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Direct Analysis of Thermophilic Bacteria in Sewage Sludge Compost

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To analyze metabolically active thermophilic bacteria, a high–temperature direct viable count (HT–DVC) method was developed and applied to sewage sludge compost made by a hyperthermal composting method. When the HT–DVC method was conducted at 60 °C and 80 °C, maximum numbers of 23.3×10^8 and 2.62×10^8 cells/(g of dry sample) of elongated cells (length >4 μm), respectively, were detected. These results indicate that the HT–DVC method can be used to enumerate even metabolically active extreme thermophiles. Strain TH, a Gram–negative, spore–forming, and extremely thermophilic bacterium, which showed growth at 55–78 °C, was isolated from the sewage sludge compost. Strain TH is closely related to *Caldaterra satsumae* YMO81. The HT–DVC method could detect strain TH inoculated into sewage sludge compost with autoclaving, but could not selectively detect the strain inoculated into the compost without autoclaving.

Keywords: high–temperature direct viable count (HT–DVC), hyperthermal composting method, sewage sludge compost, (extreme) thermophile, strain TH

INTRODUCTION

Environmental issues related to climate change that might be induced by an increase in carbon dioxide are of interest to scientists. To reduce consumption of fossil fuels, more efficient utilization of biomass, including biological waste materials, is required. Composting of organic wastes, such as animal feces, garbage, and sewage sludge, is an effective process for recycling waste material into fertilizer. Among composting techniques reported, the hyperthermal composting method reported by Kanazawa *et al*. (2003) is unique. It involves maintaining a relatively high temperature of 80 °C during the process over a long period using a specific seed compost and aeration device. The high temperature enables the production of mature compost in a shorter period compared to general composting process and allows the disinfection of pathogens, including *E. coli*, in the composting material. Thus, it is likely that many thermophilic bacteria, especially extreme thermophiles that have an optimum growth temperature near 70–80 °C (Stetter, 1998), are involved in the composting process.

Generally, the composting process can be divided into four major microbiologically important phases: (i) an initial mesophilic phase; (ii) a thermophilic phase; (iii) second mesophilic phase; and (iv) maturation and stabilization phase (Ryckeboer *et al*., 2003). Thermophilic bacteria contribute to the degradation of organic materials during the thermophilic phase (Ryckeboer *et al*., 2003). To clarify the process more precisely, the population of thermophilic bacteria must be monitored. Many studies report using culture methods for the quantitative analysis of microorganisms (Dees and Ghiorse, 2001; Diaper and Edwards, 1994; Kuroda *et al*., 2004; Saludes *et al*., 2008). However, the number of microbes counted by the methods is dependent on the medium and incubation conditions (Olsen and Bakken, 1987). To directly detect metabolically active thermophilic bacteria in compost, a high–temperature direct viable count (HT–DVC) method was developed by modifying the DVC method reported by Kogure *et al*. (1979). The main modification was addition of incubation of the compost for 24 h at 60 °C with thermostable DNA replication inhibitors (pipemidic acid and norfloxacin) and a small amount of substrate (yeast extract). The HT–DVC method enables the investigation of metabolically active thermophile populations.

This report describes the investigation of thermophilic bacteria in compost obtained by a hyperthermal composting method. During the investigation process, a quantitative analysis of metabolically active and extreme thermophiles was performed. The isolation and characterization of extreme thermophiles by culturing was also performed. The effects of the HT–DVC method were tested by inoculating an isolate to the compost.

MATERIALS AND METHODS

Compost sample

Sewage sludge compost made through a hyperthermal composting method was used as a sample for enumeration and isolation of thermophilic bacteria. The sample was passed through a 2 mm–sieve, and then placed in sterilized plastic bottles and stored at room temperature.

Enumeration of thermophilic bacteria in compost by HT–DVC method

A flowchart of the HT–DVC method is shown in **Fig. 1**. A more detailed procedure will be published in the

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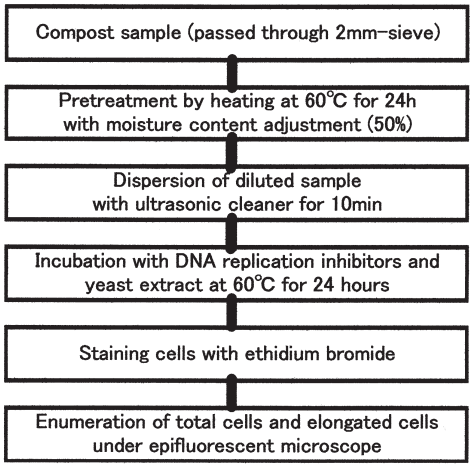

Fig. 1. A flowchart of the high–temperature direct viable count (HT–DVC) method.

future. Compost samples were pretreated at 60, 70, or 80 °C for 24 h with adjustment of moisture content (50%) to reactivate thermophilic bacteria and germinate their spores. Then, a diluted sample containing 0.02% yeast extract and 50 μg ml^{-1} of two DNA replication inhibitors (pipemidic acid and norfloxacin) was incubated at 60, 70, or 80 °C for 24 h. After incubation, bacterial cells were stained with ethidium bromide using the method of Someya (1995) and observed under epifluorescent microscopy. Cells longer than 4 μm were counted as metabolically active thermophilic bacteria. The numbers of elongated cells were net values, because a few bacteria whose length exceeded 4 μm already existed in the compost before incubation. Lengths of 100 rod–shaped cells selected randomly were measured after incubation.

Isolation of thermophilic bacteria from sewage sludge compost

The sample (approximately 0.1 g) was inoculated into 5 ml of CYS medium in glass tubes with screw caps and incubated at 70, 75, and 80 °C for 2 d statically. After incubation, the suspensions were spread onto CYS medium solidified with 1% GELRITE® gellan gum (Sigma) and 20 mM $CaCl_2$. The solidified CYS plates were then incubated under identical growth conditions. Single well–separated distinct colonies were selected and inoculated into CYS medium in glass tubes to incubate under identical growth conditions. The turbid liquid cultures were spread onto CYS gellan gum plates, and the plates were incubated. This single colony isolation procedure was repeated three times. One of the pure cultures obtained, designated strain TH, was stored in a CYS medium/DMSO mixture (10:1) at −20 °C.

Characterization of strain TH

Culture media

CYS medium was used for investigation of growth conditions. CYS medium contained (per liter) 3 g casein acid hydrolysate (Sigma); 2 g dried yeast extract (Wako); 1 g soluble starch (Sigma); 3 g NaCl; and 10 ml trace metal solution. The trace mineral solution contained (per liter) 1 g $FeSO_4 \cdot 7H_2O$; 0.12 g $NaMoO_3 \cdot 2H_2O$; 20 mg $VOSO_4 \cdot 5H_2O$; 50 mg $MnCl_2 \cdot 4H_2O$; 6 mg $ZnSO_4 \cdot 7H_2O$; 1.5 mg $CuSO_4 \cdot 5H_2O$; 80 mg $CoCl_2 \cdot 6H_2O$; 2 mg $NiCl_2 \cdot 6H_2O$; 0.25 g $MgCl_2 \cdot 6H_2O$; and 50 mg $CaCl_2$. Medium pH was adjusted to 7.5–7.7 with 1 M NaOH.

For testing utilization of carbon sources, 0.1× CY medium was used. This medium contained (per liter) 0.3 g casein acid hydrolysate; 0.2 g dried yeast extract; 0.3 g NaCl; 20 mM MOPS (separately prepared and filter–sterilized); 10 ml trace metal solution (autoclaved separately); and 2 g carbon source (separately sterilized). The pH was adjusted to 7.5–7.7 with 1 M NaOH.

Observation with light and electron microscopy

An Eclipse E600 microscope (Nikon) and digital camera (DS–5M–L1, Nikon) were used for routine observations. Gram stain reactions were determined using the modified Hucker method under light microscopy. Cells from both the exponential and stationary growth phases were used. The Leifson method was used for staining flagella (Gerhardt, 1981).

For scanning electron microscopy, cells were grown at 75 °C for 2 d. The cells were collected into a microfuge tube and fixed for 2 h with 1% glutaraldehyde in 0.01 M sodium cacodylate buffer (pH 7.0) containing 0.15 M NaCl. The fixed cells were dehydrated with a graded ethanol series (50, 60, 70, 80, 90, and 100%), each for 10 min. The dehydration with 100% ethanol was repeated four times for 5 min. Ethanol was finally replaced with t–butyl alcohol and the cells were freeze–dried. The freeze–dried cells were sputter–coated with platinum under a vacuum (30 mA, 40 sec, auto fine coater JFC–1600, JEOL). Electron microscopic observation was performed using a JEOL JSM–6701F scanning electron microscope.

Growth characterization

All growth experiments were conducted in triplicate in liquid CYS medium at 75 °C, unless otherwise stated. Growth was determined by measuring optical density at 660 nm (OD_{660}) with a spectrophotometer (U–1500, Hitachi). Effect of pH on growth was determined by adjusting the pH of the medium to 5–10 with sterilized 1 M NaOH or 1 M HCl at room temperature. Effect of temperature on growth was determined in the range of 50–80 °C. The NaCl requirement was determined in the medium containing 0–5% (w/v) NaCl.

To test for catalase activity, 5 ml H_2O_2 (3%, v/v) was poured onto the fresh culture plate and the occurrence of any oxygen bubbles was noted. The presence of oxidase was determined with tetramethyl ρ–phenylenediamine hydrochloride (Holding and Collee, 1971).

Substrate utilization of strain TH was tested using 0.1× CY medium containing substrate at a concentration of 0.2% (w/v). After incubation at 75 °C for 2 d, the OD_{660} values were compared with the OD_{660} values of control cultures that contained no added substrate except casein acid hydrolysate (0.03%) and yeast extract (0.02%). Cultures with OD_{660} values 0.04 greater than

the OD_{660} values of the controls were considered positive. The test was done in triplicate.

Quinone compositions

Strain TH cells cultivated in CYS medium at 75 °C were used for extraction of menaquinones, according to the method of Tang *et al*. (2004), with slight modification. Analysis of the menaquinones was performed using HPLC according to the method of Tasumi *et al*. (2007) except for monitoring at 270 nm. Menaquinone 7 extracted from *Bacillus subtilis* IFO 13719 was used as the standard for the analysis.

Determination of DNA G+C content

Cells were harvested by centrifugation (7,000 × *g*, 10 min), washed, and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 8.0). Egg–white lysozyme (1 mg ml^{-1}; Sigma) was added to the cell suspension, which was then incubated at 37 °C for 1 h. The lysed cells were incubated at 50 °C in 0.5% SDS and 0.2 mg ml^{-1} proteinase K (Wako) for 1 h. After two treatments with phenol:chloroform:isoamyl alcohol (25:24:1) and one chloroform extraction, DNA was precipitated with 0.1 vol. sodium acetate and 2.5 vol. absolute ethanol, and was then washed with 70% ethanol. The DNA pellet was resuspended in TE buffer, and incubated with RNaseA (10 mg ml^{-1}; Sigma) at 37 °C for 1 h. The DNA was re–precipitated, washed, and resuspended in distilled water. The DNA obtained was hydrolyzed with nuclease P1 (DNA–GC Kit, Yamasa Shoyu) as described by Matsuyama (2004). After centrifugation at 15,000 × *g* for 10 min, the treated DNA was subjected to HPLC analysis: pump, Hitachi L–2130; column, YMC–Pack ODS–AQ312 (150 mm × 6 mm i.d.), YMC, Japan; column oven, Hitachi L–2300; detector, Hitachi L–2455 diode array detector. Nucleotides were eluted with 10 mM phosphate buffer (pH 3.5) at a flow rate of 1.5 ml min^{-1} and were detected at 270 nm. A DNA–GC Kit (Yamasa Shoyu) was used as the standard.

Isolation, amplification and sequencing of 16S rRNA gene

Genomic DNA of strain TH was extracted using the FastDNA® Spin Kit for soil (Qbiogene), according to the manufacturer's protocols. The DNA dissolved in sterile double–distilled water was used as a template for amplification of the 16S rRNA gene (rDNA) by polymerase chain reaction (PCR). The primer pairs used were 65F (5'–AACACATGCAAGTCGA–3') and 1378R (5'–AAGGCCCGGGAACG–3'), modified 63F (Marchesi *et al*., 1998) and R1378 (Heuer *et al*., 1997), respectively. The reaction mixture consisted of 25 μl of Premix *Taq* (TaKaRa Bio), 0.8 μM of each primer, and 100–fold dilution of the template solution (1 μl) in a final volume of 50 μl. Cycle conditions for the amplification were as follows: an initial denaturation at 98 °C for 0.5 min, followed by 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 49 °C for 0.5 min, extension at 72 °C for 2 min, then a final extension at 72°C for 5 min with a PCR thermal cycler (Dice model, TaKaRa Bio). PCR products were purified using the QiaQuick PCR Purification kit (Qiagen) according to the manufacturer's instructions. The purified product was sequenced by Macrogen Inc. (Seoul, Korea), using an ABI3730 XL automatic DNA sequencer.

Phylogenetic analysis

The new sequence (1316 bp) was multiply aligned using Clustal W version 1.83 (Thompson *et al*., 1994) with a selection of reference sequences obtained from the Ribosomal Database Project (RDP) database. Nucleotide substitution rates were calculated after gaps and unknown bases were eliminated. A phylogenetic tree was constructed from evolutionary distance data by applying the algorithm of the neighbor–joining method (Saitou and Nei, 1987) to K_{nuc} values (Kimura, 1980). To evaluate the robustness of the branches of the inferred tree, the bootstrap resampling method of Felsenstein (1985) was used with 1,000 replicates. The neighbor–joining tree was drawn with the NJ plot program (Perrière and Gouy, 1996).

Detection of strain TH inoculated into compost sample by HT–DVC

The compost sample was autoclaved at 121 °C for 15 min. Strain TH in 0.75% NaCl solution (3.07×10^7 cells /mL) was inoculated (2 ml) into the autoclaved compost with an adjustment of the moisture content to 50%. After incubation at 75 °C for 24 h, the HT–DVC method was applied at 75 °C with addition of ofloxacin. Bacterial cell staining and observation under epifluorescent microscopy was done as mentioned above after incubation for 24 h. To check whether the HT–DVC method could detect strain TH selectively, a non–autoclaved compost sample inoculated with the strain also was used as a sample.

RESULTS

Enumeration of thermophilic bacteria in compost by HT–DVC

Effect of temperature during pretreatment and incubation on elongated cell numbers and the average cell length of 100 rod–shaped cells is shown in **Table 1**. When incubation was performed at 70 °C, cell numbers and average length were maximal at all pretreatment temperatures. This result suggests that the major active thermophilic bacteria in the compost sample had the greatest metabolic activity near 70 °C. For incubation at 60 or 70 °C, the number was maximal at 60 °C pretreatment [23.3 or 37.6×10^8 cells/(g of dry sample), **Fig. 2**]. In contrast, for incubation at 80 °C, the number was maximal at 70 °C pretreatment [2.62×10^8 cells/(g of dry sample)]. These results suggest that thermophilic bacteria elongated at 80 °C might be different from those elongated below 70 °C. In other words, pretreatment temperature of the sample influences thermophilic bacterial species activated by the incubation and detected as elongated cells.

When incubation was performed at 60 °C with pretreatment at 60 °C, elongated cells of 23.3×10^8 cells/(g of dry sample) were detected (**Table 1**). The number was 45 times greater than that estimated by the plate count

Table 1. Enumeration of thermophilic bacteria in sewage sludge compost by HT–DVC[a)]

Pretreatment temperature	Incubation temperature		
	60 °C	70 °C	80 °C
60 °C	23.3 ± 4.8 (3.35)	37.6 ± 5.3 (5.08)	1.06 ± 0.32 (2.97)
70 °C	0.69 ± 0.23 (3.17)	4.10 ± 0.12 (4.71)	2.62 ± 0.35 (4.14)
80 °C	0.61 ± 0.13 (3.14)	0.69 ± 0.61 (3.86)	0.13 ± 0.03 (2.45)

[a)] Elongated cell number under various temperatures during pretreatment and incubation is represented. Average cell length (μm) is shown in parenthesis. Elongated cell counts are expressed as × 10^8 cells/(g dry compost) ± standard deviation. Experimental figures are averages of triplicate filters.

method (data not shown). And when incubation was performed at 80 °C, 2.62×10^8 elongated cells/(g of dry sample) were detected (**Table 1**). These numbers seem quite high, considering that isolation of extreme thermophilic bacteria from the compost was not successful. Thus, it is significant that the HT–DVC method could detect more than 10^8 cells/(g dry compost) of metabolically active thermophile growing at 80 °C, which is a temperature too high to apply to the quantitative culture–dependent method.

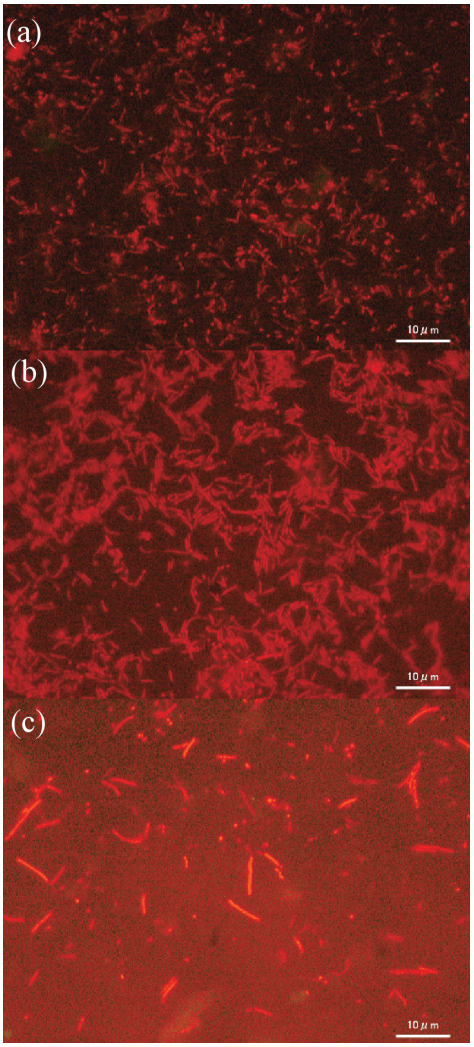

Fig. 2. Representative photographs under fluoromicroscopy of sewage sludge compost (a) before incubation; (b) after incubation at 60 °C; (c) after incubation at 70 °C. Pretreatment at 60 °C and incubation with yeast extract and DNA replication inhibitors were done for 24 h. Magnification of × 1,000; Scale bars = 10 μm.

Isolation of thermophilic bacteria from sewage sludge compost

Growth of thermophilic bacteria was observed in CYS medium inoculated with a compost sample after incubation at 70 and 75 °C for 2 d, but no growth was observed at 80 °C. An isolate, closely related to *Geobacillus stearothermophilus*, was obtained from the turbid medium at 70 °C (data not shown). When the turbid medium at 75 °C was spread onto CYS medium solidified with gellan gum and incubated under the same growth conditions, cream–colored or light brown and circular colonies were observed on the plate. Several colonies of different sizes and colors were selected and purified. Several pure cultures were obtained as described in Methods. One of the isolates, strain TH, was characterized further.

Characterization of strain TH

Strain TH cells were non–motile rods (1–5× 0.2–0.4 μm, **Fig. 3**), occurred singly or in pairs, and stained Gram–negative. Spherical terminal endospores were found in liquid culture at an early stationary phase. Strain TH was a facultative anaerobe and grew in a temperature range of 55–78 °C (optimally at 75 °C) in liquid

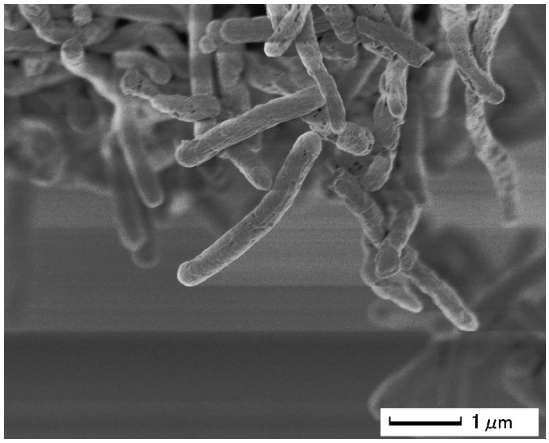

Fig. 3. Scanning electron micrographs of strain TH. Scale bar = 1 μm.

CYS medium. The isolate grew within a pH range of 5.0–9.0 (optimally pH 7.0). The isolate grew in medium containing 0–2% NaCl (optimally at 1%). At levels greater than 2.5% NaCl, growth was inhibited. For strain TH cells on a CYS gellan gum plate, both catalase and oxidase activity was negative.

Strain TH could utilize casein acid hydrolysate, yeast extract, Casamino acids, tryptone, malt extract, meat extract, aesculin, xylose, proline, tryptophan, threonine, asparagine, glutamine, tyrosine, glutamate, aspartate and glutarate in presence of casein acid hydrolysate (0.03%), and yeast extract (0.02%). No increase in growth on the following substrates was detected: peptone, polypeptone, xylan, chitin, gelatin, inulin, inositol, starch, cellulose, carboxymethylcellulose, glucose, fructose, galactose, maltose, sucrose, lactose, cellobiose, trehalose, isoleucine, methionine, phenylalanine, serine, cysteine, lysine, arginine, histidine, glycerol, pyrubate, lactate, acetate, propionate, fumarate, maleate, oxalate, phthalate, ascorbate, or benzoate. No growth was obtained with lignin, ribose, xylitol, arabinose, melibiose, L–sorbose, glycogen, glycine, alanine, valine, leucine, or citrate.

The G+C content of strain TH was 64.3 mol%. The dominant menaquinone type of strain TH was MK–7. Partial sequence of the 16S rRNA gene from strain TH was determined (1316 bp). The nearest neighbor of the strain was *Caldaterra satsumae* YMO81 (accession no. AB250968) with a sequence similarity of 99.2%. The phylogenetic position of strain TH is shown in **Fig. 4**.

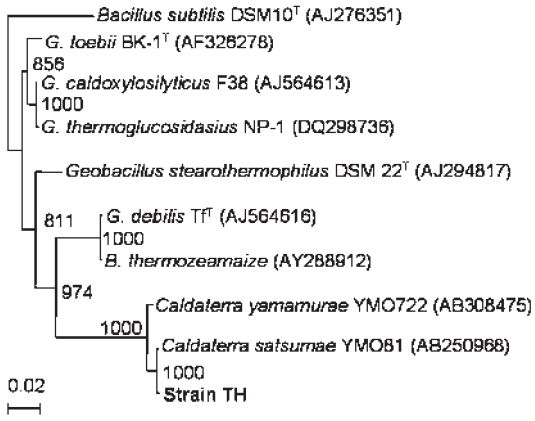

Fig. 4. Neighbor–joining tree showing the phylogenetic position of strain TH and related taxa based on 16S rRNA gene sequences, using 1300–bp sequences for the analysis. Gaps and unknown bases were eliminated from the sequences. The sequence of *Bacillus subtilis* was used to root the tree. Bootstrap confidence values obtained with 1,000 bootstrap trials are given at branching points. Scale bar = two nucleotide substitutions per 100 nucleotides.

Detection of strain TH inoculated into compost by HT–DVC

Since HT–DVC could detect metabolically active thermophilic bacteria growing at 80 °C (**Table 1**), the HT–DVC method was improved to detect more extreme thermophiles. Thus, utilization of ofloxacin together with pipemidic acid and norfloxacin increase metabolically active thermophilic bacteria detected (data not shown). Enumeration of rod–shaped and elongated cells in autoclaved sewage sludge compost inoculated with strain TH by HT–DVC is shown in **Table 2**. Elongated cells were not observed before incubation at 75 °C for 24 h. When a diluted compost sample was incubated without any inhibitors, the number of rod–shaped cells significantly increased by about 100–fold, and elongated cells were observed after the incubation (**Fig. 5a, b**). This result indicates the growth of inoculated strain TH. In contrast, the number of rod–shaped cells was nearly equal

Table 2. Enumeration of rod–shaped and elongated cells in autoclaved sewage sludge compost inoculated with strain TH by HT–DVC[a)]

incubation time (h)	concentration of each inhibitor (μg ml^{-1})	bacterial cell number	
		rod–shaped cell	elongated cell
0	0	6.94 ± 1.21	N.D.
24	0	626 ± 109	24.3 ± 4.2
24	60	6.94 ± 0.97	3.47 ± 2.11

[a)] Incubation of sample with yeast extract and DNA replication inhibitors (pipemidic acid, norfloxacin, and ofloxacin) was performed at 75 °C for 24 h. Experimental figures are expressed as × 10^7 cells/(g dry compost) and are the average of triplicate filters ± standard division. N.D. = not detected.

Table 3. Enumeration of rod–shaped cell and elongated cell in non–autoclaved sewage sludge compost inoculated with strain TH by HT–DVC[a)]

incubation time (h)	concentration of each inhibitor (μg ml^{-1})	bacterial cell number	
		rod–shaped cell	elongated cell
0	0	15.4 ± 1.7	N.D.
24	0	2690 ± 296	197 ± 21
24	60	19.3 ± 2.1	3.08 ± 1.21

[a)] See in **Table 2**.

to that before incubation with each inhibitor concentration set at 60 μg ml^{-1}. Under these conditions, total cell number also was nearly equal (data not shown), and the metabolically active strain TH could be detected as elongated cells (**Fig. 5c**).

Enumeration of rod–shaped and elongated cells in non–autoclaved sewage sludge compost inoculated with strain TH by HT–DVC also was conducted (**Table 3**). When a diluted compost sample was incubated without any inhibitors, the number of rod–shaped cells significantly increased by more than 100–fold and elongated cells were observed after the incubation. This tendency was similar to that shown in **Table 2**. However, the number of rod–shaped cells increased more than that before incubation with the inhibitors. Under these conditions, total cell number also increased by 10%. These results are caused by elongation of the coccus and germination of spores of indigenous thermophiles. Thus, elongated cells detected under these conditions would include not only strain TH but also indigenous thermophiles, including the strain TH. Thus, the HT–DVC method could not selectively detect strain TH in sewage sludge compost.

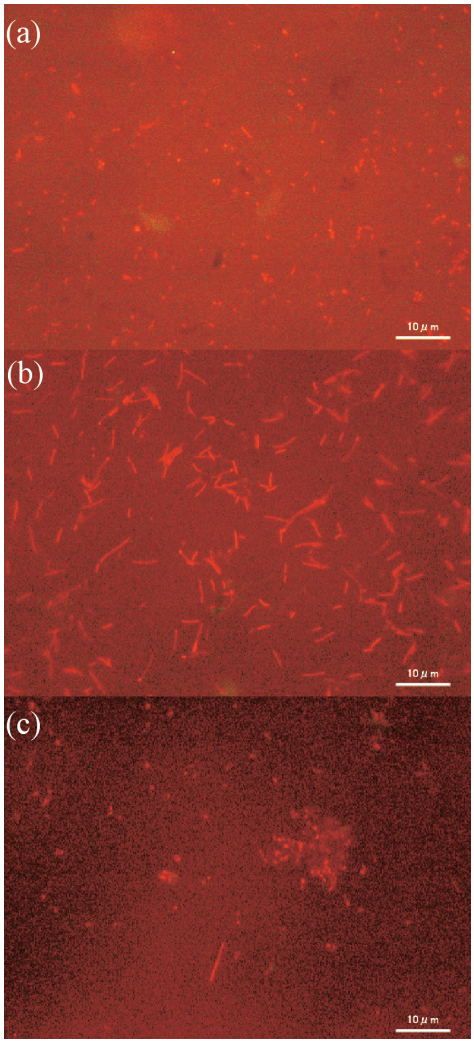

Fig. 5. Representative photographs under fluoromicroscopy of autoclaved sewage sludge compost inoculated with strain TH, as described in **Table 2** (a) before incubation; (b) after incubation with yeast extract alone; (c) after incubation with yeast extract and three inhibitors (each 60 μg ml^{-1}); Magnification of × 1,000; Scale bars = 10 μm.

DISCUSSION

Since the temperature during most periods of hyperthermal composting method is greater than 80 °C, many thermophilic bacteria, especially extreme thermophiles that have an optimum temperature for growth near 70–80 °C (Stetter, 1998), are involved in the composting process. To understand the process more thoroughly, an analysis of thermophilic bacteria in compost was done quantitatively. The HT–DVC method was useful for detecting metabolically active thermophilic bacteria growing at 60–80 °C, especially the higher temperatures at which application of the quantitative culture–dependent method is difficult to achieve.

Generally, strains related to *Geobacillus stearothermophilus*, which are considered dominant during the thermophilic phase of composting, cannot grow at temperatures of 75 °C or higher (Strom, 1985a,b; Nazina *et al.*, 2001). Since a strain related to *G. stearothermophilus* was isolated at 70 °C from sewage sludge compost made using hyperthermal composting, the strain may be involved in composting at temperatures below 75 °C. In contrast, extreme thermophiles growing at 80 °C were detected by the HT–DVC method on the order of 10^8 cells/(g of dry sample) in sewage sludge compost (**Table 1**). An attempt to isolate extreme thermophiles from the compost succeeded in the isolation of strain TH at 75 °C. Since strain TH grows slowly in CYS liquid media even at 78 °C, it may contribute to the composting process at temperatures of 75 °C or higher. For this report no isolates growing at 80 °C could be obtained, but *Caldaterra satsumae* YMO81, which is related to strain TH because of the 16S rRNA gene sequence, was isolated from compost and could grow at 80 °C (personal communication). Since thermophiles, such as *Hydrogenobacter* sp., *Symbiobacterium toebii*, and *Thermus* sp., growing at 75 °C or higher also were isolated from compost (Beffa *et al.*, 1996a,b; Rhee *et al.*, 2002), various extreme thermophiles such as *Caldaterra* strains including strain TH may be involved in the hyperthermal composting process.

Inoculation of strain TH into an autoclaved compost sample could be detected by HT–DVC (**Table 2** and **Fig. 5**). However, the HT–DVC method cannot selectively detect the strain, as shown in **Table 3**. Other culture–independent methods such as denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH) may be helpful for understanding the role of extreme thermophiles in compost. Combining HT–DVC and FISH would allow selective enumeration of metabolically active strain TH.

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