Meiothermus cateniformans sp. nov., a slightly thermophilic species from north-eastern China

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Two aerobic, non-motile and non-sporulating bacteria, strains LY1^T and L462, were recovered from a hot spring located in the Qianshan area of north-eastern China. The novel strains had Gram-negative cell walls and grew at 30–66 °C (optimum 55–60 °C) and pH 5.5–10.0 (optimum pH 8.0–8.5). 16S rRNA gene sequence similarity analysis revealed that strains LY1^T and L462 were most closely related to *Meiothermus ruber* ATCC 35948^T, *M. taiwanensis* WR-30^T and *M. cerbereus* GY-1^T, with 97.1–98.4 % sequence similarity. Phylogenetic analysis indicated that the new isolates represented a novel species by forming a distinctive lineage within genus *Meiothermus*, which was confirmed by DNA–DNA reassociation values. Strain LY1^T was obviously different from its closest relatives in a number of phenotypic characteristics, such as the inability to hydrolyse casein or to assimilate melibiose, trehalose, sucrose and lactose. Strain L462 was distinct in its ability to reduce nitrate. In addition, the novel strains were distinct in having larger amounts of anteiso-$C_{17:0}$ than their closest phylogenetic neighbours. On the basis of this polyphasic taxonomic characterization, the name *Meiothermus cateniformans* sp. nov. is proposed for the novel species, currently represented by the type strain LY1^T (=CGMCC 1.6990^T =JCM 15151^T) and strain L462 (=CGMCC 1.6989 =JCM 15150).

On the basis of phylogenetic, phenotypic and chemotaxonomic distinctiveness, the genus *Meiothermus* was created to accommodate some members of the genus *Thermus* (Nobre *et al.*, 1996). Six species of the genus *Meiothermus* have validly published names: *Meiothermus ruber* (Loginova *et al.*, 1984), *M. silvanus* and *M. chliarophilus* (Tenreiro *et al.*, 1995), *M. cerbereus* (Chung *et al.*, 1997), *M. taiwanensis* (Chen *et al.*, 2002b) and *M. timidus* (Pires *et al.*, 2005). A strain that shares 99.7 and 98.6 % 16S rRNA gene sequence similarity with the type strains of *M. ruber* and *M. taiwanensis* has been assigned to '*Meiothermus rosaceus*' (Chen *et al.*, 2002a), but this name has not been validly published.

In natural environments, *Meiothermus* strains have been found exclusively in thermal limnetic systems, predominantly in terrestrial hot springs (Pires *et al.*, 2005; da Costa *et al.*, 2006). Strains of *M. ruber* have been isolated from geothermal areas worldwide, even from man-made thermal environments, while the distribution of the other five *Meiothermus* species seems to be regional (Nobre & da Costa, 2001): for example, strains of *M. chliarophilus* and *M. taiwanensis* have been isolated only from hot springs in central Portugal and Taiwan, respectively. In this study, two strains, LY1^T and L462, were isolated from a hot spring located in the Qianshan area, Anshan, north-eastern China, where *Meiothermus* strains have never been reported before.

Enrichment and isolation were performed with *Thermus* medium (da Costa *et al.*, 2006). Water samples were filtered through membrane filters (0.22 μm), which were subsequently put into 50 ml flasks containing 20 ml *Thermus* medium. The preparations were incubated in a rotary water-bath shaker at 55 °C and 120 r.p.m. for 3 days. Turbid cultures were serially diluted and spread onto *Thermus* agar plates, which were then incubated at 55 °C until obvious colonies formed. Distinctive colonies were picked and purified at least five times before being preserved by freeze-drying. Purity of the isolates was verified by morphological examination of colonies and cells.

Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL). The temperature and pH ranges for growth were examined as described by Chung *et al.*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LY1^T and L462 are EU247891 and EU247890, respectively.

(1997) except that different buffers were used: MES (pH 5.0–6.5), MOPSO (pH 6.5–7.5), Tricine (pH 7.5–9.0) and CAPSO (pH 9.0–10.5).

Single-carbon-source assimilation tests were performed at 55 °C for 7 days as specified by Chung *et al.* (1997). Other physiological and biochemical characteristics were examined as described by Hudson *et al.* (1986), Manaia & da Costa (1991) and Chung *et al.* (1997) on *Thermus* agar plates or in *Thermus* medium at 55 °C for 4 days.

Cultures for polar lipid and lipoquinone analysis were incubated up to the exponential phase in 300 ml flasks containing 100 ml *Thermus* medium at 55 °C and 100 r.p.m. in a rotary water-bath shaker. Cells were collected and washed by centrifugation. Lipid extraction was carried out as described by Tindall (1991). Individual polar lipids were separated by one-dimensional TLC on silica gel 60 F_{254} plates (Merck) as described by Chung *et al.* (1997). Lipoquinones were extracted from freeze-dried cells and purified as described by Tindall (1989). Prepared lipoquinones were analysed with an Agilent 1200 HPLC equipped with a ZORBAX Eclipse XDB-C18 column (150 × 4.6 mm, particle size 5 μm; Agilent) (Chung *et al.*, 1997).

Cultures for fatty acid analysis were prepared as specified previously (Chung *et al.*, 1997). Fatty acid methyl esters (FAMEs) were obtained from freeze-dried cells as described by Kuykendall *et al.* (1988) except that several drops of saturated NaCl solution were added together with 0.3 M NaOH at the final step of extraction. Identification and quantification of the FAMEs, as well as numerical analysis of fatty acid profiles, were performed automatically by using the Sherlock Microbial Identification System with the standard MIS Library Generation software (MIDI, Inc.).

Genomic DNA used in PCR amplification was extracted as described by Rainey *et al.* (1996). The 16S rRNA gene was amplified with primers 27F (5′-AGAGTTTGATCC-TGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTG-TTACGACTT-3′). Sequences comprising unambiguous nucleotides between positions 28 and 1491 (Brosius *et al.*, 1978) were compared with all closely related sequences with the EzTaxon service (Chun *et al.*, 2007). Multiple sequence alignment was performed with CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with MEGA4 (Tamura *et al.*, 2007) and by the maximum-parsimony method (Fitch, 1971) with PHYLIP version 3.6 (Felsenstein, 1993).

The DNA G+C content was determined by the thermal denaturation temperature (T_m) method (Marmur & Doty, 1962) with *Escherichia coli* K-12 DNA as the calibration standard. DNA–DNA hybridization was performed with the thermal denaturation and renaturation method described by De Ley *et al.* (1970) and modified by Huß *et al.* (1983) using a Beckman DU800 spectrophotometer.

Following enrichment and isolation, six pigmented strains were recovered from a hot spring located in the Qianshan area in north-eastern China. On the basis of 16S rRNA gene sequence similarity, one strain, which was yellow-pigmented, was found to be affiliated with *Thermus igniterrae* (100 % similarity to the type strain) and three were members of *M. ruber* (99.8–100 % similarity to the type strain). The remaining two, strains LY1^T and L462, were considered to represent a potential novel *Meiothermus* species and therefore were selected for further study.

Strains LY1^T and L462 were rose- and orange-pigmented, respectively. Cells were short, non-motile and non-sporulating rods. After the end of the exponential phase, cells tended to occur in chains (Fig. 1). Gram-staining was negative. Thin-section electron micrographs also showed a typical Gram-negative cell wall structure, which consisted of a thin, electron-dense inner layer representing the peptidoglycan and a thick outer layer that connected with the former (not shown). Both of the new isolates grew at 30–66 °C and pH 5.5–10.0, with optimum growth at 55–60 °C and pH 8.0–8.5. Strain LY1^T grew well at 67 °C; however, no growth occurred at 68 °C.

Detailed results of physiological and biochemical examination are summarized in Table 1. Strain LY1^T was different from the three reference strains and even from strain L462 in physiological and biochemical characteristics. Among the five tested strains, LY1^T was the only strain that could not hydrolyse casein or assimilate sucrose, lactose, trehalose and

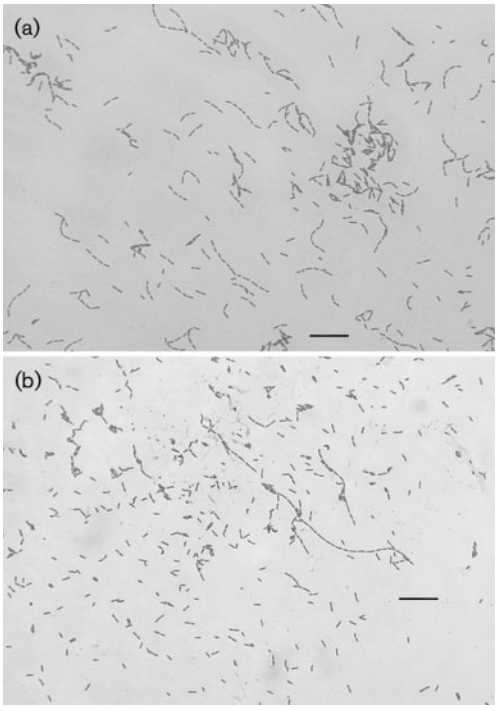

Fig. 1. Cell morphology of strains LY1^T (a) and L462 (b) grown in *Thermus* medium at 55 °C and pH 8.2. Bars, 10 μm.

Table 1. Phenotypic and genotypic characteristics of the novel isolates and the type strains of related *Meiothermus* species

Strains: 1, LY1^T; 2, L462; 3, *M. ruber* DSM 1279^T; 4, *M. taiwanensis* DSM 14542^T; 5, *M. cerbereus* DSM 11376^T. Data were taken from this study. All strains produced β-galactosidase, hydrolysed aesculin, DNA and gelatin and utilized L-arginine, L-asparagine, D-fructose, D-galactose, D-glucose, maltose, D-mannose, L-proline and pyruvate. None of the strains hydrolysed starch or xylan or utilized L-arabinose, citrate, raffinose, L-rhamnose or ribitol. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4	5
Pigmentation	Rose	Orange	Light red	Red	Red–orange
Catalase	+	+	+	+	−
Oxidase	+	+	+	+	W
Reduction of nitrate	−	+	−	−	−
Hydrolysis of casein	−	+	+	+	+
Utilization of:					
Cellobiose	W	+	+	+	+
L-Glutamate	+	+	+	W	+
L-Glutamine	−	−	+	+	−
Glycerol	−	+	+	+	−
myo-Inositol	−	+	−	+	−
Lactose	−	+	+	+	+
Malate	−	−	+	−	−
Mannitol	+	+	+	+	−
Melibiose	−	+	+	+	+
L-Serine	+	+	+	−	−
Sorbitol	+	+	+	+	−
Succinate	−	+	+	−	−
Sucrose	−	+	+	+	+
Trehalose	−	+	+	+	+
D-Xylose	+	+	+	+	−
DNA G+C content (mol%)	61.5	60.8	61.8	62.1	60.1
DNA–DNA reassociation (%) with:					
LY1^T	(100)	81.1	59.4	56.7	54.6
L462	81.1	(100)	60.9	60.6	57.5

melibiose. Strain L462 was the only strain that could reduce nitrate to nitrite and was more similar to *M. ruber* DSM 1279^T and *M. taiwanensis* DSM 14542^T than to strain LY1^T.

Polar lipids of strains LY1^T and L462 were composed of one prominent phospholipid and two prominent glycolipids (not shown), which is the typical pattern of the genus *Meiothermus*. Menaquinone 8 (MK-8) was the predominant respiratory lipoquinone of the isolates, as reported for all other members of genus *Meiothermus*. The fatty acid compositions of strains LY1^T and L462 and other related *Meiothermus* strains are shown in Table 2. For all tested strains, iso- and anteiso-branched $C_{15:0}$ and $C_{17:0}$ dominated the fatty acid composition, and iso-branched 2-hydroxy fatty acids were present in moderate amounts, which is another common chemotaxonomic characteristic of the genus *Meiothermus* (Nobre & da Costa, 2001; da Costa *et al.*, 2006). Nevertheless, anteiso-$C_{17:0}$ was present in larger amounts in strains LY1^T and L462 than in the three reference strains.

Almost-complete 16S rRNA gene sequences comprising 1455 nucleotides were determined for strains LY1^T and L462. Similarity analysis based on these and other sequences revealed that the two strains both belonged to genus *Meiothermus*. The 16S rRNA gene sequences of the isolates were identical and showed highest sequence similarity with *M. ruber* ATCC 35948^T, *M. taiwanensis* WR-30^T and *M. cerbereus* GY-1^T (98.4, 98.4 and 97.1 %, respectively). Although these values were appreciably high, it is known that the three species of the *M. ruber* clade are closely related to each other (97.4–98.7 % similarity; Chen *et al.*, 2002b; Chung *et al.*, 1997). Sequence similarity of strains LY1^T and L462 to the other *Meiothermus* species was lower (89.1–91.0 %) and was comparable to the similarity between species of the *M. ruber* clade and other *Meiothermus* species (88.4–91.4 %; Pires *et al.*, 2005; da Costa *et al.*, 2006). In the phylogenetic analysis (Fig. 2), strains LY1^T and L462 formed a sister lineage next to the *M. ruber* clade with a high bootstrap value and clearly represented a novel species within the genus *Meiothermus*. As strains LY1^T and L462 had comparably high sequence similarity with members of the *M. ruber* clade, they were considered to represent a new member of this cluster that diverged early.

The DNA G+C contents of strains LY1^T and L462 were 61.5 and 60.8 mol% (T_m), respectively. DNA–DNA reassociation values between the two isolates and *M. ruber* DSM 1279^T, *M. taiwanensis* DSM 14542^T and *M. cerbereus* DSM 11376^T are shown in Table 1. Strains LY1^T and L462

Table 2. Fatty acid compositions of the novel isolates and the type strains of related *Meiothermus* species

Strains: 1, LY1^T; 2, L462; 3, *M. ruber* DSM 1279^T; 4, *M. taiwanensis* DSM 14542^T; 5, *M. cerbereus* DSM 11376^T. Data were taken from this study. Thiosulfate was not added for the cultivation of *M. cerbereus* DSM 11376^T. Values are percentages of total fatty acids. Fatty acids amounting to less than 0.5 % in all strains are omitted; −, not detected (<0.5 %).

Fatty acid	1	2	3	4	5
iso-$C_{13:0}$	−	−	−	0.5	0.8
iso-$C_{13:0}$ 3-OH	1.2	1.0	0.8	1.4	−
iso-$C_{14:0}$	0.9	0.5	−	−	1.9
$C_{15:0}$	1.9	1.3	0.9	1.9	1.4
iso-$C_{15:0}$	28.9	30.2	30.9	37.2	34.7
anteiso-$C_{15:0}$	6.4	9.8	1.9	1.9	10.1
iso-$C_{15:0}$ 3-OH	−	−	−	−	0.8
iso-$C_{15:1}$ F	−	−	1.3	−	2.7
$C_{16:0}$	5.0	4.5	5.1	3.5	4.8
iso-$C_{16:0}$	5.4	2.8	1.5	2.7	4.6
$C_{16:0}$ 2-OH	0.5	0.6	0.8	0.7	1.0
$C_{17:0}$	3.0	1.4	0.9	2.8	−
iso-$C_{17:0}$	27.6	21.8	26.9	29.9	9.5
anteiso-$C_{17:0}$	8.8	11.0	3.0	2.7	4.6
$C_{17:0}$ 2-OH	0.5	0.5	−	0.5	0.9
iso-$C_{17:0}$ 2-OH	4.5	7.5	10.8	9.3	3.5
iso-$C_{17:0}$ 3-OH	0.7	0.7	0.8	0.5	3.6
$C_{17:1}\omega6c$	−	−	−	−	0.6
$C_{17:1}\omega8c$	−	−	0.5	−	0.5
iso-$C_{17:1}\omega9c$	−	0.7	6.6	0.9	5.2
anteiso-$C_{17:1}\omega9c$	−	−	−	−	0.8
iso-$C_{18:0}$	0.6	−	−	−	−
iso-$C_{18:0}$ diol	1.0	0.7	1.0	1.7	−
iso-$C_{19:0}$	0.7	−	−	0.6	−

had high DNA–DNA reassociation values with each other, which indicated that they represented the same species even though these two strains had obvious differences in physiological and biochemical characteristics. DNA–DNA reassociation between strain LY1^T and the reference strains was 54.6–59.4 %, while that between strain L462 and the reference strains was 57.5–60.9 %. These data together support the conclusion from the phylogenetic analysis that the isolates represent a novel species of genus *Meiothermus*.

A number of phenotypic differences could be found between the new isolates and the members of the *M. ruber* group. For strain LY1^T, its inability to hydrolyse casein and to utilize melibiose, trehalose, sucrose and lactose clearly differentiate it from the *M. ruber* group. For strain L462, although it was more similar to *M. ruber* DSM 1279^T and *M. taiwanensis* DSM 14542^T than to strain LY1^T in physiological and biochemical characteristics, phenotypic differences, especially the ability to reduce nitrate, distinguish it from the *M. ruber* group. The major distinguishing chemotaxonomic characteristic was the relatively large amount of anteiso-$C_{17:0}$ in strains LY1^T and L462.

On the basis of the genotypic and phenotypic characteristics described above, strains LY1^T and L462 are proposed to represent a novel species of genus *Meiothermus*, with the name *Meiothermus cateniformans* sp. nov.

Description of *Meiothermus cateniformans* sp. nov.

Meiothermus cateniformans (ca.te.ni.for′mans. L. n. *catena* chain; L. part. adj. *formans* forming, fashioning; N.L. part. adj. *cateniformans* chain-forming, referring to the fact that cells occur in chains after the end of the exponential growth phase).

Cells are non-motile, non-sporulating, short rods, about 0.5 μm wide and 1.0–2.5 μm long, and form chains after exponential growth. Gram-negative. After growth on *Thermus* agar for 72 h at 55 °C, colonies of strain LY1^T are rose-pigmented and 3–4 mm in diameter while those of strain L462 are orange. Optimal growth occurs at 55–60 °C and pH 8.0–8.5. The pH range for growth is pH 5.5–10.0. No growth occurs below 30 °C. The maximum growth temperature of strain LY1^T is 67 °C while that of strain L462 is 66 °C. Aerobic and heterotrophic. Catalase-, oxidase- and β-galactosidase-positive. Aesculin, DNA and gelatin are hydrolysed. Starch and xylan are not degraded. Casein is not hydrolysed by the type strain. Nitrate is reduced by strain L462. The following substrates are utilized: L-arginine, L-asparagine, cellobiose, D-fructose, D-galactose, D-glucose, L-glutamate, maltose, mannitol, D-mannose, L-proline, pyruvate, L-serine, sorbitol and D-xylose. The following compounds are not utilized by the type strain: L-arabinose, citrate, L-glutamine, glycerol, *myo*-inositol, lactose, malate, melibiose, raffinose, L-rhamnose,

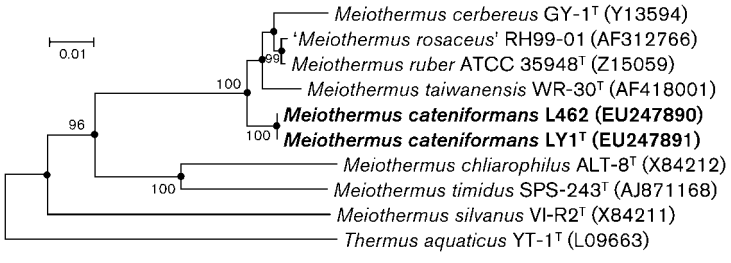

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolates and related taxa. Bootstrap values (>50 %) based on 1000 replicates are shown as percentages at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.01 substitutions per nucleotide position.

ribitol, succinate, sucrose and trehalose. The major fatty acids are iso- and anteiso-branched $C_{15:0}$ and $C_{17:0}$. Iso-branched 2-hydroxy and 3-hydroxy fatty acids are also present. The DNA G+C content of the type strain LY1^T is 61.5 mol% and that of L462 is 60.8 mol% (T_m).

The type strain LY1^T (=CGMCC 1.6990^T =JCM 15151^T) and reference strain L462 (=CGMCC 1.6989 =JCM 15150) were isolated from a hot spring located in the Qianshan area, Anshan, north-eastern China.

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