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Thermophilic bacterial communities in hot composts as revealed by most probable number counts and molecular (16S rDNA) methods

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Abstract

Thermogenic composts are known to host a variety of thermophilic micro-organisms that were recently investigated by cultural means and identified as *Thermus thermophilus*, *Bacillus* spp., and *Hydrogenobacter* spp. In this paper, we present a classical, cultural enumeration of thermophilic populations on the one hand, and a molecular investigation of the bacterial community by restriction enzyme analyses of a clone library of bacterial 16S rRNA genes on the other hand. Bacterial diversity, revealed by the clone analyses of four samples, was shown to undergo a dramatic change between the young (13–18-day) and the old (39–41-day) samples, possibly linked to the general decrease in temperature and the physicochemical evolution of organic matter during the composting process. Among the 200 clones investigated, 69 clones could be identified as *Thermus thermophilus* and thermophilic *Bacillus* spp. These results proved both taxa to be among the dominant bacterial populations at the highest temperatures reached by thermogenic composts.

Keywords: Compost; Thermophilic *Bacteria*; 16S rDNA clone library; *Thermus*; Thermophilic *Bacillus*

1. Introduction

Composting is a self-heating, aerobic, solid-phase process, during which organic waste materials are biologically degraded [1–3]. Among the factors which condition the development of microbial populations in compost, such as oxygen and nutrient availability, the temperature increase up to 65–80°C results in a rapid transition from a mesophilic to a thermophilic community [4–6]. This thermogenic phase is followed by a slow temperature decrease where the diversity of micro-organisms increases, fungi and mesophilic bacteria re-establish themselves, and further biotransformations of the organic matter occur [1,2].

Recently, we developed a set of media adapted to the enumeration of thermophilic bacterial populations. Relatively high numbers of autotrophic bacteria, growing at temperatures above 70°C, were isolated from hot composts and characterized [7], as well as large numbers of thermophilic heterotrophic

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Abbreviations: MPN, most probable number; OTU, operational taxonomic unit

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bacteria related to *Thermus thermophilus* [8] and *Bacillus* spp. [9–11].

Methods that rely on bacterial cultivation are currently thought to identify only a small fraction (0.01–10%) of the micro-organisms in natural environments [12–14]. A molecular alternative, that involves DNA extraction followed by PCR amplification and subsequent cloning of 16S rRNA genes, was developed to alleviate the limitation associated with cultural approaches, although it is anticipated that this approach may also introduce bias. This technique has been used successfully for marine bacterioplankton [15,16], soil environments [17,18], hydrothermal vent systems [19], and for a peat bog sample [20].

In this study, we investigated four hot compost samples, two of them being taken from the mid and late thermogenic phases, respectively. We carried out a most probable number (MPN) determination of thermophilic populations based on the methods previously published [7,8]. In parallel, we estimated the diversity of the community in each sample by a molecular cloning approach. The bacterial 16S rRNA genes were amplified by PCR of DNA extracted by directly lysing the micro-organisms in the compost matrix, and these amplicons were then used to construct a clone library that was subsequently analyzed by restriction enzyme profiles and partial sequencing of dominant clones. This clone library was compared to the restriction profiles of strains previously isolated from hot composts [8–10] and of sequences available in the EMBL gene database.

2. Materials and methods

2.1. Composting facility and sampling

The industrial composting facility consisted of classic open air windrows (1.5-m high) made up of 45% (v/v) grass, 25–35% kitchen and garden waste, and 10–25% shredded wood. The large pieces of wood retained by the sieving of the mature compost piles were recycled by mixing with fresh organic material and accounted for 5% of the mixture. The windrows were turned daily. Oxygen and carbon dioxide were measured using electrochemical cells (Multiwarn P detectors, Drägerwerk, Lübeck, Germany). Measurements were made immediately prior to sampling.

Compost samples were taken from the core (40 cm from the top) of four different windrows, before turning (August 1996). Each sample (approximately 1 kg) was homogenized by sterile hand-mixing and divided into two subsamples: one was used for MPN determination (inoculation within 3 h), and one was deep frozen in liquid nitrogen and stored at −80°C for subsequent DNA extraction. For the dry weight determination, about 100 g of fresh compost was dried for 24 h at 70°C under vacuum.

2.2. MPN determination

To determine the MPN of culturable thermophilic bacteria, 30 g samples of compost (fresh weight) was added to 270 ml of a sterile 0.9% NaCl solution and shaken at 150 rpm for 30 min at room temperature. The samples were then serially diluted (10^{-2}–10^{-13}) in a basal mineral medium [21] supplemented with nutrient broth and yeast extract, as previously described (MNY medium, [8]), and in the basal mineral medium supplemented with 20 mM $Na_2S_2O_3$ (BTM medium). Eight parallel 200-μl microplate wells were filled with each dilution.

MNY plates were incubated under air without shaking, either for 2 days at 60°C, or for 6 days at 75°C, to favor either heterotrophic spore-forming bacteria, or highly thermophilic, heterotrophic non-spore-formers [8]. Wells were scored positive when a distinct cell pellet was visually detected.

BTM plates for autotrophic, hydrogen- and sulfur-oxidizing bacteria were incubated without shaking for 8 days at 70°C under an atmosphere of H_2:CO_2:O_2 (25:10:3 kPa, measured at room temperature) according to Aragno [21]. To avoid confusion with oligocarbophilic growth, and taking into account that known hydrogen-oxidizing thermophilic populations in compost also oxidized thiosulfate [7], wells were checked for thiosulfate oxidation by adding 25 μl of 0.1 g l^{-1} bromocresol purple. Wells were considered positive when they turned yellow as a result of acidification. Cell types were examined with a phase-contrast microscope.

MPN values were calculated using the program of Schneider [22] and expressed per gram (dry weight) of compost.

2.3. Genomic DNA extraction and purification

All reagents and glassware were autoclaved or sterile filtered before use. Samples (including an empty tube as negative control) were extracted in duplicates by a modification described below of the procedure published by Lee et al. [23].

Frozen compost material (4.0 g (fresh weight)) was thawed and suspended in 10 ml of a 0.12 M sodium phosphate buffer (pH 8.0), left to stand at room temperature for 10 min with occasional mixing, then centrifuged at 7500 × *g* for 10 min. The supernatant was discarded and the pellet was suspended for another washing cycle. A volume of 8 ml of lysis solution I (0.15 M NaCl, 0.1 M EDTA (pH 8.0), 10 mg lysozyme ml^{-1}) were added to the washed pellet and incubated at 37°C with occasional mixing for 90 min, and then 8 ml of lysis solution II (0.1 M NaCl, 0.5 M Tris-HCl (pH 8.0), 10% sodium dodecyl sulfate) were added. The sample was frozen at −80°C for 20 min and thawed in a 65°C water bath for 20 min, and this freezing and thawing cycle was repeated three times [24]. The lysate was centrifuged at 7500 × *g* at room temperature for 10 min. The supernatant was transferred to fresh tubes and brought to a final concentration of 0.7 M NaCl and 1% CTAB (cetyltrimethyl-ammonium bromide; Serva, Heidelberg, Germany). The lysate was mixed and incubated at 65°C for 10 min, followed by extraction with an equal volume of $CHCl_3$–isopentylalcohol (24:1) and centrifugation at 3000 × *g* for 5 min. The supernatant was transferred to a fresh tube and 15 ml of 13% polyethylene glycol (MW = 6000, prepared in 1.6 M NaCl) were added. This solution was held on ice for 10 min, before centrifugation at 25 000 × *g* for 25 min. The pellet was then washed once with 70% ethanol, briefly dried at room temperature and dissolved in 750 μl of TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). A 190-μl volume of 10 M ammonium acetate was added to a final concentration of 2.5 M ammonium acetate, and the sample was incubated on ice for 10 min. The mixture was centrifuged in a microcentrifuge at 13 000 × *g* for 10 min.

To obtain amplifiable DNA, it proved necessary to repeat the CTAB step to remove contaminating substances, such as humic acids (Andrew Ogram, personal communication; [24]). Consequently, 750 μl of the supernatant were brought to a final concentration of 1% CTAB and 0.7% NaCl, incubated and extracted with $CHCl_3$–isopentylalcohol as described above. Seven hundred and fifty microliters of the upper aqueous phase was recovered and one volume of ice-cold isopropanol was added. The precipitated DNA was removed from the liquid phase after 10 min on a Pasteur pipette, gently washed in 70% ethanol, and allowed to dry in open air. The dried pellet was then suspended in 400 μl TE and allowed to dissolve overnight at 4°C.

Spectrophotometric measurements showed that the DNA extraction yielded 10–35 μg of nucleic acids per g (fresh weight) of compost. The integrity of the DNA was checked by horizontal gel electrophoresis in 0.8% agarose gels in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.3)), containing ethidium bromide (0.5 mg l^{-1}).

2.4. Amplification and cloning of 16S rDNA

The 16S rDNA was selectively amplified from the purified genomic DNA by PCR using universal oligonucleotide primers designed to anneal to conserved positions in the 3′- and 5′-regions of bacterial 16S rDNA, as previously published [8], with minor modifications: 2.5 U *Taq* DNA polymerase purchased from Appligene-Oncor (Gaithersburg, MD) was added in a total volume of 100 μl, in 1 × reaction buffer according to the manufacturer's instructions and 1–10 ng of compost DNA was used as template. Also, a final extension step at 72°C for 10 min was added to the protocol. PCR products were checked by electrophoresis in 1.3% agarose gels as described above and they appeared as single bands, about 1.4 kbp in size. To test for possible contamination of glassware and solutions by foreign DNA, the above-mentioned negative controls were used as template DNA and gave no detectable amplification signal.

PCR products were excised from 2% low melting agarose (Sigma, St. Louis, MO), the DNA was purified using a Geneclean *II* kit (Bio 101, La Jolla, CA) or a Qiaex II kit (Qiagen, Hilden, Germany) and amplicons were then ligated into a pGEM-T vector (Promega, Madison, WI). The molar ratio of the ligation reaction mixture was 3:1, in a total volume of 35 μl. This ligation mixture was used to

perform 1–7 parallel transformations in *Escherichia coli* competent cells according to the manufacturer's instructions.

Plasmid preparation of 70 randomly picked colonies of *E. coli* per sample was performed, using the alkaline lysis method followed by chloroform extraction [25,26]. Plasmid preparations were checked by electrophoresis in 0.8% agarose gels as described above. Plasmid vectors that did not contain the insert migrated faster and were discarded.

2.5. 16S rDNA restriction profile analysis

The 16S rDNA genes ligated in the pGEM-T vector were amplified as described previously [9]. Three tetrameric enzymes were used separately for the 16S rDNA restrictions. Aliquots (5 μl) of the PCR products were digested in 20 μl reaction volumes, either with 2 U *Hae*III restriction endonuclease, 2 U of *Hha*I or 2 U of *Rsa*I, according to the manufacturer's instructions (Gibco BRL, Life Sciences, Bethesda, MD). The restriction fragments were then separated by gel electrophoresis in 2.5% agarose gels as described above. Fragments shorter than 80 bp were not taken into consideration, because they were too near the detection threshold.

The Shannon diversity index H was calculated from the number of clones in each OTU with the following formula [27]:

$$N^{-1}\cdot\Sigma - N_i(\log N_i - \log N)$$

where N_i is the number of clones per OTU, and N is the total number of clones per sample.

2.6. Determination of nucleotide sequences

Partial sequences of some 16S rDNA genes ligated in the pGEM-T vector were made by Microsynth (Balgach, Switzerland) using an analytical sequencer. Two hundred and fifty to 690 nucleotides were sequenced from one side with a T7 primer (single run) and compared to 16S rDNA sequences available in the nucleotide databases by using the BLAST software [28]. These sequences were deposited at the EMBL database under accession numbers AJ011355–AJ011368.

3. Results

3.1. Compost samples

Four compost samples were taken from the hottest part of four different compost windrows. Samples I and II were taken from young windrows, whereas samples III and IV were taken from older windrows. The main features of the samples are listed in Table 1.

3.2. MPN of thermophilic bacteria

The values calculated are reported in Fig. 1. Phase-contrast microscopic observations of wells showed that the heterotrophic populations growing for 2 days at 60°C were almost exclusively rods, 2–12 μm in length, some of them forming oval endospores, as previously reported [8,9,11].

MNY plates incubated at 75°C yielded only non-spore-forming rods, filaments and rotund bodies related to *Thermus* spp. strains as previously described [8,29].

BTM plates wells scored positive for autotrophic bacteria yielded only non-spore-forming, short rods, suggesting them to be *Hydrogenobacter* spp. strains (data not shown).

Table 1
Main features of the four windrow spots sampled[a]

Sample	Age (days)	Temperature (°C)	O_2 content (v/v)	CO_2 content (v/v)
I	13	68	17.8%	3.9%
II	18	82	4.1%	23%
III	39	64	2.8%	21%
IV	41	67	13.7%	9.1%

[a]Measurements were made just prior to sampling.

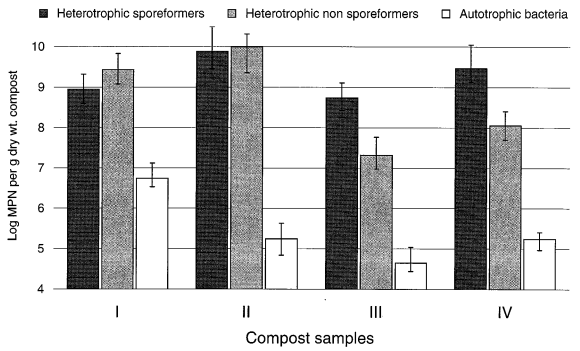

Fig. 1. Most probable numbers (MPN, single values) of three thermophilic bacterial populations in four hot compost samples. The sample features are listed in Table 1. Error bars are confidence intervals (0.05) with 10 000 bootstraps [22].

3.3. Analysis of the clone library

A total of 50 clones from each sample were chosen after 16S rDNA recombinant plasmids tested positive for PCR amplification. The clones with identical patterns for the three restriction profiles were grouped in discrete operational taxonomic units (OTUs) as shown in Fig. 2. No restriction profile among the clones resembled the *E. coli* 16S rDNA profile. The Shannon diversity indexes were 0.54 and 1.30 for compost samples I and II, and 1.56 and 1.60 for samples III and IV.

Dominant OTUs appeared in the four compost samples shown in Fig. 2 and some of them were identified (Table 2). The identification of OTUs A, E, G and L was based on the comparison with previously published restriction profiles [8,9] and confirmed by partial sequencing. The restriction patterns of OTU C matched those of the thermophilic *Bacillus* sp. strain TP-84 that we had previously isolated from hot compost [9]. The sequence of TP-84 was deposited at the EMBL database under accession number AJ002154 and showed 96.2% homology with *Bacillus thermosphaericus*[TS] (sequence X90640).

The identification of OTU F and OTU J was based on the theoretical profiles calculated from 16S rDNA sequences available in the EMBL database for the thermophilic aerobic species *Saccharococcus thermophilus* (sequence X70430) and *Rhodothermus marinus* (sequence X77140), respectively. These matches were confirmed by partial sequencing.

No other profile could be identified on the basis of

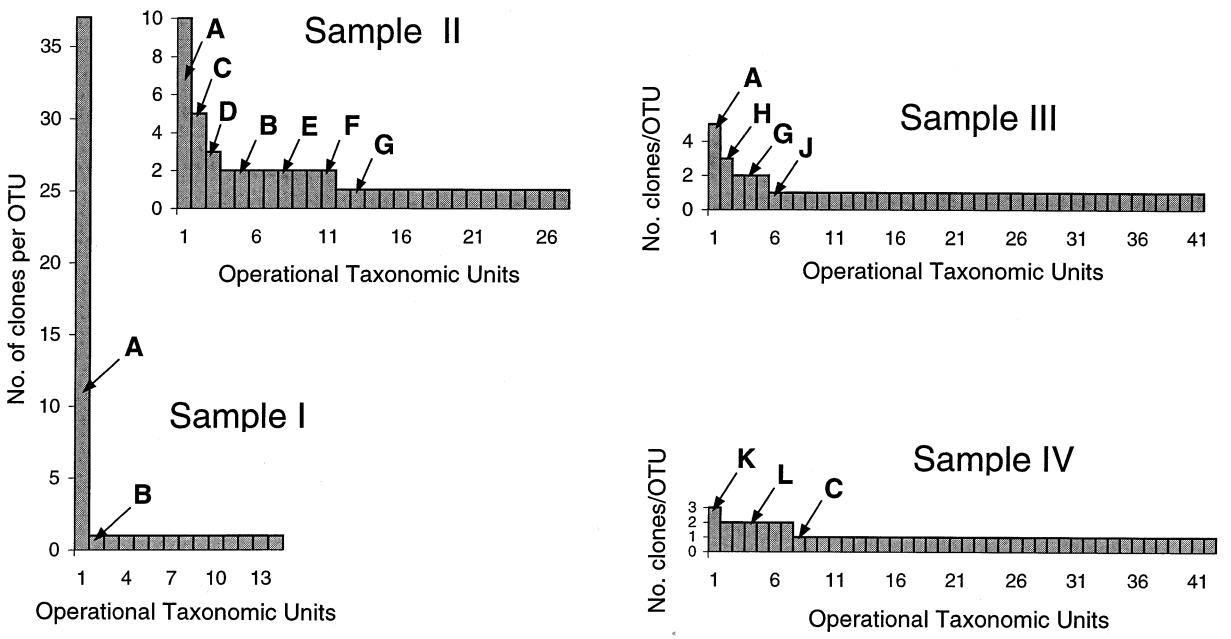

Fig. 2. Distribution among operational taxonomic units (OTUs) of bacterial 16S rDNA clones from four hot compost samples. Letters identify OTUs referred to in the text.

Table 2
Identification of OTUs as defined in Fig. 2

OTU	Identification based on restriction profiles	Closest relatives based on partial sequence homology (%)
A	*Thermus thermophilus*^{TS} [8]	*Thermus thermophilus*^{TS} (99%)
B	n.m.	*Bacillus* sp. TP-84 (98%)
C	*Bacillus* sp. TP-84 [9]	*Bacillus* sp. TP-84 (98%)
D	n.m.	*Thermus thermophilus*^{TS} (99–100%)
E	'*Bacillus thermodenitrificans*' [9]	'*Bacillus thermodenitrificans*' (99%)
F	*Saccharococcus thermophilus*^{TS}	Members of the genus *Bacillus* (92–95%) *Saccharococcus thermophilus*^{TS} (92%)
G	*Bacillus pallidus*^{TS} [9]	*Ammoniphilus oxalaticus* (98%) Members of the genus *Bacillus* (93–96%)
H	n.m.	Thermophilic members of the genera *Thermoanaerobacter*, *Clostridium*, *Desulfotomaculum*, *Bacillus* (85–90%)
J	*Rhodothermus marinus*^{TS}	*Rhodothermus marinus*^{TS} (98%)
K	n.m.	Members of Micrococcaceae and nocardioforms (89–95%)
L	*Bacillus thermoglucosidasius*^{TS} [9]	*Bacillus firmus*^{TS} (98%)

The numbers in square brackets are reference numbers for restriction profiles. TS, type strain. n.m., no match with available sequence profiles of thermophiles.

the available thermophilic aerobic Bacteria profiles (genus *Bacillus* [9], *Thermus* [8], and unpublished profiles of *Hydrogenobacter*, *Calderobacterium* or *Aquifex* strains).

4. Discussion

The MPN values for heterotrophic and autotrophic thermophiles were shown to be within the range previously reported in similar compost samples [7,8]. However, significant differences appeared between young and old compost samples. The MPN of thermophilic aerobic micro-organisms (Fig. 1) showed a 100-fold decrease in *Thermus* spp. populations between young and old samples. This is probably due to an increased competition with less thermophilic, fast-growing strains when the temperature drops below the optimal temperature range for *T. thermophilus* (70–75°C [8,30]). Thermophilic spore-forming bacterial numbers remained almost within the same order of magnitude in both samples. Nevertheless, since thermophilic *Bacillus* species cannot grow at the temperature measured in sample II (82°C) [10,31,32], the high numbers of colony forming units (approximately 7.5×10^9 per g (dry wt.)) detected in this sample were probably endospores rather than actively growing cells, as previously suggested [8]. Autotrophic hydrogen- and sulfur-oxidizer numbers were three to five orders of magnitude fewer than heterotrophs, as previously reported for hot compost [7]. Even if their numerical importance could be considered as minor among the thermophilic populations studied, their specific role might actually prove to be significant for the detoxification of sulfur compounds, as previously suggested [7].

Among the 200 restriction profiles investigated during the study, 38 clones of compost I, 22 clones of compost II, 6 clones of compost III and 1 clone of compost IV could be identified to the species level. The identification of OTUs F and G was not positively confirmed by the partial sequence data, but this could conceivably be due to the fact that only part of the 16S rRNA gene was sequenced. The partial sequence of the OTU L proved to be closer to the sequence reported for the mesophile *Bacillus firmus*^{TS} (sequence X60616) than to any other *Bacillus* sp., whereas the theoretical restriction profiles calculated from the sequence of *B. firmus*^{TS} were identical to those reported for *Bacillus thermoglucosidasius*^{TS} [9]. In this particular case, the sequencing step showed that the identification of this OTU L based on the restriction profiles was not reliable. To our knowledge, this paper is the first report of clones related to *S. thermophilus* [33] and *R. marinus* [34] to be found in environments other than sugar beet factories or marine hydrothermal vents, respectively.

The number of clones investigated determines the threshold of detection. In this case, it is limited to the

most abundant bacterial OTUs. This explains that profiles for hydrogen-oxidizers were not detected, although the strains were shown to be present by the MPN determination. More specific primers could be designed, though, to enhance the detectability of particular strains [14,35]. Moreover, it should be emphasized that biases in DNA extraction and amplification [19,35–37] can be reduced using a procedure designed to maximize the extraction of bacterial DNA, along with a subsequent PCR amplification program that proved adapted to poorly amplifiable, highly thermophilic DNA [8].

A previous work used random amplified polymorphic DNA (RAPiD) fingerprinting for characterization of compost microbial communities [36]. This study yielded characteristic fingerprints of the community at different times and suggested fast changes in population composition in the first days of the composting process in a pilot-scale reactor. Nevertheless, the RAPiD technique could not be used to identify discrete, previously known populations among the community. Recently, Hellmann and coworkers [38] and Herrmann and Shann [39] studied the microbial community changes during composting based on the phospholipid fatty acid (PLFA) analysis. These authors showed the PLFA profiles to evolve in a consistent and predictable manner, proving the profiles to be characteristic of specific stages of composting.

OTUs distinguished by a combination of three restriction profiles using different tetrameric enzymes were shown in a model data set to correspond to discrete species, with $P > 0.99$ [40]. In our study, the bacterial diversity revealed by the clone analysis was therefore shown to undergo a dramatic change between the young and the old samples. As a matter of fact, the indisputable dominance of *T. thermophilus* in the young composts (50% of the clones, OTUs A and D) was no longer observed in the older composts (Fig. 2). Also, only four OTUs (A, B, C and G) could be found in more than one sample. It has to be stressed, though, that the samples were taken from four different windrows. Consequently, the differences observed between samples I and II, for instance, account for the variability and the heterogeneity of the compost matter at the same step of the process (about 2 weeks).

The physicochemical evolution of organic matter during the composting process (such as depletion of solubles within the first weeks [1]) possibly plays a major role in this population shift. It may be that the higher diversity in the old compost (1.56/1.6 vs. 0.54/1.30) indicates that the decreasing temperatures favor the recolonization of the maturing compost by a broader range of micro-organisms. Those micro-organisms were reported to degrade more diverse and less easily degradable materials [1–4,39].

Although composting is essentially an aerobic process, anaerobic decomposition is known to occur in micro-environments of the heterogenous, oxygen-poor matrix of the compost itself [41,42]. Moreover, a decreasing gradient of temperature establishes from the core to the outer part of the compost windrow within a few hours after turning [2,43]. Given that the windrows are turned each day, the bacterial populations colonizing a particular spot in the pile daily undergo a complete redistribution, a factor which must be taken into account when discussing the biodiversity assessed in a single spot. The media and incubation conditions chosen for the MPN determination did not allow potential mesophilic or anaerobic micro-organisms to be revealed.

It should be emphasized that numbers of clones found in a clone library are a rough estimate for abundance. Indeed, the study of the true microbial community structure would require hybridization techniques applying specific oligonucleotide probes for taxa of interest. Therefore, further work is needed to better understand how bacterial populations, not only aerobic thermophiles, but also thermoresistant mesophiles and anaerobes, vary as a function of time and space in a compost pile. Our molecular approach confirms, though, that *T. thermophilus* strains and, to a lesser extent, thermophilic *Bacillus* spp. are the dominant bacterial populations at the higher temperatures reached by thermogenic composts. Interestingly, the most abundant *Bacillus* sp. strain, TP-84 (OTU C), is not related to any validly described species and could thus form a new species.

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