Tuberibacillus calidus gen. nov., sp. nov., isolated from a compost pile and reclassification of *Bacillus naganoensis* Tomimura *et al.* 1990 as *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *Bacillus laevolacticus* Andersch *et al.* 1994 as *Sporolactobacillus laevolacticus* comb. nov.

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Two thermophilic strains, designated 607^T and 606b, were isolated from a compost pile in Japan. The novel strains were Gram-positive, aerobic, spore-forming rods. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strains 607^T and 606b were closely related to *Bacillus naganoensis* (94.0–94.1 % similarity) and separated from clusters of the related genera *Bacillus* (<91.9 %) and *Sporolactobacillus* (91.0–92.5 %). In addition, some chemotaxonomic and physiological characteristics of strains 607^T and 606b differed from those of *B. naganoensis* and the two related genera. Several differences in physiological characteristics and 16S–23S rRNA gene internal transcribed spacer region nucleotide sequences were observed between strains 607^T and 606b; however, DNA–DNA hybridization indicated that these two strains belonged to the same species. From these results, it is proposed that strains 607^T and 606b represent the type species of a new genus, *Tuberibacillus calidus* gen. nov., sp. nov., with strain 607^T (=JCM 13397^T=DSM 17572^T) as the type strain. In addition, the results of phylogenetic analyses, as well as chemotaxonomic and physiological characterization, indicated that *B. naganoensis* and *Bacillus laevolacticus* did not belong to the genus *Bacillus*. Based on these results, it is proposed that *B. naganoensis* and *B. laevolacticus* should be transferred to *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *Sporolactobacillus laevolacticus* comb. nov., respectively.

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Tuberibacillus calidus* 607^T and 606b are AB231786 and AB231785, respectively. The accession numbers for the nucleotide sequences of the 16S–23S ITS regions of the *Tuberibacillus*, *Pullulanibacillus* and *Sporolactobacillus* strains determined in this work are AB231787–AB231797.

An additional phylogenetic tree, phase-contrast microscope images of cells of *Tuberibacillus calidus* sp. nov. and a table detailing major cellular fatty acids for the novel isolates and related recognized species are available as supplementary material in IJSEM Online.

The genus *Bacillus* consists of many species which display a variety of physiological and chemotaxonomic characteristics (Claus & Berkeley, 1986). Recent studies have shown that this genus is in fact made up of heterogeneous genera and several new genera have been established from this genus (Niimura *et al.*, 1990; Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998; Wainø *et al.*, 1999; Schlesner *et al.*, 2001; Fortina *et al.*, 2001; Nazina *et al.*, 2001; Yoon *et al.*, 2001). However, it has been noted that there are still several species of the genus *Bacillus* which need to be reclassified into other genera (Xu & Côté, 2003).

In our study of the microbial flora associated with the hyperthermal composting process in Japan, several mesophilic and thermophilic strains have been previously isolated and characterized (Hatayama *et al.*, 2005a, b). In this process, organic wastes were subjected to a rapid composting step at a high temperature using a hyperthermal composting machine followed by a further piling step in the atmosphere. In this latter step, the temperature of the compost piles was kept at approximately 60 °C for several weeks by the heat generated metabolically by the micro-organisms growing in the compost piles, as is the case for general composting (Fogarty & Tuovinen, 1991; Williams *et al.*, 1992). After the easily metabolized organic compounds in the piles had been thoroughly utilized by the micro-organisms, the temperature of the piles decreased to the ambient temperature, indicating the maturation of the compost piles. We assumed that the microbial flora in the piles changed as the composting process progressed, as in other composting processes (Fogarty & Tuovinen, 1991; Ishii *et al.*, 2000). We also assumed that when the easily metabolized organic compounds became scarce in the piles, such as during the later step in this process, the populations of some micro-organisms that utilize the corpses of previously dominant microbes might rise. To isolate these micro-organisms from the samples, we developed a medium containing microbial corpses as the sole nutrient source. Two thermophilic bacteria, 607^T and 606b, growing at around 60 °C, were isolated using this medium. From our phylogenetic analyses, based on 16S rRNA gene sequences and the sequences of the internal transcribed spacer (ITS) regions between the 16S and 23S rRNA genes, as well as chemotaxonomic and physiological characterizations of strains 607^T and 606b, we propose that the strains represent a new genus and novel species, *Tuberibacillus calidus* gen. nov., sp. nov.

Furthermore, during the above-mentioned analyses, we found that *Bacillus naganoensis* and *Bacillus laevolacticus* should be reclassified into other genera than the genus *Bacillus*. *B. naganoensis* was isolated as a producer of pullulanase and proposed by Tomimura *et al.* (1990). However, this species was established mainly on the basis of the results of physiological characterization and not analysed according to its phylogenetic position based on 16S rRNA gene sequence. Although Goto *et al.* (2000) determined the 16S rRNA gene sequence for *B. naganoensis*, there has been no discussion on the taxonomic position of this species. *B. laevolacticus*, originally described by Nakayama & Yanoshi (1967), was proposed by Andersch *et al.* (1994). At the time of this proposal, the close relationship of this species to the genus *Sporolactobacillus* was discussed, but it was nonetheless classified in the genus *Bacillus*. On the basis of the data described here, we propose the reclassification of *B. naganoensis* as *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *B. laevolacticus* as *Sporolactobacillus laevolacticus* comb. nov.

To isolate microbes that utilize the corpses of other microbes during the later piling step of the hyperthermal composting

process, we used plates comprising microbial corpses, termed MC agar. To make these plates and to isolate such microbes, we used a sample from the compost pile obtained when the temperature of the pile was beginning to decrease, since this sample had the potential to contain various microbes related to the succession of the microbial flora. Microbes originating from this sample were cultured in Luria–Bertani (LB) medium at 37 °C overnight and the cells were collected by centrifugation. The cells were suspended in an equal volume of distilled water and sterilized by autoclaving at 121 °C for 20 min, after the addition of 1.5 % agar. Using the MC agar made in this way, several micro-organisms growing at 60 °C were isolated from the same sample.

To determine the rough phylogenetic positions of the novel isolates, partial sequences of their 16S rRNA genes (approximately 500 bp) were analysed. DNA extraction from colonized cells was performed using the method of Zhu *et al.* (1993). PCR amplification and analysis of the partial 16S rRNA gene sequences were performed as previously described (Hatayama *et al.*, 2005a) using Eubac27F (DeLong, 1992) and 518R (Ogino *et al.*, 2001) primers. The results of a similarity search using BLASTN (Altschul *et al.*, 1997) revealed that most of the isolates were closely related to *Bacillus smithii* (>99.6 % similarity; data not shown). However, the sequences of two isolates, designated 607^T and 606b, showed high similarity (99.8 and 100 %, respectively) to that of uncultured bacterial clone F2–7 isolated from a hydrogen sulfide and methanol biofilter (GenBank accession no. AY096130), but did not show any close relationships to recognized species.

To determine the phylogenetic position of strains 607^T and 606b in more detail, PCR amplification of their 16S rRNA genes (>95 % of their full length) was performed with Eubac27F and 1492R (DeLong, 1992) primers. Sequencing and phylogenetic analyses were performed as previously described (Hatayama *et al.*, 2005a). The 16S rRNA gene sequences were aligned at positions 28 to 1490 [*Escherichia coli* numbering system (Brosius *et al.*, 1978)] using the CLUSTAL_X software package (Thompson *et al.*, 1997). Distance values were calculated using the method described by Kimura (1980). These values were then used in CLUSTAL_X to construct a phylogenetic tree by the neighbour-joining method with bootstrap values based on 1000 replications. A maximum-likelihood analysis was performed in DNAML with bootstrap values based on 100 replications using the SEQBOOT and CONSENSE programs of the PHYLIP package, version 3.65 (Felsenstein, 2005). The results were visualized using TREEVIEW software (Page, 1996). The 16S rRNA gene sequences of strains 607^T and 606b were almost identical (99.9 % similarity) and homology searches in BLASTN and FASTA (Pearson, 2000) revealed that the closest recognized species to these strains was *Bacillus naganoensis* (94.0–94.1 % similarity). The novel strains showed relatively low 16S rRNA gene sequence similarities to members of the genera *Bacillus* (below

91.9%) and *Sporolactobacillus* (91.0–92.5%). In the phylogenetic tree built via the neighbour-joining method, strains 607^T and 606b formed an independent lineage from that of *B. naganoensis*. These two lineages were not included in the clusters of the genus *Sporolactobacillus* or of the genus *Bacillus* and related genera (Fig. 1). Similar results were obtained in the phylogenetic tree generated via the maximum-likelihood method (data not shown). These phylogenetic trees also revealed that *B. laevolacticus* was not included in the cluster of the genus *Bacillus*, but was found in the cluster of the genus *Sporolactobacillus* (Fig. 1). However, Xu & Côté (2003) reported that *B. laevolacticus* grouped with *Virgibacillus pantothenicus* on the basis of the results of phylogenetic analysis based on the nucleotide sequences of 3'-end of the 16S rRNA gene (150 bp) and 5'-end of the 16S–23S rRNA gene ITS (16S–23S ITS) region (70 bp). In order to clarify the phylogenetic position of *B. laevolacticus*, PCR amplification, cloning and sequencing of 3'-end of the 16S rRNA gene and 5'-end of the 16S–23S ITS region were conducted for strains 607^T and 606b, *B. naganoensis*, *B. laevolacticus* and members of the genus *Sporolactobacillus* according to the method of Xu & Côté (2003) and a phylogenetic analysis was performed with the sequence data for members of related genera. Our phylogenetic tree, constructed via the neighbour-joining method, indicated that *B. laevolacticus* fell into the cluster of the genus *Sporolactobacillus* (see Supplementary Fig. S1 in IJSEM Online). We therefore propose that *B. laevolacticus* should be reclassified into the genus *Sporolactobacillus*. Moreover, this tree again indicated that strains 607^T and 606b and *B. naganoensis* belong to different lineages, separated from the clusters of the genera *Bacillus* and *Sporolactobacillus*. The phylogenetic analysis also showed that strains 607^T and 606b were separated into two independent lineages. It is notable that this phylogeny reflects the differences in

their nucleotide sequences at the 5'-end of the 16S–23S ITS region, rather than the 3'-end of the 16S rRNA coding region (data not shown). These phylogenetic analyses strongly suggest that strains 607^T and 606b and *B. naganoensis* do not belong to the genera *Bacillus* or *Sporolactobacillus* and that the two novel strains and *B. naganoensis* should be classified into two different genera. Based on these observations, strains 607^T and 606b, *B. naganoensis* DSM 10191^T and *B. laevolacticus* DSM 442^T were subjected to physiological and chemotaxonomic characterization using *Sporolactobacillus kofuensis* JCM 3419^T, *Sporolactobacillus inulinus* DSM 20348^T, *Sporolactobacillus nakayamae* subsp. *nakayamae* DSM 11696^T and *Sporolactobacillus terrae* DSM 11697^T as reference strains.

The MC agar plates initially used for the isolation of the novel strains were not suitable for the maintenance and characterization of strains 607^T and 606b. A medium was selected in which strains 607^T and 606b would grow well. Although strains 607^T and 606b did not show any growth in LB medium, nutrient medium (nutrient broth; Difco) or GYP medium (Yanagida *et al.*, 1997), they grew in CYC medium [33.4 g Czapek-Dox liquid medium, modified (Oxoid), 2.0 g Bacto yeast extract (Difco), 6.0 g Bacto vitamin assay Casamino acids (Difco), 1000 ml distilled water, pH 7.2] (Lacey & Cross, 1989) at 60 °C and this medium was used for further work. Colonies of strains 607^T and 606b were round, obscure-edged, translucent and cream in colour. The cells were Gram-positive rods (using Live BacLight Bacterial Gram Stain Kit; Invitrogen), 3–7 µm long and 0.3–0.5 µm wide, non-motile and occurred singly or in chains. Strains 607^T and 606b both formed oval endospores which were 0.7–1.0 µm long and 0.5–0.7 µm wide and located terminally within swollen sporangia (see Supplementary Fig. S2 in IJSEM Online). The widths of

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strains 607^T and 606b, *B. naganoensis*, *B. laevolacticus* and recognized species of related genera. Bootstrap values are calculated from 1000 replications and values >50% are shown at branch points. GenBank accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

the spores were greater than those of the cells. In CYC medium, growth of strains 607^T and 606b was observed over a pH range of 5.0–7.0 and over a temperature range of 40 to 60 °C, with optimal growth at between 50 and 55 °C.

To examine strains 607^T and 606b in more detail, their physiological characteristics were determined as described by Claus & Berkeley (1986). Lactic acid production was tested using an F-kit (D-lactic acid L-lactic acid; Roche Diagnostics). There were several differences in physiological characteristics between the novel strains and the other reference strains (Table 1). Notably, a marked difference was seen in the growth temperature: strains 607^T and 606b grow at up to 60 °C and can therefore be classified as thermophiles, whereas the reference strains, including the closest related species, *B. naganoensis*, are mesophiles.

Strains 607^T and 606b and *B. naganoensis* showed some features that distinguished them from species of the genus *Sporolactobacillus* and *B. laevolacticus*, such as having catalase activities and not producing lactic acid. Strains 607^T and 606b could also be distinguished from *B. naganoensis* by the presence of oxidase activity, the pH range for growth and the hydrolysis of starch and casein. Several differences were also detected between strains 607^T and 606b, such as nitrate reduction ability, acid production from xylose and tolerance of NaCl. *B. laevolacticus* showed almost the same features as species of the genus *Sporolactobacillus* except for the hydrolysis of starch.

Chemotaxonomic characteristics and the DNA G+C content were determined as previously described (Hatayama *et al.*, 2005b) using cells of strains 607^T and

Table 1. Differential physiological and chemotaxonomic characteristics of strains 607^T and 606b, *B. naganoensis*, *B. laevolacticus* and related recognized species

Taxa: 1, 607^T; 2, 606b; 3, *B. naganoensis* (data from Tomimura *et al.*, 1990, except for oxidase, cell-wall sugars, major menaquinone and major cellular fatty acids); 4, *B. laevolacticus* (data from Andersch *et al.*, 1994, except for catalase and cell-wall sugars); 5, *S. inulinus*; 6, *S. kofuensis*; 7, *S. nakayamae*; 8, *S. terrae*. For taxa 5–8, data are from Yanagida *et al.* (1997), except for cell-wall sugars, hydrolysis and utilization of substrates and nitrate reduction. +, Positive reaction; –, negative reaction; ND, no data available. All strains contain menaquinone-7 as the major isoprenoid quinone and are negative in tests for hydrolysis of tyrosine, utilization of citrate and propionate and phenylalanine deaminase activity.

Characteristic	1	2	3	4	5	6	7	8
Catalase activity	+	+	+	–*	–	–	–	–
Oxidase activity	+	+	–	–	–	–	–	–
Motility	–	–	–	+	+	+	+	+
Lactic acid production	–	–	ND	+	+	+	+	+
Growth temperature range (°C)	40–60	40–60	28–33	15–40	25–40	25–40	15–40	15–40
NaCl tolerance (%)	0–4	0–2	0–5	0–5	0–7	0–7	0–7	0–5
pH range	5.0–7.0	5.0–7.0	4.0–6.0	4.5–7.7	ND	ND	ND	ND
Hydrolysis of starch	–	–	+	+	–	–	–	–
Hydrolysis of casein	+	+	–	–	–	–	–	–
Nitrate reduction	+	–	–	–	–	–	–	–
Acid production from:								
Arabinose	+	+	+	–	–	–	–	–
Glucose	+	+(weak)	+	+	+	+	+	+
Lactose	–	–	+(weak)	ND	–	–	–	–
Xylose	+	–	+	–	–	–	–	–
Cell-wall sugars:								
Galactose	–	–	+	+	+	+	+	+
Glucose	–	–	+	–	+	+	+	+
Mannose	–	–	–	+	–	–	+	–
Rhamnose	–	–	+	+	–	–	+	–
Predominant cellular fatty acids:†								
C _{16:0}	–	–	–	–	ND	–	–	–
iso-C _{16:0}	–	–	+	–	ND	–	–	–
anteiso-C _{15:0}	–	–	–	+	ND	+	+	+
anteiso-C _{17:0}	+	+	–	+	ND	+	+	+
DNA G+C content (mol%)	46.0	47.3	45 ± 2	43–45	47	43	43–47	43–46

*A positive reaction was reported by Andersch *et al.* (1994).

†Major components consisting of about 50% of total cellular fatty acids are regarded as predominant cellular fatty acids in taxa 1–3.

606b, *B. naganoensis* and the other reference strains cultured in CYC medium at 55 °C, CYC medium (pH was adjusted to 5.0) at 30 °C and GYP medium at 30 °C, respectively. The cell-wall peptidoglycan of strains 607^T and 606b contained *meso*-diaminopimelic acid, alanine and glutamic acid as did that of the reference strains (data not shown). No sugars were detected in the cell walls of strains 607^T and 606b, whereas those of the reference strains contained several sugars (Table 1). The predominant cellular fatty acid of strains 607^T and 606b was anteiso-C_{17:0}, which differed from those of the reference strains (Table 1 and Supplementary Table S1). Strains 607^T and 606b and the reference strains contained menaquinone-7 as the predominant component of isoprenoid quinone and menaquinone-5 as the minor component (above 10 % peak area ratio). The DNA G + C contents of strains 607^T and 606b were 46.0 and 47.3 mol%, respectively, which were almost the same as those of the reference strains.

Our phylogenetic analyses based on 16S rRNA gene sequences and the nucleotide sequences of the 16S–23S ITS region (Fig. 1 and Supplementary Fig. S1) indicated that strains 607^T and 606b could be classified as representing a novel species in a new genus. The differences found in growth temperatures, cell-wall sugar composition, cellular fatty acids and other physiological characteristics between strains 607^T and 606b and related genera also support this proposal (Table 1). Although strains 607^T and 606b showed differences in their nitrate reduction ability, acid production from xylose, tolerance of NaCl and in the nucleotide sequences of the 16S–23S ITS regions, DNA–DNA hybridization studies, using the method of Ezaki *et al.* (1989), indicated that the two strains belong to the same species (79–100 % DNA–DNA relatedness). On the basis of these results, we propose that strains 607^T and 606b be classified as *Tuberibacillus calidus* gen. nov., sp. nov., with strain 607^T as the type strain.

On the basis of 16S rRNA gene sequence similarity (<92.9 %, data not shown) and phylogenetic analyses (Fig. 1 and Supplementary Fig. S1), *B. naganoensis* showed a low relationship to the genera *Bacillus* and *Sporolactobacillus*. The cellular fatty acid profile of *B. naganoensis* also differed from those of related species (Table 1 and Supplementary Table S1). *B. laevolacticus* was included in the cluster of the genus *Sporolactobacillus* in both phylogenetic trees (Fig. 1 and Supplementary Fig. S1) and its physiological and chemotaxonomic characteristics mostly agreed with those of other species of this genus (Table 1). Therefore, we also propose that *Bacillus naganoensis* (Tomimura *et al.*, 1990) is reclassified as *Pullulanibacillus naganoensis* gen. nov., comb. nov. and *Bacillus laevolacticus* is reclassified as *Sporolactobacillus laevolacticus* comb. nov.

Description of *Tuberibacillus* gen. nov.

Tuberibacillus (Tu.be.ri.ba.cil'lus. L. neut. n. *tuber* swelling; L. masc. n. *bacillus* a small staff; N.L. masc. n. *Tuberibacillus* a small staff with a swelling).

Cells are aerobic, thermophilic, Gram-positive rods (3–7 × 0.3–0.5 µm), occur singly or in chains and are non-motile. Oval endospores (0.7–1.0 × 0.5–0.7 µm) are formed terminally within swollen sporangia. Catalase- and oxidase-positive. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid, alanine and glutamic acid, but no sugars. The major menaquinone is MK-7. The predominant fatty acid is anteiso-C_{17:0}. The DNA G + C content is 46.0–47.3 mol%. The type species is *Tuberibacillus calidus*.

Description of *Tuberibacillus calidus* sp. nov.

Tuberibacillus calidus (ca.li'dus. L. adj. *calidus* hot, due to their growth temperature).

Displays the following properties in addition to those given in the genus description. Growth is observed on CYC medium [33.4 g Czapek-Dox liquid medium, modified (Oxoid), 2.0 g Bacto yeast extract (Difco), 6.0 g Bacto vitamin assay Casamino acids (Difco), 1000 ml distilled water, pH 7.2] under aerobic and thermophilic conditions. Colonies are round, obscure-edged, translucent and cream in colour. Growth occurs at a pH range of 5.0–7.0 and at 40–60 °C (with an optimum temperature of 50–55 °C) and 0–2 or 0–4 % NaCl. Positive reactions are observed for catalase and oxidase activities and for the hydrolysis of casein. Negative reactions are observed for the deamination of phenylalanine, hydrolysis of starch and tyrosine, utilization of citrate and propionate and the production of lactic acid. Acid is produced from glucose and arabinose, but not from lactose. Nitrate reduction and acid production from xylose are dependent on the strain. DNA G + C content is 46.0–47.3 mol%. When grown in CYC medium at 55 °C, the predominant cellular fatty acid is anteiso-C_{17:0} with iso-C_{17:0} and iso-C_{16:0} as minor components.

The type strain, strain 607^T (= JCM 13397^T = DSM 17572^T), was isolated from a compost pile undergoing a hyperthermal composting process in Okinawa Prefecture, Japan. Strain 606b is a reference strain.

Description of *Pullulanibacillus* gen. nov.

Pullulanibacillus (Pul.lu.la.ni.ba.cil'lus. N.L. n. *pullulanum* pullulan; L. masc. n. *bacillus* a small staff; N.L. masc. n. *Pullulanibacillus* a small staff hydrolysing pullulan).

Cells are aerobic, non-motile, Gram-positive rods (0.5–1.0 × 2.1–10.0 µm) with rounded or square ends and occur singly or in chains. Endospores are oval and cause swelling of the sporangia. Mesophilic and moderately acidophilic. Catalase-positive. Tests for reduction of nitrate to nitrite and nitrite to NO₂ are negative. When grown in nutrient broth containing 1 % starch, the major fatty acid is iso-C_{16:0}. The DNA G + C content is 45 ± 2 mol%. Additional characteristics found in this study are as follows: oxidase-negative, cell-wall peptidoglycan contains *meso*-diaminopimelic acid, alanine, glutamic acid, galactose, glucose and rhamnose and menaquinone-7 and menaquinone-5 are the major and minor isoprenoid quinones,

respectively. When grown in CYC (pH 5.0) medium, the predominant cellular fatty acids are C_{16:0} and iso-C_{16:0}, with iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0} as minor components. The type species is *Pullulanibacillus naganoensis*.

Description of *Pullulanibacillus naganoensis* comb. nov.

Pullulanibacillus naganoensis (na.ga.no.en'sis. N.L. gen. n. *naganoensis* of Nagano, a Japanese Prefecture).

Basonym: *Bacillus naganoensis* Tomimura *et al.* 1990.

The description is the same as that given by Tomimura *et al.*, 1990. Additional characteristics found in this study are given above in the genus description.

The type strain is DSM 10191^T (=ATCC 53909^T=LMG 12887^T).

Description of *Sporolactobacillus laevolacticus* comb. nov.

Sporolactobacillus laevolacticus [lae.vo.lac'ti.cus. N.L. adj. *laevolacticus* referring to D(-)-lactic acid, the only lactic acid produced by the organisms].

Basonym: *Bacillus laevolacticus* Andersch *et al.* 1994.

The description is the same as that given by Andersch *et al.*, 1994, except for the absence of catalase activity. Additional characteristics found in this study are as follows: cell-wall peptidoglycan contains *meso*-diaminopimelic acid, alanine, glutamic acid, galactose, mannose and rhamnose.

The type strain is DSM 442^T (=ATCC 23492^T).

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