Meiothermus hypogaeus sp. nov., a moderately thermophilic bacterium isolated from a hot spring

Koji Mori,¹ Takao Iino,¹ Jun-ichiro Ishibashi,² Hiroyuki Kimura,³
Moriyuki Hamada¹ and Ken-ichiro Suzuki¹

Correspondence

Koji Mori
mori-koji@nite.go.jp

¹NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan

²Faculty of Science, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan

³Faculty of Science, Shizuoka University, 836 Oya, Suruga-ku, Shizuoka 422-8529, Japan

A novel moderately thermophilic, red-pigmented bacterium, designated strain AZM34c11^T, was isolated from the bottom of a 1000 m-deep drilled well located in a Japanese hot spring. Cells were Gram-negative and grew optimally at 50 °C, at pH 7.6 and with 0–0.3% (w/v) NaCl. Analysis of the 16S rRNA gene sequence revealed that the isolate belonged to the genus *Meiothermus*. Levels of 16S rRNA gene sequence similarity between strain AZM34c11^T and the type strains of recognized *Meiothermus* species were 88.2–94.8%. Strain AZM34c11^T was distinguished from recognized *Meiothermus* species by its cellular fatty acid profile: iso-C_{16:0} was one of the predominant components and hydroxy fatty acids were present only in trace amounts. The strain could also be differentiated based on its growth substrate preferences and characteristic enzyme reactions. On the basis of these results, strain AZM34c11^T is considered to represent a novel species of the genus *Meiothermus*, for which the name *Meiothermus hypogaeus* sp. nov. is proposed. The type strain is AZM34c11^T (=NBRC 106114^T =DSM 23238^T).

The genus *Meiothermus* comprises strictly aerobic, non-halophilic and moderately thermophilic heterotrophs. The first species to be described was *Meiothermus ruber*, isolated from the Kamchatka Peninsula (Loginova *et al.*, 1984). *M. ruber*, *Meiothermus silvanus* and *Meiothermus chliarophilus* were originally described as members of the genus *Thermus* (Tenreiro *et al.*, 1995). However, based on differences in optimal growth temperature range, 2-hydroxy fatty acid content and 16S rRNA gene sequences, they were later reclassified within a separate genus (Nobre *et al.*, 1996). At the time of writing, the genus *Meiothermus* comprises nine recognized species (Albuquerque *et al.*, 2009, 2010; Chen *et al.*, 2002; Chung *et al.*, 1997; Pires *et al.*, 2005; Zhang *et al.*, 2010). Many strains belonging to the genus *Meiothermus* have been isolated from various natural geothermal samples as well as from man-made environments (Ekman *et al.*, 2007). The phenotypic diversity has gradually increased with the description of additional isolates of the genus *Meiothermus*: *Meiothermus cerbereus*

and some strains of *M. silvanus* require reduced sulfur compounds for growth in liquid medium (Chung *et al.*, 1997; Nobre & da Costa, 2001), *Meiothermus rufus* and *Meiothermus granaticius* lack cellular hydroxy fatty acids, which are present in other species (Albuquerque *et al.*, 2009, 2010), and *M. chliarophilus* and *Meiothermus timidus* form yellow-pigmented colonies, whereas other species form red-pigmented colonies (Pires *et al.*, 2005; Tenreiro *et al.*, 1995). With regard to 16S rRNA gene sequence divergence, phylogenetic analyses indicated that *M. silvanus*, *M. chliarophilus* and *M. timidus* are distantly related to other species of the genus, including the type species *M. ruber*. This might suggest placing them in a separate genus, but there are no suitable phenotypic differences to separate the two groups (Nobre & da Costa, 2001; Nobre *et al.*, 1996).

We isolated a novel moderately thermophilic, aerobic, *Meiothermus*-like bacterium from a water sample collected from the bottom of a 1000 m-deep drilled well in Hakuba-Himekawa hot spring located in Nagano Prefecture, Japan. The water sample was collected on the surface in June 2008 and its temperature and pH were 48 °C and 7.5. To enrich and isolate bacteria, we used AP13YO liquid medium (approx. pH 7), i.e. AP13 basal medium supplemented with (per litre) 0.1 g Bacto yeast extract (Difco) under a N₂/CO₂/O₂ [70:20:10 (v/v); 150 kPa] atmosphere. AP13 basal medium had the following composition (per litre):

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain AZM34c11^T and *Meiothermus taiwanensis* NBRC 105868^T (A and B genes) are AB586707, AB586708 and AB586709, respectively.

A supplementary table is available with the online version of this paper.

0.05 g K_2HPO_4 , 0.09 g KH_2PO_4 , 0.25 g $MgSO_4 \cdot 7H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.25 g NH_4Cl , 1 ml trace element solution (Mori *et al.*, 2008a) and 0.25 g Na_2CO_3 . The medium was prepared in serum bottles with butyl-rubber stoppers and aluminium caps under $N_2/CO_2/O_2$ and then autoclaved. For enrichment and routine cultivation, 50 ml serum bottles containing 20 ml liquid medium were used. For primary enrichment of bacteria, 1 ml hot-spring water was inoculated into 20 ml AP13YO medium and incubated at 50 °C. After 1 week of incubation, bacterial growth was confirmed by microscopy and the enrichment culture was repeatedly transferred to fresh AP13YO medium. For single strain isolation, colonies were allowed to form on Gelrite plates prepared with the same medium amended with 1 g $MgCl_2 \cdot 6H_2O$ l^{-1} and 0.6 % (w/v) gellan gum. After 1 week, uniform red colonies (approx. 0.5 mm in diameter) were observed and a single colony was picked and purified by repeating the isolation procedure several times. Strain AZM34c11^T was isolated successfully from the enriched sample. After the isolation procedure, we tested the growth of strain AZM34c11^T on *Thermus* medium (Castenholz, 1969) supplemented with (per litre) 1 g Bacto yeast extract, 1 g Bacto tryptone (Difco) and 1 g sodium glutamate to raise the growth yield, and found positive results: red colonies formed on this agar plate after 1 week of incubation at 50 °C.

Cells of strain AZM34c11^T were Gram-negative rods (0.4 µm wide and 1.7–10.0 µm long). Spore formation and motility were not observed. Cells were catalase-negative and oxidase-positive (Tamaki *et al.*, 2003).

Strain AZM34c11^T was unable to grow in the presence of nitrate (10 mM), nitrite (2 and 5 mM), fumarate (10 mM), thiosulfate (10 mM), sulfate (10 mM), sulfite (2 and 5 mM) or elemental sulfur (2 %) as an elemental acceptor in the AP13 medium with (per litre) 0.1 g Bacto yeast extract and 3.4 g sucrose under N_2/CO_2 [80:20 (v/v); 150 kPa]. Accordingly, strain AZM34c11^T was considered to be strictly aerobic, and neither fermentative growth nor respiration was observed under anaerobic conditions. Nitrate was reduced to nitrite (Dobrogosz, 1981), although no cell growth occurred. A reduced sulfur compound (thiosulfate) was not required for growth of strain AZM34c11^T in the liquid medium. Yeast extract (0.1 g l^{-1}) was necessary for growth. Strain AZM34c11^T utilized several single carbon sources for growth (Table 1). The optimum temperature, pH and NaCl concentration ranges for growth in *Thermus* medium with (per litre) 1 g Bacto yeast extract, 1 g Bacto tryptone and 3.4 g sucrose were determined by examining the time course of optical density (temperature gradient incubator with a bio-photorecorder, model TN-2612; ADVANTEC). The experiments were performed in duplicate. Strain AZM34c11^T was able to grow at 35–57 °C, with optimum growth around 50 °C. The pH range for growth was pH 5.9–8.7, with optimum growth at pH 7.6. The isolate grew optimally at NaCl concentrations of 0–0.3 % (w/v), and no growth was observed above 0.6 % NaCl.

Hydrolysis of casein, starch (Manaia & da Costa, 1991) and gelatin (Smibert & Krieg, 1981) and degradation of arbutin, aesculin (Manaia & da Costa, 1991), elastin, hide-powder azure and fibrin (Hudson *et al.*, 1986) were tested by using *Thermus* medium. Strain AZM34c11^T was positive for hydrolysis of starch and gelatin and for the degradation of hide-powder azure, but was negative for the other reactions. Additional enzyme activities were tested and identified by using the API ZYM system (bioMérieux) at 50 °C; the strain showed positive reactions for several enzymes.

Respiratory quinones were extracted from cells of strain AZM34c11^T by using the protocol described by Nakagawa & Yamasato (1993) and analysed with an LCMS-QP 8000alpha spectrometer (Shimadzu). This respiratory quinone was menaquinone-8, which has been found in all described members of the phylum *Deinococcus–Thermus*. Cellular fatty acids of strain AZM34c11^T grown at 50 °C were prepared by using the method of Sasser (1990) and were analysed with the MIDI microbial identification system (Microbial ID; Agilent Technologies); *M. cerbereus* NBRC 106473^T, *M. granaticus* NBRC 107808^T and *M. rufus* NBRC 107809^T were also analysed by using the same procedure. Strain AZM34c11^T possessed mainly iso- and anteiso-branched fatty acids (see Supplementary Table S1, available in IJSEM Online); 2- and 3-hydroxy fatty acids were detected in very minor amounts. The polar lipids of strain AZM34c11^T, *M. cerbereus* NBRC 106473^T, *M. granaticus* NBRC 107808^T and *M. rufus* NBRC 107809^T were extracted and examined by two-dimensional TLC by using a previously described method (Hamada *et al.*, 2010), and the patterns were visualized by spraying the TLC plates with 5 % phosphomolybdic acid, Dittmer–Lester reagent (Dittmer & Lester, 1964) and anisaldehyde. The polar lipid pattern of strain AZM34c11^T mainly comprised one phospholipid and one glycolipid, designated PL-2 and GL-1b, respectively (Nobre & da Costa, 2001), as with the profiles of *M. rufus* NBRC 107809^T and *M. granaticus* NBRC 107808^T, but in contrast to that of *M. cerbereus* NBRC 106473^T, which contained two glycolipids (not shown). The genomic DNA G + C content was determined by HPLC with a Shodex ODS pack F-411 (Showa Denko K.K.), after an initial nuclease P1 treatment by using a DNA-GC kit (Yamasa Shoyu) followed by alkaline phosphatase treatment (Kamagata & Mikami, 1991). The DNA G + C content of strain AZM34c11^T was 63.4 mol%.

The almost-complete 16S rRNA gene sequence of strain AZM34c11^T was determined by using a previously reported procedure (Hattori *et al.*, 2000). After alignment with the ARB program version 5.2 (Ludwig *et al.*, 2004) and the dataset version ssu_jan04.arb, the aligned data were extracted by using the ARB default filter (deinococcus_thermus_rr5_dec04; 1369 positions) and phylogenetic trees were reconstructed with the neighbour-joining (NJ) method via the CLUSTAL X program version 1.83 (Saitou & Nei, 1987; Thompson *et al.*, 1997) and with the maximum-likelihood (ML) method via the MOLPHY program version 2.3b3

Table 1. Differential characteristics between strain AZM34c11^T and recognized *Meiothermus* species

Taxa: 1, strain AZM34c11^T; 2, *M. ruber*; 3, *M. silvanus*; 4, *M. chliarophilus*; 5, *M. cerbereus*; 6, *M. taiwanensis*; 7, *M. timidus*; 8, *M. cateniformans*; 9, *M. rufus*; 10, *M. granaticius*. Data for recognized *Meiothermus* species were from Nobre & da Costa (2001), Tenreiro *et al.* (1995), Chung *et al.* (1997), Chen *et al.* (2002), Pires *et al.* (2005), Zhang *et al.* (2010) and Albuquerque *et al.* (2009, 2010). All taxa were positive for oxidase and utilization of D-glucose and D-fructose. $\pm$, Variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Pigmentation	Red	Red	Red	Yellow	Red	Red	Yellow	Red	Red	Red
Optimum growth temperature (°C)	50	60	55	50	55	55–60	55–60	55–60	55–60	45–50
Growth with 1% NaCl	–	+	–	+	+	+	+	ND	–	–
Reduction of nitrate to nitrite	+	–	+	+	–	–	+	$\pm$	+	+
Genomic DNA G+C content (mol%)*	63.4 ^a	66.0 ^b	63.6 ^a	69.9 ^a	60.9 ^a	61.9 ^a	65.1 ^a	60.8–61.5 ^b	64.1 ^a	63.5 ^a
Requirement for reduced sulfur compounds for growth in liquid medium	–	–	$\pm$	–	+	ND	ND	ND	ND	ND
Enzyme activity										
Catalase	–	+	–	–	–	+	+	+	+	+
α -Galactosidase	–	+	–	+	+	+	ND	ND	–	+
Hydrolysis of:										
Elastin	–	+	+	+	+	+	+	ND	+	ND
Fibrin	–	+	–	+	+	+	ND	ND	ND	ND
Starch	+	–	+	+	–	–	+	–	+	+
Casein	–	+	+	+	+	+	+	–	+	+
Gelatin	+	+	+	+	+	+	ND	+	+	+
Arbutin	–	+	+	+	+	ND	ND	ND	+	+
Aesculin	–	+	ND	+	+	+	ND	+	+	+
Utilization of growth substrate										
Lactose	+	+	+	+	+	+	ND	–	+	+
Trehalose	+	+	–	+	+	–	+	–	+	+
Sucrose	+	+	–	+	+	+	+	–	+	+
Cellobiose	+	+	–	+	+	+	+	+	+	+
Raffinose	–	+	–	+	–	+	+	–	–	–
D-Arabinose	–	–	–	–	–	+	+	–	+	–
L-Rhamnose	–	–	–	–	–	+	–	–	–	–
D-Xylose	+	+	+	+	–	+	+	+	+	+
Ribitol	–	–	+	–	–	+	–	–	–	–
D-Mannitol	–	+	+	+	–	+	+	+	+	+
D-Sorbitol	–	+	+	+	–	+	+	+	+	–
Glycerol	+	+	–	+	–	–	–	–	–	–
Pyruvate	+	–	+	+	+	+	+	+	+	–
Succinate	–	+	–	–	–	+	+	–	+	+
Malate	–	+	+	–	–	–	+	–	+	+
myo-Inositol	–	+	–	–	–	+	–	–	–	+
L-Asparagine	+	+	+	+	–	+	+	+	+	–
L-Glutamate	+	+	+	+	+	+	ND	+	+	–
L-Glutamine	–	+	+	+	–	+	+	–	+	+
L-Serine	–	+	+	+	–	–	+	+	–	–
L-Proline	–	+	+	+	+	+	+	+	–	–
L-Arginine	–	+	+	+	–	+	+	+	+	–

*Determined by HPLC (a) or T_m (b).

(Adachi & Hasegawa, 1995; Hasegawa *et al.*, 1985; Mori *et al.*, 2008b). The sequence determined indicated that strain AZM34c11^T belonged to the genus *Meiothermus* in the family *Thermaceae*. The NJ tree constructed on the basis of the 16S rRNA gene sequence of the new isolate and those of related strains is shown in Fig. 1. The result obtained with the

ML method was similar to that obtained with the NJ method (not shown). Levels of 16S rRNA gene sequence similarity calculated by the CLUSTAL X program between strain AZM34c11^T and the type strains of recognized *Meiothermus* species were as follows: *M. ruber*, 93.7%; *M. silvanus*, 88.4%; *M. chliarophilus*, 88.2%; *M. cerbereus*, 94.8%;

Fig. 1. NJ phylogenetic tree based on 16S rRNA gene sequences of strain AZM34c11^T and relatives. Bootstrap percentages $\geq 50\%$ are indicated at branch points (NJ/ML); –, $< 50\%$. GenBank/EMBL/DBJ accession numbers are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.

Meiothermus taiwanensis, 93.2 and 93.7 % (see below); *M. timidus*, 90.9 %; *Meiothermus cateniformans*, 93.2 %; *M. rufus*, 92.9 %; and *M. granaticius*, 94.8 %.

The 16S rRNA gene sequence of *M. taiwanensis* NBRC 105868^T was also determined by direct sequencing as described above, but a single sequence could not be determined and some positions were indeterminate. Therefore, PCR products retrieved from single colonies of *M. taiwanensis* NBRC 105868^T were cloned by using the TOPO TA Cloning kit (Invitrogen), and the sequences were then determined. As a result, two different 16S rRNA gene sequences were obtained for *M. taiwanensis* NBRC 105868^T and the sequence similarity between them was 98.9 %. The phylogenetic positions of the two sequences, A and B, in the genus *Meiothermus* are shown in Fig. 1. Both the sequences obtained in our study differed in several nucleotides from the previously registered sequence (AF418001) and, phylogenetically, they formed a complex relationship with *M. ruber* and *M. cateniformans*. The high levels of DNA–DNA relatedness among the type strains of the three species (Zhang *et al.*, 2010) support our results. However, similar results were not obtained with the 16S rRNA gene sequence of *M. ruber* NBRC 106122^T in the present study (not shown).

The phylogenetic analysis performed based on 16S rRNA gene sequences indicated that strain AZM34c11^T belonged to the genus *Meiothermus*. The 16S rRNA gene sequence of strain AZM34c11^T was different from those of all recognized species of the genus *Meiothermus*, and levels of similarity between this strain and the type strains of *Meiothermus* species were less than 95 %. Differential characteristics between strain AZM34c11^T and recognized *Meiothermus* species are summarized in Table 1. Strain AZM34c11^T exhibited several phenotypic and chemotaxonomic characters such as pigmentation, optimum growth temperature, DNA G + C content and respiratory quinone content that are typical of the genus *Meiothermus*. However, the characteristics did not match completely with those of recognized *Meiothermus* species. In particular, the utilization of sugars and amino acids as single carbon and energy sources was clearly different. The cellular fatty acid composition of strain AZM34c11^T was similar to those of recognized *Meiothermus* species: iso-branched and anteiso-branched fatty acids were

the predominant components (Supplementary Table S1). However, the amount of hydroxy fatty acids in strain AZM34c11^T was very low, which is a feature seen only in *M. rufus* and *M. granaticius* in the entire genus *Meiothermus* (Albuquerque *et al.*, 2009, 2010). In addition, iso-C_{16:0} was present in larger quantities in strain AZM34c11^T than in recognized species of the genus *Meiothermus*. These phenotypic differences support the phylogenetically distinct position of strain AZM34c11^T in the genus *Meiothermus*. Based on the data presented above, we suggest that strain AZM34c11^T represents a novel species of the genus *Meiothermus*, for which the name *Meiothermus hypogaeus* sp. nov. is proposed.

As reported by some researchers (Nobre & da Costa, 2001; Nobre *et al.*, 1996; Pires *et al.*, 2005), phylogenetic analysis based on 16S rRNA gene sequences indicated that *M. silvanus*, *M. chliarophilus* and *M. timidus* were distant from the other *Meiothermus* species at a high replicate/resampling value (Fig. 1) and levels of sequence similarity between the type strains of the three species and other *Meiothermus* species were less than 91.5 %. Although it may be reasonable to erect a new genus for the three species based on phylogenetic analysis, this is not supported by phenotypic and chemotaxonomic characteristics. The isolation of additional novel representatives related to *M. silvanus*, *M. chliarophilus* and *M. timidus* will be important in this regard.

Description of *Meiothermus hypogaeus* sp. nov.

Meiothermus hypogaeus (hy.po.gae'us. L. masc. adj. *hypogaeus* underground, living under the ground).

Cells are rod-shaped (0.4 μ m wide and 1.7–10.0 μ m long), red-pigmented, non-motile and non-sporulating. Gram reaction is negative. Catalase-negative and oxidase-positive. Nitrate is reduced to nitrite. Positive for hydrolysis of starch and gelatin and degradation of hide-powder azure. Negative for hydrolysis of casein and degradation of arbutin, aesculin, elastin and fibrin. API ZYM tests were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase, but negative for lipase (C14), leucine

arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Cells are aerobic and chemoheterotrophic, requiring yeast extract for growth. Sulfur compounds are not required for growth in liquid medium. Utilizes D-glucose, D-fructose, D-mannose, D-galactose, melibiose, maltose, lactose, trehalose, sucrose, cellobiose, D-xylose, glycerol, pyruvate, L-asparagine and L-glutamate as carbon and energy sources, but not raffinose, D-arabinose, L-rhamnose, ribitol, D-mannitol, D-sorbitol, citrate, succinate, malate, *myo*-inositol, L-glutamine, L-serine, L-proline or L-arginine. Grows at 35–57 °C; optimum growth at 50 °C. The pH range for growth is 5.9–8.7, with optimum growth at pH 7.6. Grows optimally with 0–0.3 % (w/v) NaCl; no growth occurs with more than 0.6 % (w/v) NaCl. Menaquinone-8 is the major respiratory quinone. The major cellular fatty acids are iso-C_{15:0} and iso-C_{16:0}. The major polar lipids are PL-2 and GL-1b. The genomic DNA G + C content of the type strain is 63.4 mol%.

The type strain, AZM34c11^T (=NBRC 106114^T =DSM 23238^T), was isolated from hot-spring water collected from the bottom of a 1000 m-deep drilled well in Hakuba-Himekawa, Nagano Prefecture, Japan.

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