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 KWC4T, was isolated from a composting reactor. Cells of strain KWC4T were 2.0–5.0 µm long and 0.5–0.7 µm in diameter. Strain KWC4T 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 KWC4T was moderately halotolerant and was able to grow at NaCl concentrations up to 4.4 % (w/v). The DNA G+C content of strain KWC4T 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 KWC4T belonged to the genus Thermobacillus and was related to Thermobacillus xylanilyticus. However, strain KWC4T 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 KWC4T and T. xylanilyticus was 95.7 %. The DNA–DNA hybridization value between strain KWC4T and T. xylanilyticus strain KE1T was 66 %. On the basis of phenotypic and genotypic evidence, strain KWC4T ( = DSM 18247T = JCM 13945T) 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, Flavobacteriaceae, 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 motobuenis 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 KWC4T, was isolated. 16S rRNA gene sequence analysis indicated that strain KWC4T 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 KWC4T.

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KWC4T is AB254031.

A table showing the cellular fatty acid contents of strain KWC4T, T. xylanilyticus and some representatives of the genus Paenibacillus and a phylogenetic tree based on 16S rRNA gene sequences of strain KWC4T 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 KWC4ᵀ, was assumed to be a member of a novel species by preliminary analysis of 16S rRNA gene sequences.

To characterize strain KWC4ᵀ 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 KWC4ᵀ 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 XEᵀ was kindly provided by J. P. Touzel and was used for comparison. Isopeptide 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 KWC4ᵀ 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’-GNYTACCTTGTTAGACTT, 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 dideoxy-nucleotide 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 KWC4ᵀ 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 KWC4ᵀ 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 KWC4ᵀ 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 KWC4ᵀ were aerobic, Gram-negative, spore-forming, occurred singly and occasionally in pairs or chains, and were non-motile and catalase- and oxidase-positive. Strain KWC4ᵀ 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 KWC4ᵀ 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 KWC4ᵀ grew at 32–61 °C and pH 5.6–10.1, with optimal growth occurring at 50 °C and around pH 9.0. The
The optimum NaCl concentration for growth of strain KWC4T 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 KWC4T was 60.0 mol%. The optimal pH, maximum NaCl concentration for growth and the DNA G+C content of strain KWC4T were slightly higher than those of T. xylanilyticus (Table 1). The major fatty acids of strain KWC4T were iso-16:0 (39.0%) and anteiso-15:0 (33.3%). This fatty acid composition also distinguished strain KWC4T from T. xylanilyticus and species of the genus Paenibacillus (see Supplementary Table S1 available in IJSEM Online). The major isoprenoid quinone of strain KWC4T 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 KWC4T was determined. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KWC4T was most closely related to T. xylanilyticus; their 16S rRNA gene sequences showed 95.7% similarity. The neighbour-joining tree showed that KWC4T 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 KWC4T 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 KWC4T 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 NE3 (Enright et al., 2003). In addition, when compared with other bacteria, strain KWC4T 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 KWC4T 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 KWC4T should be defined independently from T. xylanilyticus. Based on phylogenetic evidence, together with phenotypic and chemotaxonomic data, strain KWC4T 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 KWC4T and T. xylanilyticus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain KWC4T</th>
<th>T. xylanilyticus XE(T)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>32–61</td>
<td>Up to 63</td>
</tr>
<tr>
<td>Optimum temperature for growth (°C)</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>pH range</td>
<td>5.6–10.1</td>
<td>6.5–8.5</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>9.0</td>
<td>7.8</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
<td>0–4.4</td>
<td>0–3.0</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>60.0</td>
<td>57.5</td>
</tr>
<tr>
<td>Major fatty acid (% w/w)</td>
<td>iso-16:0 (39.0), anteiso-15:0 (33.3)</td>
<td>iso-16:0 (48.0), 16:0 (21.4)</td>
</tr>
<tr>
<td>Major isoprenoid quinone</td>
<td>MK-6</td>
<td>MK-7</td>
</tr>
</tbody>
</table>

*Touzel et al. (2000).*
**Description of Thermobacillus composti sp. nov.**

*Thermobacillus composti* (com.posti. 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-xylene 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 KWC4T and *Thermobacillus xylanilyticus* strain XET is 66%. The DNA G+C content of the type strain is 60.0 mol%.

The type strain, KWC4T (= DSM 18247T = JCM 13945T), was isolated from a fed-batch composting reactor.

**Acknowledgements**

We thank Dr Jean P. Touzel for providing a culture of *Thermobacillus xylanilyticus* and Dr Hans G. Truper for advice on nomenclatural etymology. We also thank Shinichiro Osa for technical assistance, Takeo Suzuki for assistance with electron microscopy and Victor S. Kuwahara for correcting the English. This work was supported by ‘University-Industry Joint Research’ Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2004-2008.

**Fig. 2.** Phylogenetic tree based on 16S rRNA gene sequences of strain KWC4T and related species constructed by the neighbor-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.
References


