanaka et al., 2014). Although most of the isolates produced catalase when grown in this medium under laboratory conditions, the physiological states of these isolates *in situ* may not be as good as those under laboratory conditions. Therefore, the presence of H_2O_2 in the medium may inhibit colony formation. In addition, the effect of gellan gum may be attributed to not

only the suitability of the medium but also the high microbial activity of part of the sample used (CS3). Although different media were used for different samples, we believe that the microbial diversity observed was due to changes in the microbiota and bacterial localization. This finding was consistent with the fluctuation in temperature, the uniformity of the substrates and the inhomogeneity of the compost.

Compost extracts or extracts from isolates have been previously used to isolate bacteria from compost (Rhee et al., 2002; Bae et al., 2005; Yabe et al., 2011). Compost extract was used to expand the known diversity of identified bacterial strains. Although growth stimulation was observed upon addition of the extract, an adverse effect was also observed. This adverse effect may also be important for isolation of undiscovered bacteria. There are several examples of the isolation of novel microorganisms by the addition of antibiotics to the isolation medium (Yabe et al., 2009, 2010, 2011a, b). Furthermore, bacteria have been successfully isolated by the addition of bacterial cell extracts (Rhee et al., 2002; Bae et al., 2005). In the present study, one isolated strain, namely, CS4-7, exhibited low similarity with a reported species (*Melghiribacillus thermohalophilus*, 91%). Various nutrient concentrations in the medium, especially low nutrient concentrations are predicted to lead to the isolation of increasingly wide ranges of bacterial species (Janssen and Yates, 2002).

Isolates sharing ≥99% similarity with *G*. *thermodenitrificans*, *Ureibacillus* spp. (*U*. *suwonensis*, *U*. *themosphaericus*) and *A*. *pallidus* were isolated from both steady-state and newly prepared small-scale composts. These genera are frequently isolated from ordinary compost at temperatures higher than 50°C (Baharuddin et al., 2010; Li et al., 2014). Among the above described taxa, a *G*. *thermodenitrificans*-like strain was most frequently observed, and a similar observation was reported previously in ordinary compost (Li et al., 2014). This suggests that the *G*. *thermodenitrificans*-like strain plays a central role in high-temperature compost as well as ordinary compost. In this study, *A*. *pallidus* was isolated mostly from early-phase compost. On the other hand, *Ureibacillus* spp. was isolated mostly from steady-state compost. The introduction of a mixture of the three genera at the start of composting may accelerate composting or elevate temperature in both the early-phase and steady state.

In the process of identifying bacteria that play key roles in the initiation of high-temperature compost, adjustment of moisture conditions and aeration from the bottom successfully led to the generation of high-temperature compost without the addition of substrates (that is, wastes). Therefore, the origin of the microbiota was considered to be the composted mixture of tree materials (mostly tree roots and bark). In the present study, *G*. *thermodenitrificans*, *A*. *pallidus* and *Bacillus borbori*-related strains were frequently isolated. These strains are candidate bacteria for increased heat production during initiation of composting. There are clear indications of the mechanisms underlying the production of heat during the composting process. One direct possibility is that heat-producing bacteria are present in the compost. Heat-producing bacteria, such as *Pseudomonas putida*, have been previously reported (Tabata et al., 2013). Ordinary compost that lacks aeration does not reach temperatures higher than 90°C. Therefore, it is possible that excessive heat production is uncoupled from metabolism via stimulation by complex conditions including aeration. Energy dispersal from continuous bacterial processes may be useful for continuous operation without the production of residual biomass (Lapara et al., 2000).

Conclusions

Although the frequency of isolation of mesophilic microorganisms was lower in high-temperature compost (only one strain identified as *B. subtilis*) than in moderate-temperature compost, the upper limit of the growth temperature (approximately 70°C) of the isolates in the present study was much lower than the *in situ* temperature (90–108°C). There are several possible explanations for this discrepancy: 1) the *in situ* compost environment is different from the culture conditions in the laboratory; 2) the temperature range in which the bacteria exhibit metabolic activity but not propagation is different from that in which bacterial metabolism is coupled with propagations; 3) the bacteria that exhibited propagation at localized low-temperature sites (for example, at the surface) were occasionally introduced into the interior of the compost, and we isolated surviving microorganisms that were not able to grow at temperatures higher than 80°C. Based on the above possibilities, further studies are needed to resolve the contradiction that none of the isolates from the high-temperature compost (≥90°C) could grow at temperatures ≥80°C. Isolates that shared ≥99% similarity with *G*. *thermodenitrificans*, *Ureibacillus* spp. (*U*. *suwonensis*, *U*. *themosphaericus*) and *A*. *pallidus* were isolated from both steady state and newly prepared small-scale composts. Introduction of a mixture of these species at the start of composting may accelerate the initiation of high-temperature composts in the absence of materials such as compost seed, from which the expected microbiota originates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors thank Mr. Takayuki Motomura for providing

the opportunity and supporting the analysis of the high-temperature compost.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. Journal Molecular Biology 215:403-410.

Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews 59:143-169.

Antunes LP, Martins LF, Pereira RV, Thomas AM, Barbosa D, Lemos LN, Silva GM, Moura LM, Epamino GW, Digiampietri LA, Lombardi KC, Ramos PL, Quaggio RB, de Oliveira JC, Pascon RC, Cruz JB, da Silva AM, Setubal JC (2016). Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. Scientific Report 6:38915.

Bae J-W, Rhee S-K, Park JR, Kim B-C, Park Y-H (2005). Isolation of uncultured anaerobic thermophiles from compost by supplementing cell extract of *Geobacillus toebii* in enrichment culture medium. Extremophiles 9:477-485.

Baharuddin AS, Razak MNA, Hock LS, Ahmad MN, Abd AS, Rahman NAA, Shah UKM, Hassan MA, Sakai K, Shirai Y (2010). Isolation and characterization of thermophilic cellulaseproducing bacteria from empty fruit brunches-palm oil mill effluent compost. American Journal Applied Science 7:56-62.

Barrow GI, Feltham RKA (editors) (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3*rd edn. Cambridge University Press, Cambridge.

Cerda A, Artola A, Font X, Barrena R, Gea B, Sánchez A (2017). Composting of food wastes: status and challenges. Bioresource Technology 8, 57-67.

Cihan AC, Ozcan B, Tekin N, Cokmus C (2011). *Geobacillus thermodenitrificans* subsp. calidus, subsp. nov., a thermophilic and □-glucosidase producing bacterium isolated from Kizilcahaman, Turkey. Journal of General and Applied Microbiology 57:83-92.

Coorevits A, Logan NA, Dinsdale AE, Halket G, Scheldeman P, Heyndrickx M, Schumann P, Van Landschoot A, De Vos P (2011). *Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*. International Journal Systematic Evolutionary Microbiology 61:1954-1961.

Dees PM, Ghiorse WC (2001). Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. FEMS Microbiology Ecology 35:207-216.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Fortina MG, Pukall R, Schumann P, Mora D, Parini C, Manachini PL, Stackebrandt E (2001). *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson et al. 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. International Journal Systematic and Evolutionary Microbiology 51:447-455.

Hatayama K, Shoun H, Ueda Y, Nakamura A (2005). *Planifilum fimeticola* gen. nov., sp. nov. and *Planifilum fulgidum* sp. nov., novel members of the family '*Thermoactinomycetaceae*' isolated from compost. International Journal Systematic and Evolutionary Microbiology 55:2101-2104.

Hatayama K, Shoun H, Ueda Y, Nakamura A (2006). *Tuberibacillus calidus* gen. nov., sp. nov., isolated from a compost pile and reclassification of *Bacillus naganoensis* Tominura et al. 1990 as *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *Bacillus laevolacticus* Andersch et al. 1994 as *Sporolactobacillus laevolacticus* comb. nov. International Journal Systematic and Evolutionary Microbiology 56:2545-2551.

Hirota K, Aino K, Yumoto I (2013). *Amphibacillus iburiensis* sp. nov., an alkaliphile that reduces an indigo dye. International Journal Systematic and Evolutionary Microbiology 63:4303-4308.

Janssen PH, Yates PS (2002). Improved culturability of soil bacteria and isolation in pure cultures representative of the phylum-level diversity of soil bacteria. Applied and Environmental Microbiology 68:2392-2396.

Kim B-Y, Lee S-Y, Weon H-Y, Kwon S-W, Go S-J, Park Y-K, Schuman P, Fritze D (2006) *Ureibacillus suwonensis* sp., nov., isolated from cotton waste composts. International Journal of Systematic and Evolutionary Microbiology 56:663-666.

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.

Kumar S (2011). Composting of municipal solid waste. Critical Reviews in Biotechnology 31:112-136.

Kumar S, Stecher G, Tamura K (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874.

Lapara TM, Konopka A, Alleman JE (2000). Energy spilling by thermophilic aerobes in potassium-limited continuous culture. Water Research 34:2723-2726.

Li R, Li L, Huang R, Sun Y, Mei X, Shen B, Shen Q (2014). Variations of culturable thermophilic microbe numbers and bacterial communities during the thermophilic phase of composting. World Journal Microbiology and Biotechnology 30:1737-1746.

López-González JA, Suárez-Estrella F, Vargas-García MC, López MJ, Jurado MM, Moreno J (2015). Dynamics of bacterial microbiota during lignocellulosic waste composting: Studies upon its structure, functionality and biodiversity. Bioresource Technology 175:406-416.

Moriya T, Hikota T, Yumoto I, Ito T, Terui Y, Yamagishi A, Oshima T (2011). *Calditerricola satsumensis* gen. nov., sp. nov. and *Calditericola yamamurae* sp. nov., extreme thermophiles isolated from a high temperature compost. International Journal Systematic and Evolutionary Microbiology 61:631-636.

Ohno M, Shiratori H, Park MJ, Saitoh Y, Kumon Y, Yamashita N, Hirata A, Nishida H, Ueda K, Beppu T (2000). *Symbiobacterium thermophilum* gen. nov., sp. nov., a symbiotic thermophile that depends on co-culture with a Bacillus strain for growth. International Journal Systematic and Evolutionary Microbiology 50:1829-1832.

Oshima T, Moriya T (2008). A preliminary analysis of microbial and biochemical properties of high-temperature compost. Annals of the New York Academy of Sciences 1125:338-344.

Rappé MS, Giovannoni SJ (2003). The uncultured microbial majority. Annual Review of Microbiology 57:369-394.

Paster BJ, Russell MK, Alpagot T, Lee AM, Boches SK, Galvin JL, Dewhirst FE (2002). Bacterial diversity in necrotizing ulcerative periodontitis in HIV-positive subjects. Annals of Periodontology 7:8-16.

Rhee SK, Jeon CO, Bae JW, Kim K, Song JJ, Kim JJ, Lee SG, Kim HI, Hong SP, Choi YH, Kim SM, Sung MH (2002). Characterization of *Symbiobacterium toebii*, an obligate commensal thermophile isolated from compost. Extremophiles 6:57-64.

Poudel P, Miyamoto H, Miyamoto H, Okugawa Y, Tashiro Y, Sakai K (2014). Thermotolerant *Bacillus kokeshiformis* sp. nov. isolated from marine animal resources compost. Int. International Journal Systematic and Evolutionary Microbiology 64:2668-2674.

Ryckeboer J, Mergaert J, Vaes K, Klammer S, De Clercq D, Coosemans J, Insam H, Swings J (2003) A survey of bacteria and fungi occurring during composting and self-heating processes. Annals of Microbiology 53:349-410.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sakai M, Deguchi D, Hosoda A, Kawauchi T, Ikenaga M (2015). *Ammoniibacillus agariperforans* gen. nov., sp. nov., isolated from compost. International Journal Systematic and Evolutionary Microbiology 65:570-577.

Siddiqi MZ, Muhammad SS, Choi KD, Im WT (2016). *Compostibacter hankyongensis* gen. nov., sp. nov., isolated from compost. International Journal Systematic and Evolutionary Microbiology 66:3681-3687.

Strom PF (1985). Identification of thermophilic bacteria in solid-waste composting. Applied and Environmental Microbiology 60:3049-3054.

Tabata K, Hida F, Kiriyama T, Ishizaki N, Kamachi T, Okuma I (2013). Measurement of soil bacterial colony temperatures and isolation of a high heat-producing bacterium. BMC Microbiology 13:56.

Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, Tanaka M, Nakatsu CH, Kamagata Y (2014). A hidden pitfall in the preparation of agar media undermines microorganism cultivability. Applied and Environmental Microbiology 80:7659-7666.

Tashiro Y, Tabata H, Itahara A, Shimizu N, Tashiro K, Sakai K (2016). Unique hyper-thermal composting process in Kagoshima City forms distinct bacterial community structures. Journal Bioscience Bioengineering 122:606-612.

Teather RM, Wood PJ (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Applied and Environmental Microbiology 43:777-780.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Research 22:4673-4680.

Wang CM, Shye CL, Ho SP, Chiou SH (2007). Species diversity and substrate utilization pattern of thermophilic bacteria communities in hot aerobic poultry and cattle manure composts. Microbial Ecology 54:1-9.

Wang Y-Q, Yuan Y, Yu Z, Yang G-Q, Zhou S-G (2013). *Bacillus borborid* sp. nov., isolated from an electrochemically active biofil, Current Microbiology 67:718-724.

Wu H, Lian Y, Liu B, Ren Y, Qin P, Huang F (2014). *Thermotunica guangxiensis* gen. nov., sp. nov., isolated from mushroom residue compost. International Journal Systematic and Evolutionary Microbiology 64:1593-1599.

Yabe S, Kato A, Hazaka M, Yokota A (2009). *Thermaerobacter compostii* sp. nov., a novel extremely thermophilic bacterium isolated from compost. Journal of General and Applied Microbiology 55:323-328.

Yabe S, Aiba Y, Sakai Y, Hazaka M, Yokota A (2010). *Thermosporothrix hazakensis* gen. nov., sp. nov. isolated from compost and description of *Thermosporotrichaceae* fam. nov. within the class *Ktedonobacteria*. International Journal Systematic and Evolutionary Microbiology 60:1794-1801.

Yabe S, Aiba Y, Sakai Y, Hazaka M, Yokota A (2011a). *Thermasporomyces composti* gen. nov., sp. nov., a novel thermophilic actinomydete isolated from compost. International Journal of Systematic and Evolutionary Microbiology 61:86-90.

Yabe S, Aiba Y, Sakai Y, Hazaka M, Yokota A (2011b). Isolation of thermophilic bacteria from compost on antibiotic-supplemented medium. Microbiology and Culture Collections (in Japanease) 27:73-78.

Yang G, Chen J, Zhou S (2015). *Novibacillus thermophilus* gen. nov., sp. nov., a Gram-staining-negative and moderately thermophilic member of the family *Thermoactinomycetaceae*. International Journal Systematic and Evolutionary Microbiology 65:2591-2597.

Yoshii T, Moriya T, Oshima T (2013). Bacterial and biochemical properties of newly invented aerobic, high-temperature compost. Satyanarayana T (eds) Thermophilic microbes in environmental and industrial biotechnology: Biotechnology of thermophiles, 2nd edn. Springer, Dordrecht Heidelberg New York London pp. 119-135.

Zarilla KA, Perry JJ (1987) *Bacillus thermolevorans* sp. nov. A species of obligately thermophilic hydrocarbon-utilizing endospore-forming bacteria. Systematic Applied Microbiology 9:258-264.

Zhang Z, Wang Y, Ruan J (1998). Reclassification of *Thermomonospora* and *Microteyraspora*. International Journal Systematic Evolutionary Microbiology 48:411-422.