Thermobacillus composti sp. nov., a moderately thermophilic bacterium isolated from a composting reactor

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A Gram-negative, rod-shaped, spore-forming and moderately thermophilic bacterium, strain KWC4^T, was isolated from a composting reactor. Cells of strain KWC4^T were 2.0–5.0 µm long and 0.5–0.7 µm in diameter. Strain KWC4^T grew aerobically at 32–61 °C, with optimal growth occurring at 50 °C. It grew at pH 5.6–10.1, with optimal growth at around pH 9.0. The optimum NaCl concentration for growth was almost 0 % (w/v), but strain KWC4^T was moderately halotolerant and was able to grow at NaCl concentrations up to 4.4 % (w/v). The DNA G + C content of strain KWC4^T was 60.0 mol%. The major fatty acids were iso-16 : 0 (39.0 %) and anteiso-15 : 0 (33.3 %). Based on 16S rRNA gene sequence similarity data, strain KWC4^T belonged to the genus *Thermobacillus* and was related to *Thermobacillus xylanilyticus*. However, strain KWC4^T had a 38 bp insertion sequence located near the 3' end of its 16S rRNA gene that was not present in *T. xylanilyticus*. The 16S rRNA gene sequence similarity value between strain KWC4^T and *T. xylanilyticus* was 95.7 %. The DNA–DNA hybridization value between strain KWC4^T and *T. xylanilyticus* strain XE^T was 66 %. On the basis of phenotypic and genotypic evidence, strain KWC4^T (= DSM 18247^T = JCM 13945^T) is the type strain of a novel species, for which the name *Thermobacillus composti* sp. nov. is proposed.

In recent years, the importance of the use of biotechnology for organic solid waste treatment has increased exponentially since biological processes appear to be cost-effective and have a less negative environmental impact than other treatment processes. Fed-batch composting (FBC) is an efficient way to treat organic waste without generating harmful compounds such as dioxins that are produced by incineration. In composting processes, thermophilic and mesophilic micro-organisms have important functions in terms of nutrient recycling and decomposition of complex organic substrates. Several culture-dependent microbial studies involved in the composting process have been reported and various species of bacteria, e.g. members of families such as the *Bacillaceae*, *Clostridiaceae*, *Flavobacteri-*

aceae, *Neisseriaceae*, *Nocardiopsaceae* and *Staphylococcaceae*, have been isolated. Moreover, many viable but non-culturable micro-organisms have also been detected by culture-independent methods from the contents of composting reactors (Dees & Ghiorse, 2001; Haruta *et al.*, 2002; Narihiro *et al.*, 2004). Therefore, composting reactors may contain many novel species of bacteria. Recently, some novel species have been isolated from FBC reactors. For example, *Paenibacillus motobuensis* was derived from a composting machine utilizing soil (Iida *et al.*, 2005) and *Cerasibacillus quisquiliarum* was isolated from a semi-continuous decomposing system for kitchen refuse (Nakamura *et al.*, 2004).

During a screen for novel species from an FBC reactor, a moderately thermophilic bacterium, strain KWC4^T, was isolated. 16S rRNA gene sequence analysis indicated that strain KWC4^T represented a novel species of the genus *Thermobacillus*. The genus *Thermobacillus* was proposed by Touzel *et al.* (2000) to contain Gram-negative, spore-forming, aerobic, non-motile, rod-shaped thermophiles. To date, only a single species, *Thermobacillus xylanilyticus*, has been described (Touzel *et al.*, 2000). This paper reports the isolation, characterization and taxonomic classification of strain KWC4^T.

Abbreviations: FBC, fed-batch composting; SEM, scanning electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KWC4^T is AB254031.

A table showing the cellular fatty acid contents of strain KWC4^T, *T. xylanilyticus* and some representatives of the genus *Paenibacillus* and a phylogenetic tree based on 16S rRNA gene sequences of strain KWC4^T and related species constructed using the maximum-likelihood method are available as supplementary material with the online version of this paper.

An FBC reactor for household use, the 'Namagomi-eater' (TK401-T, Matsushita Electric Works), was used for the composting reaction. The working volume of the reactor was 15 l and the temperature was usually maintained above 40 °C and reached about 60 °C during active degradation of organic matter. The biomass carrier comprised 5 l (1.2 kg wet wt) wood chips (about 0.5–2.0 mm). The artificial organic waste [500 g dog food containing 80 % (w/w) water] was loaded daily into the reactor. The contents were gently mixed twice per minute by automated paddles. The sample from which the micro-organism was isolated was taken from the optimal conditioned reactor in which the rate of decomposition of organic matter was about 17 g l⁻¹ day⁻¹. The temperature and pH of the sample were 47 °C and 8.6, respectively. The diluted sample was plated onto modified Brock's basal salts (MBS) (Kurosawa *et al.*, 1998) supplemented with 0.2 % (w/v) yeast extract, pH 7.5, solidified by 0.7 % (w/v) Gelrite and incubated at 50 °C for 24 h. Colonies were isolated and purified by repeating single-colony isolation. One of the isolates, strain KWC4^T, was assumed to be a member of a novel species by preliminary analysis of 16S rRNA gene sequences.

To characterize strain KWC4^T phenotypically, standard tests were performed, including Gram staining, cell morphology, motility, catalase and oxidase production and acid or gas production from glucose (Barrow & Feltham, 1993). Cell morphology and motility were examined by using a phase-contrast microscope (Axioskop 40; Carl Zeiss) and a scanning electron microscope (SEM) (JSM-5600LV; JEOL). For SEM observation of the cells, samples of exponential-phase cultures were applied onto carbon-coated SEMPoreB filters (JEOL Datum) and then washed with distilled water. After freeze-drying, samples were metal-shadowed with platinum and examined in high-vacuum mode at 10 kV.

The potential for growth at various initial pH values was determined using MBS supplemented with 0.5 % (v/v) yeast extract at 50 °C. The growth temperature and optimal NaCl concentrations were determined using the same medium at pH 9.0. Anaerobic growth was examined by using an AnaeroPack jar (Mitsubishi Gas Chemical). Enzymic activities were analysed by the API ZYM kit (bioMérieux) according to the manufacturer's instructions except for the incubation temperature, which was adjusted to 50 °C. Utilization of D-cellobiose, dextrin, erythritol, D-fructose, D-galactose, glucose, lactose, maltose, D-mannose, melibiose, D-sorbitol, sucrose, trehalose and D-xylose were tested. Hydrolysis of casein, starch and xylan were also examined. Cells for fatty acid analysis were grown on MBS plates at 50 °C for 24 h. Fatty acid methyl esters were prepared and identified following the Sherlock Microbial Identification system instructions (MIDI). The resultant esters were separated using a GC (HP6890; Hewlett Packard). The G + C content of DNA of strain KWC4^T was determined by HPLC (LC-10; Shimadzu) using genomic DNA digested with nuclease P1 (DNA-GC kit; SEIKAGAKU) (Katayama-Fujimura *et al.*, 1984). DNA–DNA hybridization experiments were carried

out three times independently as described by Ezaki *et al.* (1989). *Thermobacillus xylanilyticus* strain XE^T was kindly provided by J. P. Touzel and was used for comparison. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and were analysed by using fast-atom bombardment-MS (EI/FAB mate BU25; JEOL) with diethanolamine as a matrix for the negative MS.

The 16S rRNA gene of strain KWC4^T was amplified by PCR using the bacterial universal primers B27F (forward; 5'-AGAGTTTGATCMTGGCTCAG, positions 8–27 based on *Escherichia coli* numbering) and U1492RM (reverse; 5'-GGYTACCTTGTTACGACTT, positions 1510–1492 based on *E. coli* numbering). The following thermal cycle was used for 25 cycles: 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min. DNA sequencing was carried out by the dideoxynucleotide chain-termination method with Texas-red-labelled primers using the ThermoSequenase Primer Cycle Sequencing kit (GE Healthcare Biosciences) and an automated DNA sequencer (SQ5500E; Hitachi). The 16S rRNA gene sequence of strain KWC4^T was compared with available 16S rRNA gene sequences in the NCBI nucleotide sequence database using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) (Altschul *et al.*, 1990). Twenty-nine 16S rRNA gene sequences of related species were aligned using CLUSTAL W (Thompson *et al.*, 1994) and all sites with gaps in any sequences and regions of the PCR primers were removed from the alignment. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods and algorithms were integrated in the PHYLIP package (Felsenstein, 1993). The stability of relationships was assessed by performing bootstrap analyses of the neighbour-joining data based on 1000 resamplings.

Colonies of strain KWC4^T on MBS plates were 0.5–1.0 mm in diameter, light-cream in colour, round and half-opaque, but sometimes exhibited an irregular, flat morphology and had rather undulate margins. Cells of strain KWC4^T were 2.0–5.0 µm long and 0.5–0.7 µm in diameter (Fig. 1). Endospores were formed at the middle of the cell (data not shown). Cells of strain KWC4^T were aerobic, Gram-negative, spore-forming, occurred singly and occasionally in pairs or chains, and were non-motile and catalase- and oxidase-positive. Strain KWC4^T utilized D-cellobiose, D-fructose, D-galactose, D-xylose, D-mannose, lactose, melibiose, starch, trehalose and xylan; *T. xylanilyticus*, the type species of the genus, also utilized these substrates. Strain KWC4^T also utilized D-glucose, maltose, sucrose and dextrin. Alkaline phosphatase, esterase, leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase activities were detected; no lipase, valine arylamidase, chymotrypsin or α-mannosidase activities were found. Acid or gas production from glucose was negative.

Strain KWC4^T grew at 32–61 °C and pH 5.6–10.1, with optimal growth occurring at 50 °C and around pH 9.0. The

Fig. 1. SEM images of cells of strain KWC4^T. Bars, 1 μm.

optimum NaCl concentration for growth of strain KWC4^T was almost 0 % (w/v), but it was moderately halotolerant and was able to grow at NaCl concentrations up to 4.4 % (w/v). The DNA G+C content of strain KWC4^T was 60.0 mol%. The optimal pH, maximum NaCl concentration for growth and the DNA G+C content of strain KWC4^T were slightly higher than those of *T. xylanilyticus* (Table 1). The major fatty acids of strain KWC4^T were iso-16:0 (39.0 %) and anteiso-15:0 (33.3 %). This fatty acid composition also distinguished strain KWC4^T from *T.*

xylanilyticus and species of the genus *Paenibacillus* (see Supplementary Table S1 available in IJSEM Online). The major isoprenoid quinone of strain KWC4^T was MK-6(H₈), in contrast to *T. xylanilyticus*, which had MK-7 as the major isoprenoid quinone (Touzel *et al.*, 2000).

The almost full-length 16S rRNA gene sequence (1528 bp) of strain KWC4^T was determined. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KWC4^T was most closely related to *T. xylanilyticus*; their 16S rRNA gene sequences showed 95.7 % similarity. The neighbour-joining tree showed that KWC4^T and *T. xylanilyticus* form a monophyletic cluster with a bootstrap value of 100 % (Fig. 2). This cluster was also distinct from other genera, i.e. *Cohnella* and *Paenibacillus*, in the maximum-likelihood tree (see Supplementary Fig. S1 available in IJSEM Online). Interestingly, strain KWC4^T has a 38 bp insertion sequence near the 3' end of the 16S rRNA gene compared with the sequence of *T. xylanilyticus*. The 16S rRNA gene sequences of strain KWC4^T and *T. xylanilyticus* showed a similarity value of 98.3 % when the insertion sequence was removed from the alignment. This unusual insertion sequence was also found in the 16S rRNA gene sequence of *Paenibacillus nematophilus* strain NEM3 (Enright *et al.*, 2003). In addition, when compared with other bacteria, strain KWC4^T and *T. xylanilyticus* have a 36 bp insertion sequence located near the 5' end of the 16S rRNA gene. This insertion sequence may be unique for the genus *Thermobacillus*. DNA–DNA hybridization experiments were also performed between strain KWC4^T and *T. xylanilyticus* strain XE^T. The DNA–DNA reassociation value was 66 % (SD 5.4 %). This value is relatively high for members of different species, but phenotypic signatures suggest that strain KWC4^T should be defined independently from *T. xylanilyticus*. Based on phylogenetic evidence, together with phenotypic and chemotaxonomic data, strain KWC4^T is proposed as a representative of a novel species of the genus *Thermobacillus*, *Thermobacillus composti* sp. nov.

Table 1. Comparison of phenotypic characteristics of strain KWC4^T and *T. xylanilyticus*

Characteristic	Strain KWC4 ^T	<i>T. xylanilyticus</i> XE ^T *
Temperature range for growth (°C)	32–61	Up to 63
Optimum temperature for growth (°C)	50	55
pH range	5.6–10.1	6.5–8.5
Optimum pH	9.0	7.8
NaCl range (% w/v)	0–4.4	0–3.0
DNA G+C content (mol%)	60.0	57.5
Major fatty acid (% w/v)	iso-16:0 (39.0), anteiso-15:0 (33.3)	iso-16:0 (48.0), 16:0 (21.4)
Major isoprenoid quinone	MK-6	MK-7

*Touzel *et al.* (2000).

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of strain KWC4^T and related species constructed by the neighbour-joining method with *Escherichia coli* as the outgroup species. GenBank accession numbers of the sequences used in this study are shown in parentheses. The numbers at each node are bootstrap values performed with 1000 replicates. Bootstrap values less than 50 % are not shown. Bar, 10 % nucleotide substitution.

Description of *Thermobacillus composti* sp. nov.

Thermobacillus composti (com.pos'ti. N.L. gen. neut. n. *composti* of/from compost).

Cells are non-motile, spore-forming rods (approx. 2.0–5.0 µm long). Aerobic and Gram-negative. Catalase- and oxidase-positive. Colonies are light-cream in colour, round and half-opaque, but sometimes exhibit an irregular, flat morphology. Grows at 32–61 °C and pH 5.6–10.1, with optimal growth at 50 °C and around pH 9.0. Optimal NaCl concentration is almost 0 %, but is able to grow at NaCl concentrations up to 4.4 % (w/v). The following compounds are utilized: D-cellobiose, dextrin, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, starch, sucrose, trehalose, D-xylose and xylan. Erythritol, sorbitol, and casein are not utilized. The major fatty acids are iso-16:0 (39.0 %) and anteiso-15:0 (33.3 %).

The major isoprenoid quinone is MK-6(H₈). DNA–DNA relatedness between strain KWC4^T and *Thermobacillus xylanilyticus* strain XE^T is 66 %. The DNA G + C content of the type strain is 60.0 mol%.

The type strain, KWC4^T (= DSM 18247^T = JCM 13945^T), was isolated from a fed-batch composting reactor.

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