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Obligately and facultatively autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria isolated from hot composts

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Abstract A variety of autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria were isolated from thermogenic composts at temperatures of 60–80°C. All were penicillin G sensitive, which proves that they belong to the Bacteria domain. The obligately autotrophic, non-spore-forming strains were gram-negative rods growing at 60–80°C, with an optimum at 70–75°C, but only under microaerophilic conditions (5 kPa oxygen). These strains had similar DNA G+C content (34.7–37.6 mol%) and showed a high DNA:DNA homology (70–87%) with *Hydrogenobacter* strains isolated from geothermal areas. The facultatively autotrophic strains isolated from hot composts were gram-variable rods that formed spherical and terminal endospores, except for one strain. The strains grew at 55–75°C, with an optimum at 65–70°C. These bacteria were able to grow heterotrophically, or autotrophically with hydrogen; however, they oxidized thiosulfate under mixotrophic growth conditions (e.g. pyruvate or hydrogen plus thiosulfate). These strains had similar DNA G+C content (60–64 mol%) to and high DNA:DNA homology (> 75%) with the reference strain of *Bacillus schlegelii*. This is the first report of thermogenic composts as habitats of thermophilic sulfur- and hydrogen-oxidizing bacteria, which to date have been known only from geothermal manifestations. This contrasts with the generally held belief that thermogenic composts at temperatures above 60°C support only a very low diversity of obligatory heterotrophic thermophiles related to *Bacillus stearothermophilus*.

Key words Compost · Thermophilic bacteria · *Hydrogenobacter* · *Bacillus schlegelii* · Sulfur- and hydrogen-oxidizing bacteria

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Introduction

Composting is a self-heating, aerobic, solid-phase biodegradative process of organic waste materials (Waksmann et al. 1939; Finstein and Morris 1975; De Bertoldi et al. 1983). The composting process at the microbial level involves several interrelated factors, i.e., metabolic heat generation, temperature, ventilation (oxygen input), moisture content, and available nutrients.

Temperature reflects both the prior and the current rate of microbial activity. The temperature increase involves a rapid transition from a mesophilic to a thermophilic microflora. The compost ecosystem is limited by excessive heat accumulation. The terminal phase of composting is a cooling and maturation stage. The amount of readily available nutrients becomes a limiting factor that causes a decline in microbial activity and heat output. A high diversity of bacteria, fungi, and Actinomycetes has been reported during the short initial mesophilic phase and the cooling or maturation phase (Finstein and Morris 1975; De Bertoldi et al. 1983). However, the present knowledge of microbial diversity during the thermogenic (> 60°C) phase is surprisingly poor. The high temperatures reached (60–80°C) are often considered to reduce the microbial diversity dramatically (Strom 1985a, b; Nakasaki et al. 1985a, b; Fujio and Kume 1991). At the highest temperatures considered (65–69°C), previous studies focused only on obligately heterotrophic bacteria, and only strains related to *Bacillus stearothermophilus* were identified (Strom 1985a, b).

Microbial diversity could be expected during the thermogenic phase, where degradation and mineralization of complex organic matter also takes place (Schulze 1962; Nakasaki et al. 1985b). For example, autotrophic sulfur oxidizers oxidize (and, thus, detoxify) the hydrogen sulfide generated through the mineralization of organic sulfur compounds, whereas hydrogen-oxidizing bacteria use the molecular hydrogen produced by fermentative reactions during transitions from aerobic to anaerobic decomposition (Dugnani et al. 1986; Beffa et al. 1995). Such

transitions occur readily in the heterogeneous, biologically active, and oxygen-poor matrix in composts (Miller 1989).

The purpose of this study was to provide a better understanding of the taxonomic and functional diversity of highly thermophilic, facultatively or obligately autrotrophic bacteria during the thermogenic stage of the composting process (> 60° C).

Materials and methods

Compost facilities and sampling

The industrial composting facilities studied (10 sites) represent the main types of composting systems used in Switzerland, such as classical open-air windrows, boxes in a semi-closed hall with automated turning and aeration, or closed bioreactors with automated aeration. Organic materials subjected to composting varied considerably and consisted mainly of green waste, wood chips, and kitchen waste, or sewage sludge. The compost facilities were located 30–250 km from the authors' laboratory. None of the compost facilities studied used seeding with a commercial compost-starter containing thermophilic bacteria.

Enrichment and culture procedures

Enrichment, parallel serial dilutions, and cultures were performed in basal mineral medium (Aragno 1991). One gram fresh-weight organic material from hot (60–80° C) compost was placed in 10 ml of sterile basal mineral medium and shaken at 150 rpm for 30 min at room temperature. The parallel serial dilutions (10^{-1}–10^{-10}) and cultures were incubated at 70° C for 1–14 days under microaerophilic conditions (5 kPa oxygen). Autotrophic hydrogen-oxidizing bacteria were grown under an atmosphere of $H_2/CO_2/O_2$ (35 kPa : 10 kPa : 5 kPa, measured at room temperature) according to Aragno (1991). Autotrophic sulfur-oxidizing bacteria were grown with 20 mM thiosulfate or 5 g l^{-1} crystalline elemental sulfur (S^0) under an atmosphere of $N_2/CO_2/O_2$ (35 kPa : 10 kPa : 5 kPa, measured at room temperature). $NaHCO_3$ (60 mM) or a few grains of $CaCO_3$ were added to prevent excessive acidification under sulfur-oxidizing conditions.

Pure colonies were isolated by successive plating on the same media solidified with 13 g l^{-1} agar-agar (Oxoid, Fakola AG, Basel, Switzerland) with or without 20 mM thiosulfate. Gas mixtures were the same as for liquid cultures except that O_2 was at 2.5 kPa. Distinct colonies appeared after 1–7 days. Obligately autotrophic strains that oxidize both hydrogen and sulfur were able to form colonies only when the solid medium was supplemented with 20 mM thiosulfate, as previously reported for hydrogen-oxidizing, thermophilic bacteria (Alfredsson et al. 1986). The purity of each culture was routinely checked microscopically and by plating on solid mineral and organic media. We report the minimal and maximal values of the parallel serial dilutions observed in hot compost samples from 10 different compost facilities.

Heterotrophic growth of pure strains of spore-forming bacteria was assessed in liquid basal mineral medium supplemented with 0.2% w/v organic compounds (acetate, pyruvate, D-glucose) or with 0.8% w/v nutrient broth (Merck, Darmstadt, Germany) under an atmosphere of N_2/O_2 (45 kPa:5 kPa, measured at room temperature) and under normal air conditions. Heterotrophic growth in liquid cultures of pure strains of non-spore-forming, obligately autotrophic bacteria was determined under an atmosphere of N_2/O_2 (45 kPa:5 kPa, measured at room temperature) in basal mineral medium at 65° C supplemented with 0.1% (w/v) organic compounds. The organic compounds tested were as follows: D-arabinose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, D-xylose, soluble starch, acetate, citrate, formate, fumarate, β-hydroxybutyrate, gluconate, DL-lactate, malate, pyruvate, succinate, L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-tryptophan, L-valine, ethanol, isopropanol, methanol, nutrient broth (Merck, Darmstadt, Germany), and yeast extract (Merck, Darmstadt, Germany).

The growth of obligately autotrophic, hydrogen-oxidizing thermophilic bacteria (e.g., *Hydrogenobacter thermophilus*) was severely inhibited by the presence of 20 mM pyruvate (Shiba et al. 1984). Accordingly, the effect of 2.5 and 25 mM pyruvate on the autotrophic growth of pure strains of non-spore-forming autotrophic bacteria was determined in the basal mineral medium under an atmosphere of $H_2/CO_2/O_2$ (35 kPa:10 kPa:5 kPa, measured at room temperature). Cultures were incubated at 75° C during 10 days.

Reference or type strains

Thermophilic bacteria related to the genus *Hydrogenobacter* were isolated from the following geothermal sources: *Hydrogenobacter thermophilus* strain TK-H (Kyshu, Japan; Kawasumi et al. 1984); *Hydrogenobacter* strain MF-3 (Etna, Italy; Aragno 1992), *Hydrogenobacter* strain T-3 (Tuscany, Italy; Bonjour and Aragno 1986), and *Hydrogenobacter* strain H-1 (Borgarfjördur, Iceland; Kristjansson et al. 1985); *Calderobacterium hydrogenophilum* strain Z-829, (Kamtchatka, USSR; Kryukov et al. 1983). *Bacillus schlegelii* type strain (DSM 2000, Deutsche Sammlung von Mikroorganismen und Zellkulturen) was isolated from a cold environment (lake sediment, Le Loclat, Switzerland; Schenk and Aragno 1979).

Characterization of isolates

Cell numbers and types were estimated by phase-contrast microscopical examination of enrichment cultures performed from serial dilutions and by the ability to form colonies on solid media, with either thiosulfate or hydrogen as electron source. Morphology and cell size were estimated on actively H_2-growing cultures at 70° C using phase-contrast microscopy. Sensitivity to penicillin was tested during 5 days of incubation at 65° C in liquid media containing 10 mg l^{-1} benzyl-penicillin (Fluka Chemie, Buchs, Switzerland) according to the procedure described by Schenk and Aragno (1979). Temperature dependent growth was tested between 50° C and 85° C at 5° C intervals.

DNA base composition and hybridization

DNA was isolated by the procedure (slightly modified) described by Jenni et al. (1987). Cells (1–2 g fresh weight) were collected by centrifugation at 6,000 × g for 20 min at 4° C and washed with 15 ml of 10 mM Tris-HCl buffer (pH 7.2) supplemented with NaCl (0.9% w/v). Washed cells were resuspended in 16 ml MUP buffer (7.5 M urea, 120 mM NaH_2PO_4, 120 mM Na_2HPO_4, final pH 7.5) and broken by ultrasonic treatment using a Branson model 450 sonicator for 10 min at 35–40 W on ice. The broken cells were mixed with 5 g hydroxylapatite (Fluka AG, Buchs, Switzerland) equilibrated with MUP buffer and left for 1 h with occasional agitation at room temperature. The hydroxylapatite was decanted, washed with additional MUP buffer, and decanted again. The hydroxylapatite-DNA complex was then poured onto a GF/A filter (Whatman, Springfield Mill, UK) in a vacuum-filtration system. The hydroxylapatite-DNA was washed twice with 10 ml of MUP buffer and then 4 times with 10 ml of 14 mM sodium phosphate buffer (pH 6.8). The DNA was eluted with 400 mM sodium phosphate buffer (pH 6.8). The DNA fractions were pooled and concentrated with 2-butanol to about 4 ml. The purified DNA was then dialyzed against saline citrate buffer (15 mM NaCl – 1.5 mM Na-citrate). The ratio of absorbance of the DNA preparations at 260 and 280 nm was around 1.9, which indicates that they were essentially free of proteins. The concentrated DNA solutions were stored at –20° C. DNA

mol% G+C content was determined by the melting point (T_m) method (Marmur and Doty 1962) and calculated according to the formula of Owen and Hill (1979). DNA-DNA homologies were measured spectrophotometrically by following the renaturation rates at T_m −20° C according to De Ley et al. (1970).

Protein gel electrophoresis

Cells (about 1 g fresh weight) growing actively under autotrophic conditions (hydrogen) were collected and washed as described above. Washed cells were resuspended in 1–2 ml of 100 mM Tris-HCl buffer (pH 8.0). Cells were broken by ultrasonic treatment using a Branson model 450 sonicator for 0.5–1.5 min at 10–20 W on ice. The broken-cell suspension was centrifuged at 12,000 × g for 10 min at 4° C and the supernatant (cell-free extract) was stored at −20° C. Proteins (25–30 µg per lane) were resolved by denaturing SDS-PAGE (5% stacking gel and 12.5% resolving gel) at 22° C according to Hames and Rickwood (1990). Gels were stained with Coomassie Brilliant blue R-250 after electrophoresis. Molecular mass standards (14.4–200 kDa) were purchased from Bio-Rad Laboratories (Hercules, Calif., USA).

Respiratory activity measurements

Cells for respiratory activity measurements were taken from actively growing cultures, washed, and resuspended gently in basal mineral medium according to Beffa et al. (1991a). The respiratory activities were measured polarographically with an oxygen electrode (Hansatech, model CBH_2, adjustable volume) at 60° C as described previously (Beffa et al. 1991b, 1992). Oxygen consumption was calculated on the basis of 134 nmol O_2 ml^{-1} in air-saturated medium at 60° C according to Beffa et al. (1991b). The final cell concentration in the respiratory cuvette was 0.02–0.2 mg protein ml^{-1}. The respiratory substrates were supplied as follows (final concentration): 0.35 µg H_2 ml^{-1}. 10 mM thiosulfate; 10 mM hydrophilic S^0 (free of thiosulfate, obtained as described previously; Beffa et al. 1991a), 15 mM pyruvate. KCN (0.1 M) was freshly dissolved in 0.2 M sodium phosphate buffer at pH 7.5 and used at 1 mM final concentration. Cells were pre-incubated for 3 min at 60° C prior to addtion of the substrate, and the respiratory activities were measured from constant respiratory slopes. Results are expressed as nmol O_2 (mg protein)$^{-1}$ min^{-1}; corrected for the low endogenous oxygen uptake.

Analytical methods

Growth was followed turbidimetrically at 436 nm using 1-cm cuvettes in a Perkin-Elmer Lambda 6 spectrophotometer. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Results and discussion

We report here the first evidence for the occurrence of sulfur- and hydrogen-oxidizing, facultatively or obligately chemoautotrophic bacteria growing between 70° C and 75° C in thermogenic (60–80° C) composts. Thermophilic bacteria numbers in compost samples were estimated by enrichment of serial dilutions: 10^4–10^6 cells (g compost dry weight)$^{-1}$ for non-spore-forming, obligately autotrophic sulfur- and hydrogen-oxidizing bacteria, and 10^5–10^8 cells (g compost dry weight)$^{-1}$ for facultatively autotrophic hydrogen-oxidizing bacteria forming spherical spores.

Obligately autotrophic bacteria could grow only under microaerophilic conditions (<10 kPa oxygen). They could not grow with the organic compounds and media tested as the sole energy and carbon sources. All isolates except strain THS-13 grew autotrophically on hydrogen in the presence of 25 mM pyruvate. In addition, organic compounds did not seem to significantly inhibit autotrophic growth with hydrogen and thiosulfate because the enrichments of these bacteria from compost samples that probably contained high concentrations of organic matter scored positive even at the first serial dilution. In contrast, the growth of obligately autotrophic, thermophilic hydrogen-oxidizing bacteria isolated from geothermal environments (e.g., *Hydrogenobacter thermophilus* TK-6) was strongly inhibited, particularly by 20 mM pyruvate (Shiba et al. 1984).

Facultatively autotrophic bacteria were able to grow on organic compounds or nutrient broth under microaerophilic and normal air pressure conditions. Bacteria related to the latter were unable to grow with inorganic reduced sulfur compounds or sugars as the sole electron donors.

All strains presented in this study were penicillin G sensitive, which proves that they belong to the Bacteria

Table 1 Main characteristics of hydrogen- and sulfur-oxidizing autotrophic, thermophilic Bacteria strains isolated from hot compost piles (+ growth, − no growth)

Strains	Shape and size	Spores	Sensitivity to penicillin	Gram stain	Motility	Growth temperature (° C)		mol % G+C	Growth substrates					
						(min)	(max)		H_2	Thiosulfate	S^0	Acetate	Pyruvate	Glucose
Obligate autotrophic sulfur- and hydrogen-oxidizers														
THS-11	Rod 0.5 × 2–3.5 µm	−	+	−	+	60	80	35	+	+	+	−	−	−
THS-13	Rod 0.5 × 2–3.5 µm	−	+	−	+	60	80	34.7	+	+	+	−	−	−
THS-25	Rod 0.5 × 2–3.5 µm	−	+	−	+	60	80	35.6	+	+	+	−	−	−
TS-63	Rod 0.5 × 3–6 µm	−	+	−	−	60	80	37.6	+	+	+	−	−	−
TS-17	Rod 0.5 × 1.5–3 µm	−	+	−	+	60	80	36	+	+	+	−	−	−
Facultative autotrophic hydrogen-oxidizers														
THS-44	Rod 0.6 × 3–6 µm	+	+	±	−	55	75	60	+	−	−	+	+	−
TH-102	Rod 0.6 × 2.5–7 µm	−	+	±	+	55	75	60.4	+	−	−	+	+	−

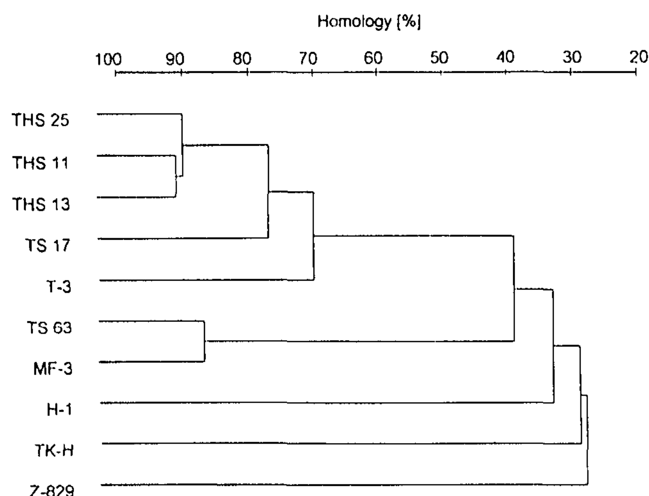

Fig. 1 Percent homology based on DNA:DNA hybridization between five strains isolated from compost (THS-25, THS-11, THS-13, TS-17, TS-63) and reference strains related to *Hydrogenobacter* isolated from geothermal areas in the world. Values are means of 3–5 runs. *Hydrogenobacter thermophilus* strain TK-H (Kyshu, Japan; Kawasumi et al. 1984); *Hydrogenobacter* strain T-3 (Tuscany, Italy; Bonjour et Aragno 1986, Aragno 1992); *Hydrogenobacter* strain MF-3 (Etna, Italy; Aragno 1992); *Hydrogenobacter* strain H-1 (Borgarfjördur, Iceland; Kristjansson et al. 1985); *Calderobacterium hydrogenophilum* strain Z-829 (Kamtchatka, Rusia; Kryukov et al. 1983)

domain. The main features of these strains are presented in Table 1.

All sulfur- and hydrogen-oxidizing, obligately autotrophic strains had a 35–37.6 mol% G+C content (Table 1), which is similar to those published for the strains related to *Hydrogenobacter* that have been isolated from geothermal areas (see Aragno 1991 and 1992 for a review). Strains THS-11, THS-13, THS-25, and TS-17 shared a high DNA:DNA homology (71–92%) with each other and with *Hydrogenobacter* reference strain T-3 (Fig. 1). This strain belongs to a DNA:DNA homology group found in geothermal springs in Italy and in the USA (Aragno 1991, 1992; M. Marchiani, Laboratory of Microbiology, University of Neuchâtel, Switzerland, personal communication). Strain TS-63 showed no significant homology with strain T3, but a high homology (86%) with *Hydrogenobacter* reference strain MF-3, which belongs to another DNA: DNA homology group found in geothermal springs in Italy and in the Azores (Aragno 1991, 1992; M. Marchiani, personal communication).

Protein profiles showed that *Hydrogenobacter*-related strains divide into two groups (Fig. 2). Strains THS-11, THS-13, THS-25, and TS-17 showed patterns similar to that of strain T-3. However, slight differences among these strains appeared among proteins of 40–45 kDa. Strain TS-63 showed a profile similar to that of strain MF-3 if one takes into account that the differences observed probably depend on band intensities. Strains Z-829, TK-H, and H-1 showed significantly different profiles (data not shown). These results confirmed the DNA:DNA hybridization results.

Studies recently have been carried out on the phylogenetic position of the genus *Hydrogenobacter*, based on the 16S rRNA complete sequence similarities between three strains of *Hydrogenobacter* (including strain T-3) and *Aquifex pyrofilus* (strain Kol5a) (Pitulle et al. 1994). The

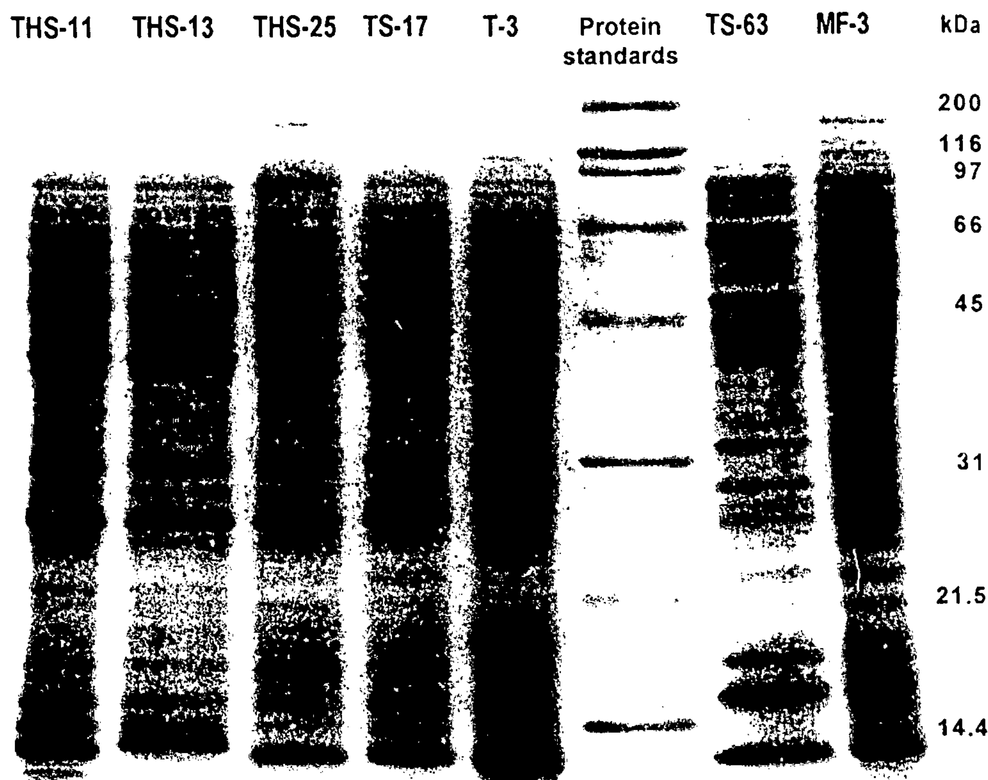

Fig. 2 Coomassie brilliant blue-stained SDS-polyacrylamide gel showing proteins of total cell extracts of thermophilic bacteria related to the genus *Hydrogenobacter* isolated from geothermal areas (strains T-3 and MF-3) and from hot composts (strains THS-11, THS-13, THS-25, TS-17, and TS-63) Protein molecular size markers are indicated on the right

Table 2 Respiratory activities of five strains isolated from compost [nmol O_2 consumed (mg protein)$^{-1}$ min^{-1}]. Cells were grown for 2–3 days at 70°C in basal mineral medium supplied with thiosulfate, Na-pyruvate, or H_2 as the energy and electron source

Strains	Growth substrates	Respiratory substrates and activities [nmol O_2 (mg protein)$^{-1}$ min^{-1}]			
		Hydrogen	Thiosulfate	Elemental sulfur	Pyruvate
THS-25	Hydrogen	132	97	12	< 1
	Thiosulfate	< 1	488	358	< 1
TS-17	Hydrogen	118	< 1	< 1	< 1
	Thiosulfate	< 1	514	436	< 1
TS-63	Hydrogen	120	< 1	< 1	< 1
	Thiosulfate	< 1	694	135	< 1
THS-44	Hydrogen	154	266	< 1	< 1
	Pyruvate	67	330	< 1	82
TH-102	Hydrogen	113	22	< 1	< 1
	Pyruvate	148	75	< 1	91

present authors have confirmed that the genera *Aquifex* and *Hydrogenobacter* are closely related and support the proposal that the *Aquifex-Hydrogenobacter* complex be placed in the new order "*Aquificales*" and that the genera *Aquifex* and *Hydrogenobacter* belong to the same family, the "*Aquificaceae*" (Burggraf et al. 1992).

Our results present the first evidence that highly thermophilic, chemolithoautotrophic Bacteria related to the genus *Hydrogenobacter* occur in hot composts, which are short-term, high-temperature habitats and are not confined to the highly specialized, sparse geothermal habitats.

The eight facultatively autotrophic strains isolated from hot composts all showed similar G+C (60–63 mol%) and high DNA homology (75–90%) with each other and with the reference strain of *Bacillus schlegelii* DSM 2000. This confirms that all the strains isolated belong to the same species regardless of their geographical origin and environment (Aragno 1992). Table 1 shows the main taxonomic and metabolic features of one of the spore-forming strains, THS-44, and those of the non-spore-forming strain, TH-102. *Bacillus-schlegelii*-related isolates also proved to grow well in nutrient broth under air.

When grown with thiosulfate as the sole energy source, strains isolated from compost and related to *Hydrogenobacter* possessed high thiosulfate- and elemental sulfur-oxidizing activities (Table 2). In contrast to strains TS-17 and TS-63, strain THS-25 possessed constitutive thiosulfate-oxidizing activity when grown on hydrogen. All respiratory activities were almost totally inhibited (> 85%) by 1 mM KCN, an inhibitor of the terminal cytochrome oxidase of the respiratory chain.

The number of types of thermophilic (optimum temperature > 65°C), aerobic, chemolithoautotrophic sulfur-oxidizing bacteria is very limited. *Hydrogenobacter* and related isolates, *Aquifex pyrofilus* (Huber et al. 1992) and *Thermothrix thiopara* (Caldwell et al. 1976; Mason et al. 1987) are the only well-characterized Bacteria to date. *T. thiopara* is however, no longer available from strain col-

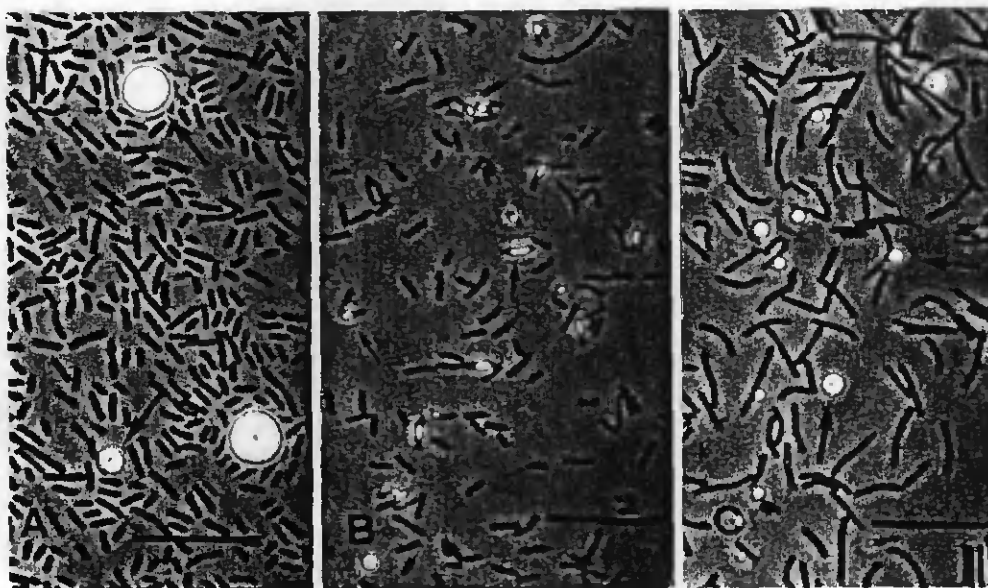

Fig. 3 Phase-contrast micrographs of three strains isolated from compost and related to *Hydrogenobacter* (A TS-17; B THS-25; C TS-63) Cultures were incubated at 65°C on solid basal mineral medium supplemented with thiosulfate and $H_2 + CO_2 + O_2$ as gas phase. When grown with H_2 and thiosulfate, cells released sulfur which appeared as white, refringent pellets (*large arrows*) and/or sheaths (*small arrows*) *Bar*: 10 μm

lections, and seems to have been lost (Kristjansson et al. 1994). Thermophilic, aerobic, autotrophic sulfur-oxidizing Bacteria are now most probably represented only by the strains related to *Hydrogenobacter* spp. and *Aquifex pyrofilus*.

The strains related to *Bacillus schlegelii* (THS-44 and TH-102) did not grow autotrophically with inorganic sulfur compounds as the sole energy and electron source. They possessed a constitutive thiosulfate-oxidizing activity but lacked elemental sulfur-oxidizing activity (Table 2). Some mesophilic heterotrophs have been reported to be able to oxidize thiosulfate (Mason and Kelly 1987). These bacteria may play a dominant role in the oxidation of sulfur compounds in soils and marine environments (Vishniac and Santer 1957; Tuttle and Jannasch 1972). To date, strains related to *B. schlegelii* constitute the first evidence of thermophilic, heterotrophic spore-forming bacteria able to oxidize sulfur compounds.

All autotrophic hydrogen oxidizers that grow on solid medium with thiosulfate alone or with thiosulfate plus hydrogen released sulfur, which appeared as white, refringent sheaths and/or pellets (Fig. 3). Reference strains of *Hydrogenobacter* and *B. schlegelii* grown under the same conditions also released S^0 in the growth medium (Beffa et al. 1993).

The results presented in this study contrast with the generally held belief that thermogenic composts at temperatures above 60° C support only a very low diversity of obligately heterotrophic thermophiles related to *Bacillus stearothermophilus* (Strom 1985a, b). The presence of high numbers of thermophilic, obligately and facultatively sulfur- and hydrogen-oxidizing bacteria in hot composts suggest that they may play a part in mineralization, and particularly in inorganic sulfur compound oxidation during the thermogenic phase (> 60°) of the composting process.

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Rapid Identification of Heterotrophic, Thermophilic, Spore-Forming Bacteria Isolated from Hot Composts

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The restriction enzyme profiles of 16S ribosomal DNAs (rDNAs) amplified by PCR from thermophilic heterotrophic bacterial strains isolated from composts were compared with those of reference strains. This allowed us to assign all but 1 of 16 strains to four different *Bacillus* species (namely, *Bacillus stearothermophilus*, *Bacillus pallidus*, *Bacillus thermoglucosidasius*, and "*Bacillus thermodenitrificans*"). This study showed that PCR restriction analysis of 16S rDNA contributes to rapid and reliable identification of newly isolated strains belonging to recognized species.

A few studies have reported the presence of thermophilic bacteria in hot compost (3, 4, 7, 19, 20). Strom (19, 20) isolated more than 750 heterotrophic spore-forming strains from compost; very few of these strains grew at temperatures above 60°C, and growth at 65°C was restricted to *Bacillus coagulans* (type A) and *Bacillus stearothermophilus*. Until recently, only strains related to *B. stearothermophilus* were identified from the hottest compost samples screened (65 to 69°C) (7, 19, 20). The great diversity of thermophilic bacteria related to the genus *Bacillus* has frequently been emphasized (16, 22), but it appears that only a few of the isolates have properly been identified to date. The morphology of sporulating cells, the shape of colonies, and growth abilities have proved to be insufficient for unequivocal identification of *Bacillus* strains (10, 11). The purpose of the present study was to identify heterotrophic, thermophilic, spore-forming strains isolated from hot composts by using a rapid molecular method based on the restriction profiles of 16S ribosomal DNA (rDNA) amplified by PCR.

Serial dilutions of compost sample suspensions were carried out in five different media. B and DN media consisted simply of nutrient broth (Merck, Darmstadt, Germany); DN medium was supplemented with 2 g of KNO_3 per liter. GA, P, and PN media were synthetic media composed of a basal mineral medium (1) supplemented with various growth substrates at a concentration of 2 g liter^{-1} [GA medium contained D-glucose and sodium acetate; P medium contained sodium pyruvate; PN medium contained sodium pyruvate and $(NH_4)_2SO_4$)]. The cultures were incubated under air at 65°C for 1 to 6 days, and pure colonies were isolated by repeated streaking on the same media solidified with agar. Colonies varying in appearance were picked deliberately to try to increase the number of different species isolated (the second and third letters of the compost strain designations in Fig. 1 refer to the isolation medium). Pure strains were then routinely cultivated at 60°C on B medium supplemented with 2 g yeast extract per liter and solidified with agar (NAY medium). The type and reference strains are listed in Table 1.

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Metabolic tests were carried out at 55°C with API 20 NE strips (BioMérieux, Marcy-l'Etoile, France) by using a few fresh colonies suspended in the basal mineral medium supplemented with 0.1 g of yeast extract per liter and 0.1 g of peptone per liter, unless indicated otherwise. Starch hydrolysis was tested in the basal mineral medium as described by Smibert and Krieg (17). Anaerobic growth (denitrification) was tested on NAY medium plates supplemented with 10 g of KNO_3 per liter. Cultures were incubated for 4 days at 60°C in desiccator jars; oxygen was eliminated by using an Anaerocult IS bag (Merck).

DNA extraction and purification were carried out as previously described (4), except that the cells were treated with lysozyme prior to guanidium thiocyanate DNA extraction (13). The 16S rDNA was selectively amplified by using oligonucleotide primers designed to anneal to bacterial 16S rRNA genes as previously described (4). The reaction conditions were as follows: 1 to 5 ng of template DNA, 0.8 U of Goldstar *Taq* DNA polymerase (Eurogentec, Seraing, Belgium), 5 μl of 10× Goldstar PCR buffer, 1.5 mM (final concentration) $MgCl_2$, 0.25 μM forward primer, 0.25 μM reverse primer, and each deoxynucleoside triphosphate at a concentration of 170 μM were combined in a total volume of 50 μl. Amplification was carried out in a model PTC-100 thermal cycler (MJ Research, Inc., Watertown, Mass.) with the following program: a preliminary denaturation step was carried out at 95°C for 1 min and was followed by 35 cycles consisting of 30 s at 94°C (denaturation), 30 s at 62°C (except for the three first touchdown cycles, which were successively at 68, 66, and 64°C), and 1 min at 72°C (extension). For restriction enzyme digestion, 50 ng of the PCR product was mixed with 2 U of *Hae*III or 1 U of *Hin*fI (New England Biolabs, Inc., Beverly, Mass.), *Taq*I, or *Rsa*I (Gibco BRL) and incubated for 4 h according to the manufacturer's instructions. PCR products and restriction digests were separated by electrophoresis as previously described (4).

Phenotypic characterization. The strains studied were isolated from 2- to 80-day-old composts as previously described (4); the sample temperatures ranged from 57 to 78°C. All of the strains were rods and formed oval endospores. Spore position varied from central to terminal. These strains were initially supposed to belong to the genus *Bacillus*, according to the description of Gordon et al. (8). Their maximum growth temperatures were between 65 and 72°C. Except for the *Bacillus pallidus* group, as pointed out previously (22), all of the strains

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Species	Strain[a]	EMBL 16S rDNA sequence accession no.
B. stearothermophilus	DSM 22^T (= ATCC 12980^T) (6)[b]	X60640 (2)
B. stearothermophilus	DSM 494 (12)	NA[c]
B. thermoglucosidasius	DSM 2542^T (= ATCC 43742^T) (21)	X60641 (2)
Bacillus sp.	DSM 6499 (18)	NA
"*B. thermodenitrificans*"	DSM 465 (= ATCC 29492) (9)	Z26928(14)
B. pallidus	DSM 3670^T (= ATCC 51176^T) (15)	Z26930(14)
Bacillus sp.	DSM 2349 (5)	Z26929(14)

[a] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, Md.
[b] The numbers in parentheses are reference numbers.
[c] NA, not available.

could grow anaerobically with nitrate as a respiratory substrate. Glucose, mannose, and maltose were utilized as growth substrates by all but two strains, as reported previously for most thermophilic bacilli (22), and starch was hydrolyzed by most strains (data not shown).

PCR restriction analysis (PRA) of 16S rDNA. The restriction fragment length polymorphism profiles of 16S rDNAs (digested with *Hae*III) of the strains isolated from composts and of the reference strains listed in Table 1 formed five groups with distinctive patterns (Fig. 1). To avoid confusion with primer dimer bands, and because of the detection threshold, restriction fragments shorter than 90 bp were disregarded.

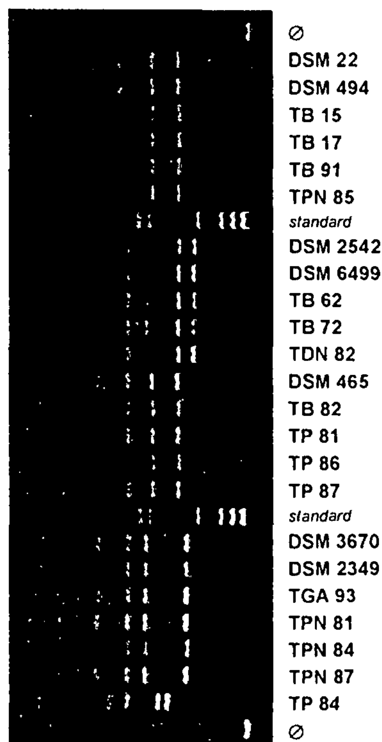

FIG. 1. PRA profiles of 16S rDNAs, selectively amplified and digested with *Hae*III, of *Bacillus* reference strains and of 16 strains isolated from hot composts. ∅, undigested amplification product; *standard*, ΦX174RF digested with *Hae*III. See Table 1 for reference strains.

These profiles permitted us to group all but 1 of the 16 strains with the seven reference strains belonging to four species (namely, *B. stearothermophilus*, *B. pallidus*, *Bacillus thermoglucosidasius* and "*Bacillus thermodenitrificans*"). The profiles obtained with restriction enzymes *Hin*fI and *Rsa*I showed no distinctive patterns for the strains tested (data not shown).

Theoretical restriction profiles calculated from 16S rDNA sequences available in the EMBL nucleotide sequence database were compared with the results obtained for our strains and for the reference strains. Within the first group, the profiles of reference strains DSM 22 and DSM 494 matched the theoretical profile calculated from the corresponding sequence of *B. stearothermophilus* (EMBL accession no. X60640). In the second group, the profile of reference strain DSM 2542 matched the theoretical profile of the sequence EMBL X60641. No 16S rDNA sequence could be found for strain DSM 6499. However, the restriction profiles did show that this strain was related to *B. thermoglucosidasius* DSM 2542, as described previously (18).

In the third group, the profile of the reference strain "*B. thermodenitrificans*" DSM 465 matched the theoretical profile of the corresponding sequence EMBL Z26928, except for the calculated 206-bp fragment that appeared to be 240 ± 5 bp long on the gel, and this was also true for the four related compost strains. This may have been due to the lack of recognition of a restriction site that caused a 25-bp fragment to remain attached to the 206-bp fragment. *Taq*I restriction profiles confirmed that this group was distinct from the three other groups (data not shown).

In the fourth group, the profiles of reference strains DSM 3670 and DSM 2349 matched the theoretical profiles of the corresponding sequences EMBL Z26930 and EMBL Z26929, respectively. Strain DSM 2349 could be linked to *B. pallidus* DSM 3670, as has been proposed in previous studies (14, 22).

The profile of strain TP-84 could not be related to any 16S rDNA sequence available for thermophilic bacilli.

PRA profiles are a powerful tool for identifying new strains related to heterotrophic thermophilic bacilli. The results obtained showed, however, that in some cases (e.g., the "*B. thermodenitrificans*" group [see above]) the digestion profiles of new strains should be compared with profiles obtained experimentally from reference strains, and the restriction profiles calculated from published sequences should not be relied on.

By using five different isolation media, we showed that there is a taxonomically and metabolically diverse population of heterotrophic thermophilic spore-forming bacteria in thermogenic composts. Few growth tests had diagnostic value for any single group of strains; only the strains related to *B. pallidus* had common features (particularly the absence of growth under denitrificating conditions) that clearly distinguished them from the other groups. Strain TP-84, despite its lack of reactivity with most of the substrates tested, like *Bacillus thermocloacae* strains (22), could not be related to this species by its PRA profile. Phenotypic tests can therefore not be relied on for taxonomic grouping of new *Bacillus* strains, while PRA of 16S rDNA appears to be a promising tool for rapid and reliable identification of newly isolated strains of recognized species.

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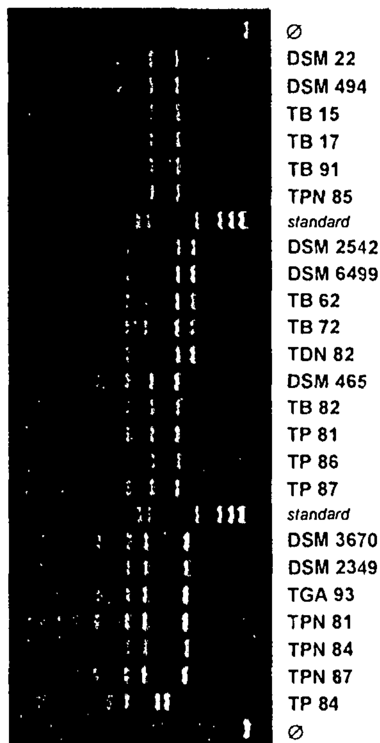

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In the fourth group, the profiles of reference strains DSM 3670 and DSM 2349 matched the theoretical profiles of the corresponding sequences EMBL Z26930 and EMBL Z26929, respectively. Strain DSM 2349 could be linked to *B. pallidus* DSM 3670, as has been proposed in previous studies (14, 22).

The profile of strain TP-84 could not be related to any 16S rDNA sequence available for thermophilic bacilli.

PRA profiles are a powerful tool for identifying new strains related to heterotrophic thermophilic bacilli. The results obtained showed, however, that in some cases (e.g., the "*B. thermodenitrificans*" group [see above]) the digestion profiles of new strains should be compared with profiles obtained experimentally from reference strains, and the restriction profiles calculated from published sequences should not be relied on.

By using five different isolation media, we showed that there is a taxonomically and metabolically diverse population of heterotrophic thermophilic spore-forming bacteria in thermogenic composts. Few growth tests had diagnostic value for any single group of strains; only the strains related to *B. pallidus* had common features (particularly the absence of growth under denitrificating conditions) that clearly distinguished them from the other groups. Strain TP-84, despite its lack of reactivity with most of the substrates tested, like *Bacillus thermocloacae* strains (22), could not be related to this species by its PRA profile. Phenotypic tests can therefore not be relied on for taxonomic grouping of new *Bacillus* strains, while PRA of 16S rDNA appears to be a promising tool for rapid and reliable identification of newly isolated strains of recognized species.

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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. © 1999 Published by Elsevier Science B.V. All rights reserved.

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.

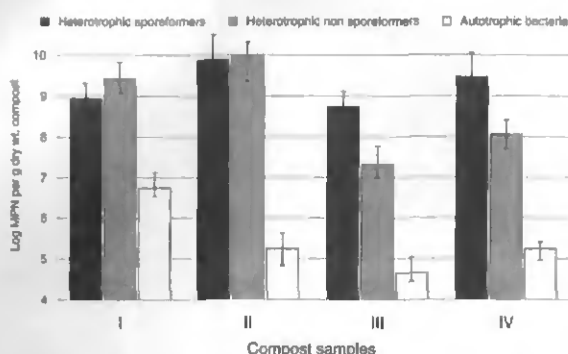

Fig. 1. Most probable numbers (MPN, single values) of three thermophilic bacterial populations in four hot compost samples. The sample features are listed in Table 1. Error bars are confidence intervals (0.05) with 10 000 bootstraps [22].

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

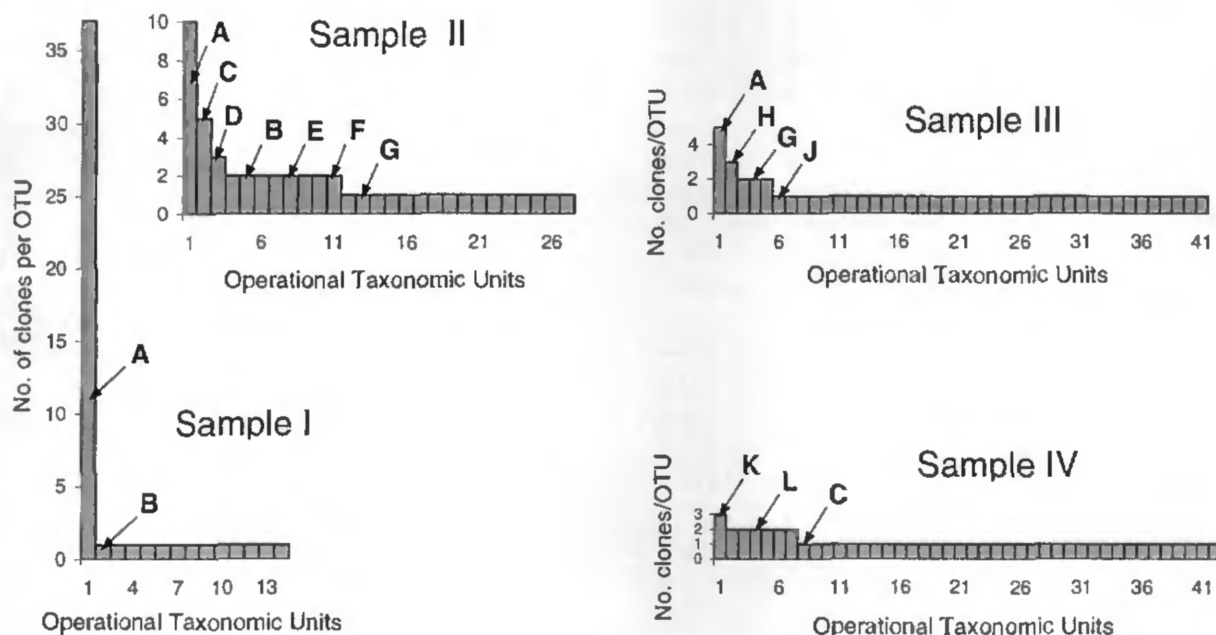

Fig. 2. Distribution among operational taxonomic units (OTUs) of bacterial 16S rDNA clones from four hot compost samples. Letters identify OTUs referred to in the text.

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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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. © 1999 Published by Elsevier Science B.V. All rights reserved.

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.

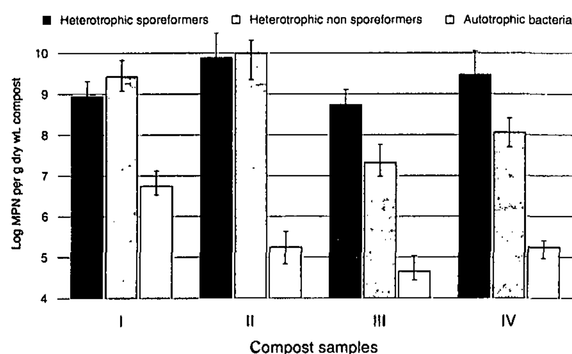

Fig. 1. Most probable numbers (MPN, single values) of three thermophilic bacterial populations in four hot compost samples. The sample features are listed in Table 1. Error bars are confidence intervals (0.05) with 10 000 bootstraps [22].

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

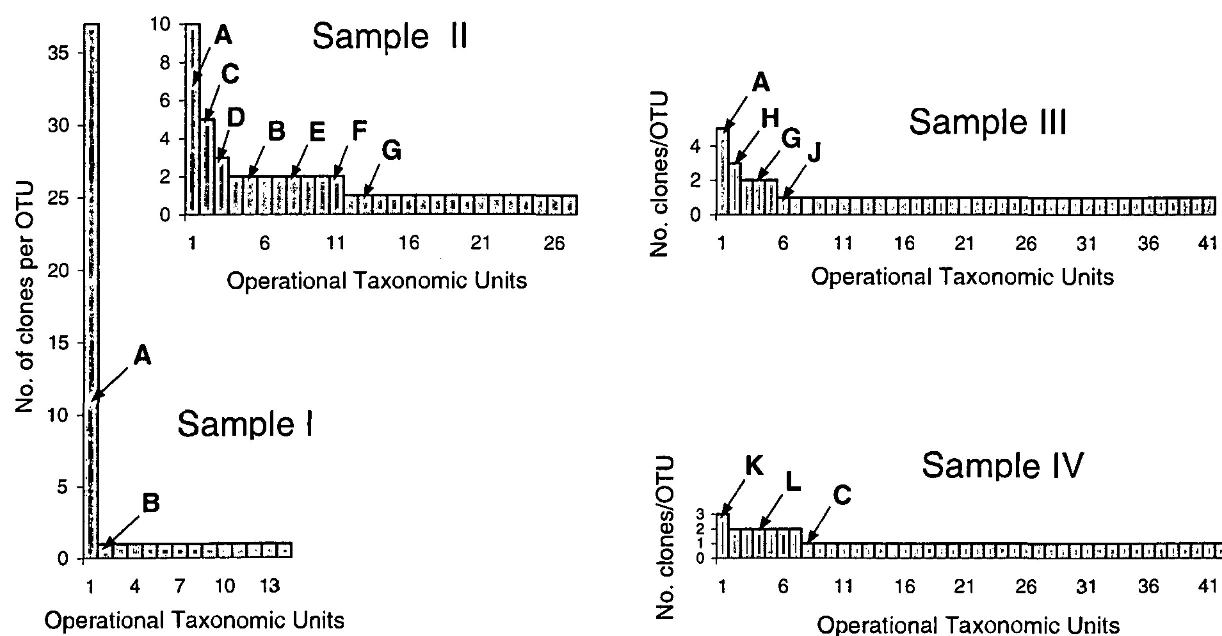

Fig. 2. Distribution among operational taxonomic units (OTUs) of bacterial 16S rDNA clones from four hot compost samples. Letters identify OTUs referred to in the text.

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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