Limnochorda pilosa gen. nov., sp. nov., a moderately thermophilic, facultatively anaerobic, pleomorphic bacterium and proposal of Limnochordaceae fam. nov., Limnochordales ord. nov. and Limnochordia classis nov. in the phylum Firmicutes

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A novel facultatively anaerobic bacterium, strain HC45T, was isolated from sediment of a brackish meromictic lake in Japan, Lake Harutori. Cells were pleomorphic, and filamentous bodies were 5–100 μm in length. For growth, the optimum pH was 7.0 and the optimum temperature was 45–50 °C. The G+C content of the genomic DNA was 71 mol%. iso-C<sub>15</sub>:<sub>0</sub> and anteiso-C<sub>15</sub>:<sub>0</sub> were the major components in the cellular fatty acid profile. The predominant respiratory quinone was MK-7. Strain HC45T shared very low 16S rRNA gene sequence similarity with cultivated strains (<85%). Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolate was distantly related to members of the family Symbiobacteriaceae and family XVII Incertae Sedis in the class Clostridia, and they formed a cluster separate from canonical species of the phylum Firmicutes. These results indicated that strain HC45T should not be placed in any existing class of the phylum Firmicutes. On the basis of phylogenetic and phenotypic characterization, Limnochorda pilosa gen. nov., sp. nov. is proposed with HC45T (=NBRC 110152<sup>T</sup>=DSM 28787<sup>T</sup>) as the type strain, as the first representative of novel taxa, Limnochordales ord. nov., Limnochordaceae fam. nov. in Limnochordia classis nov.

The phylum Firmicutes comprises, at the time of writing, five classes, Bacilli, Clostridia, Negativicutes, Thermolitho- bacteria and Erysipelotrichia. This phylum includes many bacteria possessing diverse characteristics (phylogenetically, phenotypically, chemotaxonomically and pathogenically), especially in the class Clostridia. Detailed phylogenetic studies have shown that many organisms of the phylum Firmicutes need to be reclassified (Garrity et al., 2005; Yutin & Galperin, 2013). Moreover, reclassifications of phylum- or class-level taxa have been often carried out in the phylum Firmicutes [e.g. the phyla ‘Synergistetes’ (Jumas-Bilak et al., 2009) and ‘Tenericutes’ (Brown, 2010), and classes Negativicutes (Marchandin et al., 2010) and Erysipelotrichia (Ludwig et al., 2009a)]. The taxonomic status of many bacteria in the phylum Firmicutes remains controversial, because of the polypathy of this group (Ludwig et al., 2009b). In this study, a novel facultative anaerobe representing a novel class within the phylum Firmicutes was isolated and characterized.

Strain HC45<sup>T</sup> was isolated from sediment of Lake Harutori, a meromictic lake situated in Kushiro, north-eastern Japan (Kubo et al., 2014). The sediment sample was obtained and processed as described previously (Watanabe et al., 2015). The procedure used for culturing of strain HC45<sup>T</sup> is summarized in Fig. S1 (available in the online Supplementary Material). To establish the first enrichment culture, approx. 1 ml of sediment slurry was inoculated into 40 ml of bicarbonate-buffered sulfide-reduced defined basal medium containing sulfate (Widdel & Bak, 1992). One millilitre of cyclohexane solution [2 % (v/v) in 2,2,4,4,6,8-heptamethylnonane, which served as the carrier phase (Rabus et al., 1993)] was added to the medium as a sole carbon and energy source. The headspace of the bottles was filled with N<sub>2</sub>/CO<sub>2</sub> (80 : 20, v/v) and incubation was carried out in the dark at 45 °C. After three transfers to the same medium, the carbon and energy source was successively changed to fumarate (10 mM), H<sub>2</sub> + CO<sub>2</sub> (H<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub>, 50 : 40 : 10; 2 atm total pressure)
and glucose (10 mM). The resulting culture was inoculated into sulfate-free basal medium supplemented with fumarate, and immediately incubated at 80 °C for 15 min prior to further incubation at 45 °C. Finally, a pure culture of strain HC45<sup>T</sup> was obtained with the extinction dilution method, using 0.01 % peptone, 1 g yeast extract 1<sup>−1</sup> and 10 mM glucose as substrate. Culture purity was ascertained routinely by microscopy and checked by denaturing gradient gel electrophoresis of the 16S rRNA gene (Muyzer et al. 1996) for cultures grown after experiments for physiological characterization.

Phenotypic tests for the characterization of strain HC45<sup>T</sup> were performed by using R2A liquid medium (Daigo) supplemented with 2 % NaCl (NaCl-R2A medium) under aerobic conditions, unless otherwise specified. Cell morphology of strain HC45<sup>T</sup> was observed by phase-contrast microscopy (Axioplan 2; Zeiss). Fine structures of the cell surface were observed by scanning electron microscopy (JSM-7001F; JEOL). For electron microscopic observation, cultivated cells were collected by centrifuge and fixed with 2.0 % (v/v) glutaraldehyde in 1 × PBS buffer solution at room temperature for 30 min. After dehydration in a graded ethanol series, the specimen was dried using the critical point drying technique. The dried tissues were sputter-coated with platinum and viewed under the scanning electron microscope.

Gram-staining was performed by using a Gram staining kit (Fluka) as described in the manufacturer’s instructions. Catalase activity was assessed by 3 % H<sub>2</sub>O<sub>2</sub> solution using cells collected by centrifugation.

The DNA G + C content of strain HC45<sup>T</sup> was determined by using a Yamasa GC kit (Yamasa Shoyu) as described previously (Katayama-Fujimura et al., 1984). Analyses of cellular fatty acids, respiratory quinone and amino acid components of the cell wall were carried out by the identification services of Techno Suruga Laboratory. Cellular fatty acids were identified with the Sherlock Microbial Identification System (MIDI) (Sherlock Version 6.0; MIDI database TSBA40), and respiratory quinones were analysed using HPLC.

Each culturing experiment for physiological characterization was carried out in triplicate at 45 °C except for tests of growth temperature. The temperature range for growth was determined by culture incubation at eight different temperatures ranging from 28 to 60 °C. To determine the pH range for growth, NaCl-R2A media buffered with 20 mM MOPS, Tricine, MES or CAPSO were prepared. The pH of each medium was adjusted with HCl or NaOH, and growth was tested at 10 different pH values ranging from 5.5 to 9.5. The range of NaCl concentrations for growth was tested at eight different concentrations ranging from 0 to 6.0 % (w/v). Aerobic growth in liquid media was tested with NaCl-R2A and MB2216 (Difco). Colony formation was tested on NaCl-R2A agar plates and on MB2216 agar under aerobic conditions, and in an agar shake dilution tube (Widdel & Bak, 1992) using basal medium supplemented with 10 mM glucose under anaerobic conditions.

The utilization of substrates under oxic conditions was tested by using a modified version of the basal medium used for isolation, which contained no NaHCO<sub>3</sub> or Na<sub>2</sub>S. Instead of NaHCO<sub>3</sub>, 20 mM MOPS was used as a pH buffer. Anaerobic growth was tested in the sulfate-free basal medium supplemented with 10 mM glucose. Fermentative growth was tested in the medium without additional electron acceptors. Utilization of electron acceptors was tested in the medium with additional nitrate (10 mM) or sulfate (28 mM). Sulfate reduction was evaluated based on sulfide production as monitored spectrophotometrically (Cord-Ruwisch, 1985). Nitrate reduction was evaluated by changes in anion concentrations determined with an ion chromatograph.

Genomic DNA was purified by using a Wizard genomic DNA purification kit (Promega). 16S rRNA genes were amplified with primers 27f and 1492r (Lane, 1991). PCR amplification was carried out using Takara Ex Taq DNA polymerase (Takara), and PCR products were directly sequenced by using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The obtained sequence (1461 bp) was aligned with reference sequences from the public database using the program CLUSTAL X version 2.1 (Larkin et al., 2007), and phylogenetic trees were reconstructed with the program MEGA version 5.1 (Tamura et al., 2011).

Cells of strain HC45<sup>T</sup> were pleomorphic, and almost all were filaments with a width of approximately 0.3–1.5 μm (Figs. 1 and 2). Length of filaments varied widely (50–100 μm), depending on the growth phase and growth conditions. In long filaments, swollen regions were often observed (Figs. 1b and 2a). In addition, spherical bodies (1.5–10 μm in diameter) and amorphous nodules were observed mainly in old cultures (Fig. 1). The spherical structures had one or two filiform protrusions (Figs. 1b and 2b), but they were not motile. Endospore-like structures were also observed (Fig. S2). Scanning electron microscopy indicated that the isolate had a shaggy cell surface (Fig. 2).

Cells of the isolate were Gram-stain-negative. Catalase was not produced. The growth temperature and pH are detailed in Table 1. Growth of strain HC45<sup>T</sup> was observed in media containing 0.5–4.0 % NaCl with optimum growth in the presence of 2 % NaCl. Colonies were not formed on the agar plate media, but pale pink colonies were formed in agar shake tubes under anoxic conditions. The fatty acid profile of the strain was characterized by high proportions of iso-C<sub>15</sub>: 0 (31.0 %) and anteiso-C<sub>15</sub>:0 (31.7 %). The other fatty acids detected were present at less than 5 % (Table S1). The amino acid component of the cell wall could not be determined despite a trial with cell-wall fraction collected from 15 litres of active culture, suggesting that the isolate had a thin cell wall in comparison with typical Gram-positive bacteria. The G + C content of the genomic DNA of strain HC45<sup>T</sup> was 71 mol%. The predominant respiratory quinone was MK-7. The phenotypic characteristics of strain HC45<sup>T</sup> are summarized in Table 1.
The strain grew with and utilized the following organic substrates (mM, except where stated): glucose (10), man- nose (4), fructose (5), maltose (5), trehalose (2), cellobiose (2), galactose (5), maltose (5), sucrose (4), sorbitol (4), melibiose (5) and yeast extract (0.5 g l\(^{-1}\)). The following substrates could not support growth of the strain (mM): citrate (5), malate (5), lactate (10), \(N\)-acetylglucosamine (4), L-arabinose (5), ethanol (20) and Casamino acids (0.5 g l\(^{-1}\)). Slight growth was observed with (mM): Bacto-tryptone (0.5 g l\(^{-1}\)), acetate (5), pyruvate (5) and fumarate (5). Anaerobic growth on glucose was observed under fermentation conditions, but dissimilatory reduction of sulfate or nitrate was not observed.

Analysis of the 16S rRNA gene sequence revealed that strain HC45\(^{T}\) had exceedingly low similarity with cultivated strains (\(<85\%\)). *Moorella humiferrea* 64-FGQ\(^{T}\) showed the highest sequence similarity (85\%) to strain HC45\(^{T}\) among cultivated bacteria in public databases (GenBank/EMBL/ DDBJ). The most closely related environmental sequences to strain HC45\(^{T}\) were bacterial clones FS1689 (length of sequence=1469 bp; sequence similarity=96 \%) and FS1639 (1471 bp; 95 \%) from a composting unit (Partanen et al., 2010). An environmental sequence closely related to strain HC45\(^{T}\) (>90 \% 16S rRNA gene sequence similarity) was collected from compost soil, activated sludge and sewage. The environmental sequences and strain HC45\(^{T}\) formed a cluster with high bootstrap value (99 \%) in the phylogenetic tree reconstructed using the maximum-likelihood method (Fig. 3; see also Fig. S3). This cluster including strain HC45\(^{T}\) was consistently observed in trees reconstructed with the neighbour-joining and minimum-evolution methods (Figs S4 and S5). It was also shown that strain HC45\(^{T}\) and four genera (’Caldinitratiruptor’, *Symbiobacterium*, *Sulfobacillus* and *Thermaerobacter*) were clearly separated from all classes of the phylum *Firmicutes* (Figs. 3, S4 and S5). According to Da Costa et al. (2009),

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**Fig. 1.** Phase-contrast micrographs showing the cell morphology of strain HC45\(^{T}\) grown on NaCl-R2A liquid medium for 1 week (a) and for 1 month (b). Open arrows indicate spherical structures. Solid arrow and arrowhead indicate the swollen region and amorphous nodule, respectively. Bars, 10 \(\mu\)m.

**Fig. 2.** Scanning electron micrographs of cells of strain HC45\(^{T}\) grown in NaCl-R2A liquid medium at 45 °C for 15 days. Swollen region (a) and spherical body (b) are observed. Bars, 1 \(\mu\)m.
Table 1. Differential properties between strain HC45T and four genera (‘Caldinitratiruptor’, Symbiobacterium, Sulfobacillus and Thermaerobacter) distantly related to the new isolate

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain HC45T</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene sequence similarity to strain HC45T (%)</td>
<td>–</td>
<td>81–82</td>
<td>84–85</td>
<td>76–76.5</td>
<td>82</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative/positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Major quinones</td>
<td>MK-7</td>
<td>MK-6</td>
<td>ND</td>
<td>MK-7</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>69</td>
<td>65–69</td>
<td>69–73</td>
<td>46–62</td>
<td>70</td>
</tr>
<tr>
<td>NaCl requirements</td>
<td>+</td>
<td>–</td>
<td>±</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Temperature range/optimum (°C)</td>
<td>30–55/45–50</td>
<td>40–70/55–60</td>
<td>50–80/70–75</td>
<td>4–60*/40–55</td>
<td>50–75/65</td>
</tr>
<tr>
<td>pH range/optimum</td>
<td>6.5–9/7</td>
<td>6–10/7.5</td>
<td>5–10/7–8.5</td>
<td>1.1–5.5/2–2.4</td>
<td>6.3–7.9/7</td>
</tr>
<tr>
<td>Major cellular fatty acids</td>
<td>iso-C15 : 0, 0 valerate</td>
<td>C16 : 0, iso-C15 : 0, 0 valerate</td>
<td>iso-C17 : 0, 0 valerate</td>
<td>iso- and anteiso-branched fatty acids, ω-cyclohexane fatty acids†</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Spore germination of Sulfobacillus disulfidooxidans SD-11T occurred at 4 °C.
†ω-Cyclic fatty acids were not detected in Sulfobacillus sibiricus N1T.

On the basis of phylogenetic and physiological characteristics, we propose that strain HC45T represents a novel species of a new genus. We propose the name Limnochorda pilosa gen. nov., sp. nov. with HC45T (=NBRC 110152T = DSM 28787T) as the type strain. We also propose to establish novel taxa, Limnochordales ord. nov., Limnochordaceae fam. nov. in Limnochordia classis. nov.

Description of Limnochorda pilosa gen. nov.

Limnochorda (Lim.no.chor’da. Gr. n. limnos lake; L. fem. n. chorda chord; string; N.L. fem. n. Limnochorda string of lake, referring to the isolation source of the type species).

Cells are pleomorphic filaments and stain Gram-negatively. Catalase is not produced. Moderately thermophilic. Facultatively anaerobic. The major respiratory quinone is MK-7. Branched-chain fatty acids are the major cellular fatty acids. The G+C content of the genomic DNA of the type species is 71 mol%.

The type species is Limnochorda pilosa.

Description of Limnochorda pilosa sp. nov.

Limnochorda pilosa (pi.lo’sa. L. fem. adj. pilosa hairy, rough, bristly, referring to the shaggy cell surface of the organism).

Has the following characteristics in addition to those given for the genus. Cells are 0.3–1.5 μm in width and 5 μm and more in length. The cell surface is shaggy. Endospore-like structures are observed. The temperature range for growth is 30–55 °C, with optimum growth occurring at...
45–50 °C. The pH range for growth is 6.0–8.8, with an optimum at pH 7.0. The NaCl concentration for growth is 0.5–4.0 %. The major fatty acids are iso-C<sub>15</sub>:0 and anteiso-C<sub>15</sub>:0. Sulfate and nitrate are not used as an electron acceptor for growth with organic substrates. Utilizes glucose, mannose, fructose, maltose, trehalose, cellobiose, galactose, maltose, sucrose, sorbitol, melibiose and yeast extract. Acetate, fumarate, pyruvate and tryptone support slight growth. Citrate, lactate, malate, N-acetylglucosamine, L-arabinose, ethanol and Casamino acids are not used.

The type strain, HC45<sup>T</sup> (=NBRC 110152<sup>T</sup>=DSM 28787<sup>T</sup>), was isolated from meromictic lake sediment (Lake Harutori, Japan). The DNA G+C content of the type strain is 71 mol%.

**Description of Limnochordaceae fam. nov.**

Limnochordaceae (Lim.no.chor.da.ce ae. N.L. fem. n. Limnochordaa type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. Limnochordaceae family of the genus Limnochordaa).

The description is the same as for the genus Limnochordaa. The type genus is Limnochordaa gen. nov.

**Description of Limnochordales ord. nov.**

Limnochordales (Lim.no.chor.da'les. N.L. fem. n. Limnochordaa type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. Limnochordales order of the genus Limnochordaa).
The description is the same as for the genus Limnochorda. The type genus is *Limnochorda* gen. nov.

**Description of Limnochorda classis nov.**

*Limnochorda* (Lim.no.cho’rd.i.a. N.L. fem. n. Limnochorda type genus of the type order of the class; suff. -ia ending to denote a class; N.L. fem. pl. n. Limnochorda the Limnochorda class).

The description is the same as for the genus *Limnochorda*. The type order is *Limnochordales* ord. nov.

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**References**


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