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

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Running head: *Limnochorda pilosa* gen. nov., sp. nov.

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The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of strain HC45^T are AB992259 (16S rRNA gene).

Abstract

A novel facultative anaerobic bacterium, strain HC45^T 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 45-50ºC. The G+C content of the genomic DNA was 71 mol%. Iso-$C_{15:0}$ and anteiso-$C_{15:0}$ were the major components in the cellular fatty acid profile. Predominant respiratory quinone was MK-7. The strain HC45^T had exceedingly low similarity of the 16S rRNA gene with cultivated strains (85% or less). Phylogenetic analysis based on the 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 separated cluster from canonical species of the phylum *Firmicutes*. These results indicated that it was not adequate to place the strain HC45^T 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 the type strain HC45^T (=NBRC 110152^T =DSM 28787^T), as the first the representative of novel taxa, *Limnochordales* ord. nov., *Limnochordaceae* fam. nov. in *Limnochordia* clasis. nov.

The phylum *Firmicutes* is comprised of 5 classes, *Bacilli*, *Clostridia*, *Negativicutes*, *Thermolithobacteria* and *Erysipelotrichia*. This phylum includes many bacteria possessing diverse characteristics (phylogenetically, phenotypically, chemotaxonomically, and pathogenically), especially in the class *Clostridia*. Detailed phylogenetic studies showed that many organisms of the phylum *Firmicutes* have need to be reclassified (Garrity, 2005; Yutin & Galperin, 2013). Moreover, reclassifications of the phylum- or class-level taxa have been often carried out in the phylum *Firmicutes* (e.g. the phyla '*Synergistetes*' [Jumas-Bilak et al., 2009], '*Tenericutes*' [Brown, 2010], the class *Negativicutes* [Marchandin et al., 2010], *Erysipelotrichia* [Ludwig et al., 2009a]). Taxonomic status of many bacteria in the phylum *Firmicutes* remains controversial, because of the polyphyly of this group as previously described (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^T 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 culturing procedure of strain HC45^T is summarized in Fig. S1 as a flowchart. To establish the first enrichment culture, approx. 1 ml sediment slurry was inoculated into 40 ml of bicarbonate-buffered sulfide-reduced defined basal medium containing sulfate (Widdel & Bak, 1992). One milliliter of cyclohexane solution (2% [vol・vol^{-1}] 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_2/CO_2 (80:20 [vol・vol^{-1}]) 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), $H_2 + CO_2$ ($H_2/N_2/CO_2$, 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, pure culture of the strain HC45^T was obtained with extinction dilution method, using 0.01% peptone, 1 g l^{-1} yeast extract and 10 mM glucose as substrate. Culture purity was ascertained routinely by microscopy and checked by denaturing gradient gel electrophoresis of 16S rRNA gene (Muyzer et al. 1996) for cultures grown after experiments for physiological

characterization.

Phenotypic tests for the characterization of HC45^T were performed by using R2A liquid medium (Daigo) supplemented with 2% NaCl (NaCl-R2A medium) under aerobic conditions, unless otherwise specified. Cell morphology of the strain HC45^T was observed by a phase-contrast microscope (Axioplan 2; Zeiss). Fine structures of cell surface were observed by scanning electron microscope. For the 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 by using JEOL JSM-7001F scanning electron microscope.

The Gram-staining was performed by using Gram staining kit (Fluka) as described in the manufacturer's instruction. Catalase activity was also assessed by 3% H_2O_2 solution using cells collected by centrifugation.

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

Each culturing experiment for physiological characterization was carried out in triplicate at 45°C except for test of growth temperature. Temperature range for growth was determined by culture incubation at 8 different temperatures ranging from 28 to 60°C. To determine 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 8 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 the NaCl-R2A agar plate and MB2216 agar under aerobic conditions, and in agar shake dilution tube (Widdel & Bak, 1992) using basal

90 medium supplemented with 10 mM glucose under anaerobic conditions.

91 The utilization of substrates under oxic conditions were tested by using modified version of the basal
92 medium used for isolation, which contained no $NaHCO_3$ or Na_2S. Instead of $NaHCO_3$, 20 mM MOPS was
93 used as pH buffer. Anaerobic growth was tested in the sulfate-free basal medium supplemented with 10 mM
94 glucose. Fermentative growth was tested in the medium without additional electron acceptor. Utilization of
95 electron acceptor was tested in the medium containing with nitrate (10 mM) or sulfate (28 mM). Sulfate
96 reduction was evaluated as sulfide production monitored with spectorophotometric method (Cord-Ruwisch,
97 1985). Nitrate reduction was evaluated by changes in anion concentrations determined with an ion
98 chromatography.

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

105 Cells of strain HC45^T were pleomorphic, and almost all were filaments with a width of approximately
106 0.3–1.5 μm (Fig. 1, Fig. 2). Length of filaments varied largely (5–100 μm), depending on the growth phase
107 and growth conditions. In long filaments, swollen regions were often observed (Fig. 1B, 2A). In addition,
108 spherical bodies (1.5–10 μm in diameter) and amorphous nodule were observed mainly in old cultures (Fig. 1).
109 The spherical structures had one or two filiform protrusions (Fig 1B, 2B), but they are not motile.
110 Endospore-like structures were also observed (Fig. S2). Scanning electron microscopy indicated that the
111 isolate had shaggy cell surface (Fig. 2).

112 Cells of the isolate were Gram-negatively stained. Catalase was not produced. The growth temperature and
113 pH are shown in Table 1. Growth of strain HC45^T was observed in the media containing 0.5–4.0% of NaCl
114 and the optimum was 2%. Colonies were not formed on the agar plate media, but pale-pink colored colonies

were formed in agar shake tubes under anoxic conditions. The fatty acid profile of the strain was characterized by high concentrations of iso-$C_{15:0}$ (31.0%) and anteiso-$C_{15:0}$ (31.7%). The other fatty acids detected were less than 5% (Table S1). The amino acid component of cell wall could not be determined despite a trial with cell wall fraction collected from 15-liter active culture, suggesting that the isolate had thin cell wall in comparison with typical Gram-positive bacteria. The G+C content of the genomic DNA of strain HC45^T was 71 mol%. Predominant respiratory quinone was MK-7. The phenotypic characteristics of strain HC45^T are summarized in Table 1.

The strain grew and utilized the following organic substrates (mM, except where stated): glucose (10), mannose (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). 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). Slight growth was observed with Bacto-tryptone (0.5 g/l), 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% or less). *Moorella humiferrea* 64-FGQ^T showed the highest sequence similarity to HC45^T among cultivated bacteria the public database (85%) (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). Environmental sequence closely related to strain HC45^T (>90% 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 constructed by maximum-likelihood method (Fig. 3). This cluster including strain HC45^T was consistently observed in trees reconstructed by neighbour-joining method and minimum-evolution method (Fig. S4, S5). It was also shown that strain HC45^T and 4 genera (*Caldinitratiruptor*, *Symbiobacterium*, *Sulfobacillus* and *Thermaerobacter*) were clearly separated from all of

the classes of the phylum *Firmicutes* (Fig. 3, S4, S5). According to the Bergey's Manual of Systematic Bacteriology, the genera *Thermaerobacter* and *Sulfobacillus* are classified into the family XVII *Incertae Sedis* within the class *Clostridia* (Da Costa et al, 2009). The genus *Symbiobacterium* is placed in the family XVIII *Incertae Sedis* within the same class, but the family *Symbiobacteriaceae* was recently proposed (Beppu & Ueda, 2009; Shiratori-Takano et al., 2014). The 16S rRNA gene phylogeny showed that the genus *Symbiobacterium* is related to the phylum *Actinobacteria* rather than members of the phylum *Firmicutes* (Ohno et al, 2000; Ludwig et al, 2009b), but the relatedness of the class *Clostridia* was also shown by genome-based analyses (Ueda et al., 2004, Nishida et al., 2009). These genera may represent novel phylum/phyla, as discussed in the Bergey's Manual of Systematic Bacteriology (Da Costa et al., 2009; Spanevello & Patel, 2009; Beppu & Ueda, 2009). The cluster including strain HC45^T and environmental sequences was distantly related to the 4 genera mentioned above, although supported only by low bootstrap values (Fig 3, S4, S5).

Characteristics of strain HC45^T are given in Table 1 along with the 4 genera (*Caldinitratiruptor, Symbiobacterium, Sulfobacillus* and *Thermaerobacter*) distantly related to strain HC45^T. They are commonly able to grow under the presence of oxygen, although other members of the class *Clostridia* are strictly anaerobe. Strain HC45^T and members of the genera *Thermaerobacter*, *Caldinitratiruptor* and *Symbiobacterium* possess high G+C content of the genomic DNA (65-72 mol%), but *Firmicutes* species are generally characterized by genomic DNA mol% lower than 50 (Schleifer, 2009). These phylogenetic and phenotypic characteristics strongly suggested that the organisms listed in Table 1 should not be classified into the class *Clostridia* or other classes in the phylum *Firmicutes*.

On the basis of phylogenetic and physiological characteristics, we propose that the strain HC45^T represents a novel species of novel genus. We propose the name *Limnochorda pilosa* gen. nov., sp. nov. with the type strain HC45^T (=NBRC 110152^T =DSM 28787^T). We also propose to establish novel taxa, *Limnochordales* ord. nov., *Limnochordaceae* fam. nov. in the *Limnochordia* clasis. nov., with the strain HC45^T.

Description of *Limnochorda* 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, reffering to the isolation source of the type species).

Cells are pleomorphic filaments and stain Gram-negatively. Catalase is not produced. Moderately thermophilic. Facultative anaerobe. The major respiratory quinone is MK-7. Branched-chain fatty acids are major cellular fatty acid. The G+C content of genomic DNA of 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).

Cells are pleomorphic filaments (0.3-1.5 μm in width, and 5 μm and more in length). The cell surface is shaggy. Endospore-like structures are observed. Cells stained Gram-negative. Catalase is not produced. The temperature range for growth was 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%. Major respiratory quinone was MK-7. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Sulfate and nitrate are not used as electron acceptor for growth with organic substrate. Glucose, mannose, fructose, maltose, trehalose, cellobiose, galactose, maltose, sucrose, sorbitol, melibiose, and Yeast Extract were used. Acetate, fumarate, pyruvate and tryptone supported slight growth. Citrate, lactate, malate, N-acetylglucosamine, L-arabinose, ethanol and Casamino acids were not used. The DNA G+C content of the type strain is 71 mol%.

The type strain, HC45^T (=NBRC 110152^T =DSM 28787^T), was isolated from meromictic lake sediment (Lake Harutori, Japan).

Description of *Limnochordaceae* fam. nov.

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

The description is the same as for the genus *Limnochorda*.

Type genus is *Limnochorda* gen. nov.

Description of *Limnochordales* ord. nov.

Limnochordales (Lim.no.chor.da'les. N.L. fem. n. *Limnochorda* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Limnochordales* order of the genus *Limnochorda*).

The description is the same as for the genus *Limnochorda*.

Type genus is *Limnochorda* gen. nov.

Description of *Limnochordia* classis nov.

Limnochordia (Lim.no.chor'di.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. *Limnochordia* the *Limnochorda* class).

The description is the same as for the phylum *Limnochordae*.

Type order is *Limnochordales* ord. nov.

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342

343 **Figure legends**

344

345 Fig. 1 Phase-contrast micrographs showing the cell morphology of strain HC45^T grown on NaCl-R2A liquid
346 medium for a week (A) and for a month (B). Open arrows indicate the spherical structures. Solid arrow and
347 triangle indicate the swollen region and amorphous nodule, respectively. Bar, 10 µm.

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349 Fig. 2 Scanning electron micrographs of *Limnochorda pilosa* cells grown on NaCl-R2A liquid medium at
350 45°C for 15 days. Swollen region (A) and spherical body (B) are observed.

351

352 Fig. 3 Maximum-likelihood tree based on 16S rRNA gene sequences of the strain HC45^T, related environtal
353 sequences and representatives from all classes in the phylum *Firmicutes*. This phylogenetic tree is based on a
354 comparison of 1369-1449 nucleotides. Numbers in parentheses represent the number of strains in each
355 collapsed branch. Bootstrap values (percentages of 1000 replications) only 50% or more are shown at nodes.
356 The same tree showing all entries is shown in Supplementary Fig. S3.

357

358 Table 1. Differential properties of HC45^T and 4 genera (*Caldinitratiruptor*, *Symbiobacterium*, *Sulfobacillus*
359 and *Thermaerobacter*) distantly related to the isolate.
360 Genera: 1, *Symbiobacterium* (data from Beppu & Ueda, 2009; Shiratori-Takano et al., 2009); 2,
361 *Thermaerobacter* (data from Spanevello & Patel, 2009; Tanaka et al., 2006; Yabe et al., 2009); 3,
362 *Sulfobacillus* (data obtained from Da Costa et al., 2009; Dufresne et al., 1996; Melamud et al., 2003); 4,
363 *Caldinitratiruptor* (data obtained from Fardeau et al., 2010) . +, growth; –, no growth; v, variable; N. D., not
364 determined.

365

	Limnochorda pilosa HC45^T	1	2	3	4
16S rRNA gene sequence similarity to strain HC45^T (%)	–	81–82	84–85	76–76.5	82
Gram stain	Negative	Negative	Negative/Positive	Positive	Negative
Major quinones	MK-7	MK-6	N. D.	MK-7	N. D.
DNA G+C content (%)	69	65–69	69–73	46–62	70
NaCl requirements	+	–	+/–	–	–
Temperature range /optimum(℃)	30–55/45–50	40–70/55–60	50–80/70–75	4–60[*1]/40–55	50–75/65
pH range/optimum	6.5–9/7	6–10/7.5	5–10/7–8.5	1.1–5.5/2–2.4	6.3–7.9/7
Major cellular fatty acid	iso-$C_{15:0}$, anteiso-$C_{15:0}$	$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$	iso-$C_{17:0}$, $C_{14:1}$	iso- and anteiso-branched fatty acids, ω-Cyclohexane fatty acids[*2]	N. D.

[*1] Spore germination of *Sulfobacillus disulfidooxisans* SD-11^T occured at 4℃.

[*2] ω-Cyclic fatty acids was not detected in *Sulfobacillus sibiricus* N1^T.

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