Extremophiles (2005) 9:81–84
DOI 10.1007/s00792-004-0419-y

NOTE

Norio Kurosawa · Yuko H. Itoh · Takashi Itoh

# *Thermus kawarayensis* sp. nov., a new member of the genus *Thermus*, isolated from Japanese hot springs

Received: 26 April 2004 / Accepted: 20 August 2004 / Published online: 17 September 2004


**Abstract** A long-rod-shaped thermophilic microorganism, strain KW11, was isolated from a hot springs located in the Kawarayu, Gunma, Japan. Cloning and preliminary sequence analysis of 16S rDNA showed that this isolate belongs to the genus *Thermus*. The cells were 10–20 μm long, about 0.8 μm in diameter, and produced no pigment in contrast with most of the *Thermus* species previously reported. KW11 was an aerobic heterotroph and grew at temperatures ranging from 40–73°C, with optimal growth occurring at 68°C. The pH range for growth was from 5.8–8.9, with optimal growth around pH 7. KW11 was sensitive to ampicillin, penicillin G, kanamycin, and streptomycin. The G+C content of DNA was 69 mol%. The main fatty acids were 16:0 (52.9%), iso-15:0 (22.1%), and iso-17:0 (15.6%). The 16S rDNA sequence of KW11 showed 96.0, 95.8, and 95.4% similarity with the sequences of *T. aquaticus*, *T. igniterrae*, and *T. thermophilus*, respectively, and less than 95% with other *Thermus* species. The physiological differences and phylogenetic evidence indicated that strain KW11 represents *T. kawarayensis*, a novel species of the genus *Thermus*. The type strain is isolate KW11$^T$ (JCM12314, DSM16200).

Communicated by K. Horikoshi

N. Kurosawa (✉)
Department of Environmental Engineering for Symbiosis,
Faculty of Engineering, Soka University,
1-236 Tangi-cho, Hachioji, Tokyo 192-8577,
Japan
E-mail: kurosawa@t.soka.ac.jp
Tel.: +81-426-918175
Fax: +81-426-918175

Y. H. Itoh
Department of Bioinformatics,
Faculty of Engineering, Soka University,
1-236 Tangi-cho, Hachioji, Tokyo 192-8577,
Japan

T. Itoh
Japan Collection of Microorganisms,
RIKEN (The Institute of Physical and Chemical Research),
2-1 Hirosawa, Wako, Saitama 351-0198, Japan

**Keywords** Hot springs · *Kawarayensis* · Novel species · 16S rDNA · *Thermus*

Since *Thermus aquaticus* was described by Brock and Freeze (1969), many *Thermus* isolates (which have reached over 50 strains) have been isolated from thermal environments from all over the world. However, only eight species have been described validly, because most of the isolates are very closely phylogenetically related to one another and belong to the one of the species previously described as follows: *T. antranikianii* (Chung et al. 2000), *T. aquaticus* (Brock and Freeze 1969), *T. brokianus* (Williams et al. 1995), *T. filiformis* (Hudson et al. 1987), *T. igniterrae* (Chung et al. 2000), *T. oshimai* (Williams et al. 1996), *T. scotoductus* (Kristjansson et al. 1994), and *T. thermophilus* (Oshima and Imahori 1974; Williams et al. 1995). Japan has many hot springs, which have a wide variety of chemical compositions and are suitable places for the isolation of thermophilic microorganisms. However, only *T. thermophilus* has been validated as a member of the genus *Thermus* from Japanese hot springs. During a recent attempt to discover novel thermophilic microorganisms from Japanese hot springs, we isolated a member of the genus *Thermus*, strain KW11, which was phylogenetically distant from not only *T. thermophilus*, but also with other *Thermus* species. In this paper, we describe the isolation, characterization, and phylogenetic position of the isolate KW11, and propose this isolate be named *T. kawarayensis*, a novel species of the genus *Thermus*.

The strain KW11 was obtained from a water sample taken from the hot springs at Kawarayu, Gunma, Japan. Temperature and pH of the environmental sample were 63°C and 7.5, respectively. A 10-ml sample of water was supplemented with 0.1% yeast extract and incubated at 70°C for 2 days for enrichment. The turbid culture was streaked onto KW medium (0.7 g $Na_2SO_4$, 0.7 g $CaCl_2{\times}2H_2O$ , 0.35 g $Na_2B_4O_2{\times}10H_2O$ , and 2 g/l yeast extract, pH 7.0) solidified by 0.7% Gelrite, and

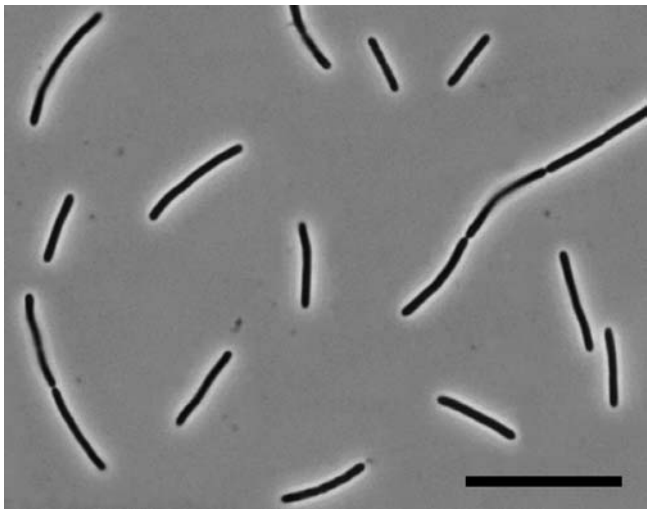

**Fig. 1** Phase contrast micrograph of strain KW11. *Bar* = 20 μm

incubated at 70°C for 3 days. A single colony that appeared on the plate was re-streaked onto a KW plate and incubated at 70°C for 2 days for purification of the microorganism. The purified strain KW11 was used for further study. *T. aquaticus* YT-1$^T$ (DSM625) and *T. thermophilus* HB8$^T$ (DSM579) were provided from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Unless otherwise noted, these two type strains and the isolate KW11 were routinely cultivated in M74 medium (2 g NaCl, 2 g yeast extract, 4 g/l polypeptone, pH 7.5) at 70°C.

The colonies of KW11 on rich media such as KW or M74 plates were colorless, in contrast to the fact that most strains of *Thermus* species form yellow- to deep-yellow-colored colonies, which are derived from carotenoidal pigments, except for *T. scotoductus* (Kristjansson et al. 1994). The shapes of the KW11 colonies are smooth and compact (not spreading type) as are most of the *Thermus* species. The cells were long-rod type (10–20 μm long), about 0.8 μm in diameter (Fig. 1), and were Gram negative. The temperature range for the growth of KW11 was from 40–73°C, with optimal growth at 68°C (Fig. 2a). This optimal temperature for growth is about 10°C lower than that of *T. thermophilus*. Growth pH range was from 5.8–8.9, and the optimum pH was around 7 (Fig. 2b). No growth was observed at pH 5.3 and pH 9.4. The doubling time was 2.8 h under optimal condition.

Oxidase activity was determined by the oxidation of 1% aqueous tetramethyl *p*-phenylenediamine on filter paper, and catalase activity was determined by the formation of bubbles with a 3% hydrogen peroxide solution at room temperature. The strain KW11 showed both oxidase and catalase activities.

The G + C content of the DNA of the strain KW11 determined by reverse- phase HPLC of the DNA digested with nuclease P1 (Tamaoka and Komagata 1984) was 68.9 mol%. This value is similar to the corresponding values of *T. thermophilus*, but higher than

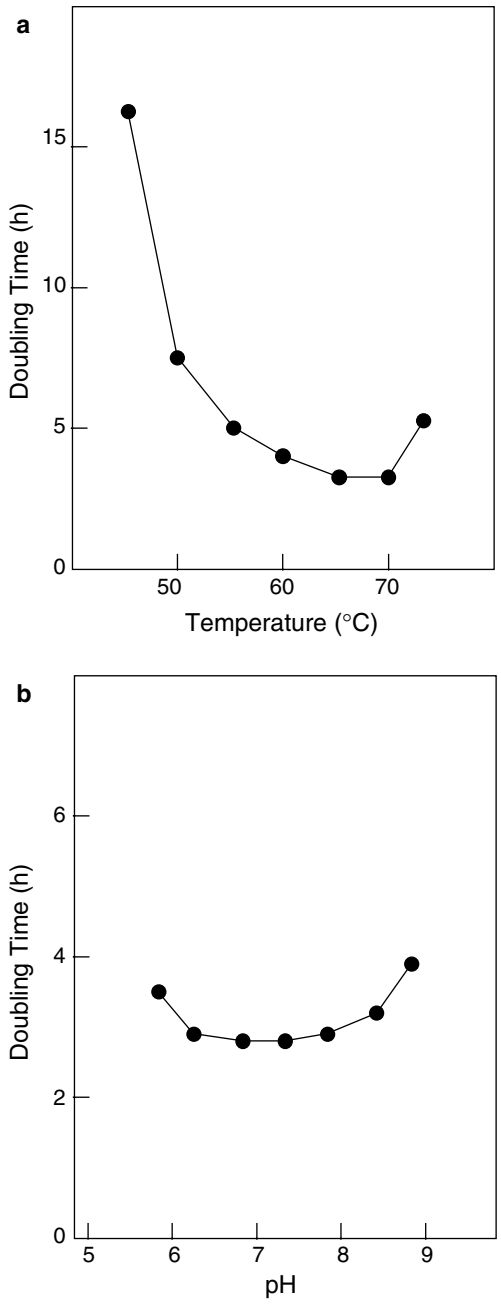


**Fig. 2** Growth rates of strain KW11 at various temperatures (**a**) and pH (**b**). KW11 was cultured in M74 medium, pH 7.5, at various temperatures. The pH of the media were adjusted by the addition of 1 M HCl or 1 M NaOH and were measured at 70°C

those of other type strains of the genus *Thermus*, including *T. aquaticus* (63–65 mol%).

For fatty acid analysis, KW11 fatty acid methyl esters were obtained from fresh wet cells by saponification, methylation, and extraction according to the technical note no. 101 provided by Microbial ID (http://www.microbialid.com/). The fatty acid methyl esters were separated and analyzed by using a Hewlett-Packard model 6890 gas chromatography system. The fatty acid composition of strain KW11 was 16:0 (52.9%),

iso-15:0 (22.1%), iso-17:0 (15.6%), anteiso-15:0 (2.6%), anteiso-3-OH-15:0 (2.6%), iso-3-OH-17:0 (2.2%), and anteiso-17:0 (2.2%), with minor components such as iso-16:0 (0.7%), 18:0 (0.7%), and iso-14:0 (0.2%). The 16:0 content of KW11 (52.9%) is remarkably higher than the ones of known *Thermus* species (ranging from 4.1–16.2%) (Chung et al. 2000). On the other hand, the levels of iso-16:0 and iso-17.0 of strain KW11 are significantly lower than the known *Thermus* species except for iso-17:0 of *T. filiformis*.

16S rDNA of the isolate KW11 was amplified by PCR using a primer set as follows: sense primer, 5′-TTTCTCGAGTTTGATCCTGGCTCAG and antisense primer, 5′-TTTCTCGAGGYTACCTTGTTACGACTT. The following thermal cycle was used for 25 cycles: 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min. The PCR product and its smaller restriction fragments were sequenced by the dideoxynucleotide chain termination method. The 16S rDNA sequence of KW11 showed a 96.0% similarity with *T. aquaticus* and lower values with the remaining seven type strains (90.3–95.8%). These values are low enough to exclude the possibility of assigning strain KW11 to a previously described *Thermus* species (Stackebrandt and Goebel 1994). To clarify the phylogenetic position of KW11 within the members of the genus *Thermus*, a phylogenetic tree was constructed based on the 16S rDNA sequence data using the maximum-likelihood method. The phylogenetic position of KW11 was branched from the internal branch between *T. thermophilus* and *T. aquaticus* and was clearly distinguished from all the known *Thermus* species on the tree (Fig. 3).

Additionally, we have examined the halotolerance of KW11 to compare with those of *T. aquaticus* and *T. thermophilus*. *T. thermophilus* can grow in a medium containing 3% NaCl. In contrast with *T. thermophilus*, KW11 grows only in a medium containing less than 1.1% NaCl, and this property is almost the same as *T. aquaticus* (Fig. 4). We also examined the sensitivity of KW11 to antibiotics (ampicillin, penicillin G, kanamycin, and streptomycin, 10–100 μg/ml), and compared it with those of *T. aquaticus* and *T. thermophilus*. KW11 and *T. aquaticus* were shown to be sensitive to all the antibiotics mentioned above, in contrast with *T. thermophilus*, which was resistant to streptomycin at a concentration of 10 μg/ml.

On the basis of 16S rDNA phylogeny and chemotaxonomy, it is clear that the isolate KW11 belongs to the genus *Thermus*, and low 16S rDNA sequence similarity values to known *Thermus* species (90.3–96.0%) indicate that this strain represents a novel species in the genus (Stackebrandt and Goebel 1994). The fatty acid composition is also very specific among the *Thermus* species. G + C content of DNA, colorless colonies, and halotolerance of the strain KW11 also can be used to differentiate the strain from some other *Thermus* species at the phenotypic level. Therefore, based on these physiological and phylogenetic evidences, the isolate KW11 is clearly distinguishable from all the eight species of the genus

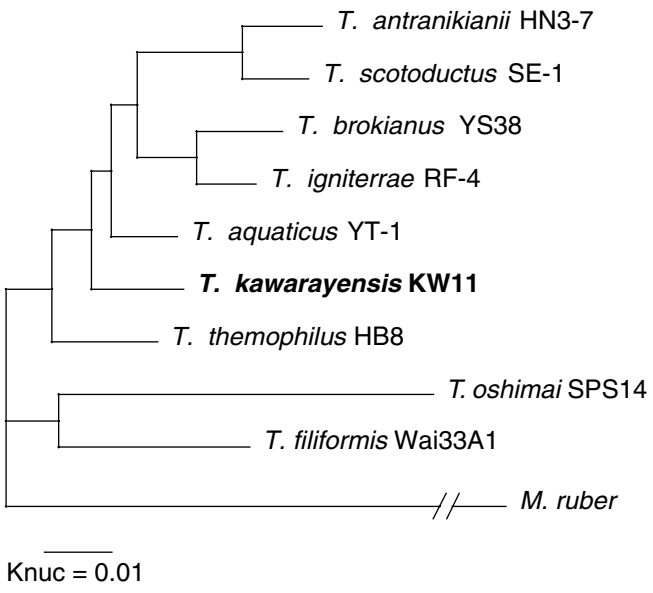


**Fig. 3** Phylogenetic tree based on the 16S rDNA sequences, constructed by the maximum-likelihood method. The sequences of the isolate KW11 and the type strains were aligned using the Clustal W program (Higgins and Clustal 1998), and all sites with gaps in any sequences and the regions of the PCR primers were removed from alignment. Pairwise distances between all sequences were estimated by Kimura's two-parameter method (Kimura 1980). A total of 1,383 positions of the 16S rDNA sequence data were applied for PHYLIP program (Felsenstein 1993). The accession numbers of nucleotide sequence data: *Thermus kawarayensis* KW11, AB071811; *T. antranikianii*, Y18411; *T. aquaticus*, L09663; *T. brokianus*, Z15062; *T. filiformis*, L09667; *T. igniterrae*, Y18406; *T. oshimai*, Y18416; *T. scotoductus*, AF032127; *T. thermophilus*, X07998; and *Meiothermus ruber*, L09672

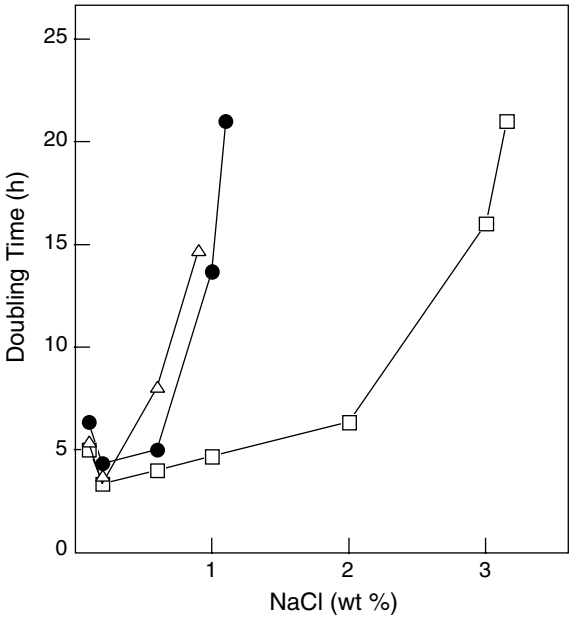


**Fig. 4** Growth rates of strain KW11 (*circles*), *T. aquaticus* (*triangles*), and *T. thermophilus* (*squares*), in the M74 medium at various concentrations of NaCl

*Thermus*, and is proposed as a new species of the genus *Thermus*, *T. kawarayensis* (type strain KW11$^T$).

## References


Brock TD, Freeze H (1969) *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J Bacteriol 98:289–297

Chung AP, Rainey FA, Valente M, Nobre MF, da Costa MS (2000) *Thermus igniterrae* sp. nov. and *Thermus antranikianii* sp. nov., two new species from Iceland. Int J Syst Evol Microbiol 50:209–217

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author, Department of Genetics, University of Washington

Higgins D, Clustal W (1998) Multiple sequence alignment. http://clustalw.genome.ad.jp/. Cited 5 Feb 1998

Hudson JA, Morgan HW, Daniel RM (1987) *Thermus filiformis* sp. nov., a filamentous caldoactive bacterium. Int J Syst Bacteriol 37:431–436

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kristjansson JK, Hjorleifsdottir S, Marteinsson T, Alfredsson GA (1994) *Thermus scotoductus*, sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. X1. Syst Appl Microbiol 17:44–50

Oshima T, Imahori K (1974) Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from Japanese thermal spa. Int J Syst Bacteriol 24:102–112

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Tamaoka J, Komagata K (1984) Determination of DNA base composition by reversed phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128

Williams RAD, Smith KE, Welch SG, Micallef J, Sharp RJ (1995) DNA relatedness of *Thermus* strains, description of *Thermus brokianus* sp. nov., and proposal to reestablish *Thermus thermophilus* (Oshima and Imahori). Int J Syst Bacteriol 45:495–499

Williams RAD, Smith KE, Welch SG, Micallef J (1996) *Thermus oshimai* sp. nov., isolated from hot springs in Portugal, Iceland, and the Azores, and comment on the concept of a limited geographical distribution of *Thermus* species. Int J Syst Bacteriol 46:403–408