***Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan**

¹ Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi City, Iwate 026-0001, Japan

² Marine Biotechnology Institute, Shimizu Laboratories, 1900 Sodeshi, Shimizu City, Shizuoka 424-0037, Japan

³ Taisei Corporation Technology Center, 344-1 Nase-cho Totsuka-ku, Yokohama, Kanagawa 245-0051, Japan

Yoh Takahata,^{1†} Miyuki Nishijima,² Toshihiro Hoaki³ and Tadashi Maruyama¹

Author for correspondence: Yoh Takahata. Tel: +81 458147226. Fax: +81 458147257.
e-mail: yoh.takahata@sakura.taisei.co.jp

Two hyperthermophilic bacteria, strains RKU-1^T and RKU-10^T, which grew optimally at 80 °C, were isolated from the production fluid of the Kubiki oil reservoir in Niigata, Japan. They were strictly anaerobic, rod-shaped fermentative heterotrophs. Based on the presence of an outer sheath-like structure (toga) and 16S rDNA sequences, they were shown to belong to the genus *Thermotoga*. Cells of strain RKU-1^T were 2–7 µm by 0.7–1.0 µm, with flagella. They grew at 47–88 °C on yeast extract, peptone, glucose, fructose, ribose, arabinose, sucrose, lactose, maltose, starch and cellulose as sole carbon sources. Cells of strain RKU-10^T were 2–7 µm by 0.8–1.2 µm, with flagella. They grew at 48–86 °C on yeast extract, peptone, glucose, galactose, fructose, mannitol, ribose, arabinose, sucrose, lactose, maltose and starch as sole carbon sources. While strains RKU-1^T and RKU-10^T reduced elemental sulfur to hydrogen sulfide, their final cell yields and specific growth rates decreased in the presence of elemental sulfur. Thiosulfate also inhibited growth of strain RKU-1^T but not strain RKU-10^T. The G+C contents of the DNA from strains RKU-1^T and RKU-10^T were 46.8 and 46.1 mol%. Phenotypic characteristics and 16S rDNA sequences of the isolates were similar to those of *Thermotoga maritima* and *Thermotoga neapolitana*, both being hyperthermophilic bacteria isolated from hydrothermal fields. However, the isolates differed from these species in their minimum growth temperatures, utilization of some sugars, sensitivity to rifampicin and the effects of elemental sulfur and thiosulfate on growth. The low levels (less than 31%) of DNA reassociation between any two of these hyperthermophilic *Thermotoga* strains indicated that the isolates were novel species. Analysis of the *gyrB* gene sequences supported the view that the isolates were genotypically different from these reference species. The isolates were named *Thermotoga petrophila* sp. nov., with type strain RKU-1^T (= DSM 13995^T = JCM 10881^T), and *Thermotoga naphthophila* sp. nov., with type strain RKU-10^T (= DSM 13996^T = JCM 10882^T).

Keywords: *Thermotoga petrophila*, *Thermotoga naphthophila*, hyperthermophilic bacteria, oil reservoir

INTRODUCTION

A large number of hyperthermophiles with optimum

growth temperatures higher than 80 °C have been isolated from various hot environments (Stetter, 1996). While most of them are members of the domain

[†] **Present address:** Taisei Corporation Technology Center, 344-1 Nase-cho Totsuka-ku, Yokohama, Kanagawa 245-0051, Japan.

Abbreviation: ASW, artificial sea water.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Thermotoga petrophila* RKU-1^T, *Thermotoga naphthophila* RKU-10^T, *Thermotoga neapolitana* DSM 4359^T and *Thermotoga thermarum* DSM 5069^T are AB027016, AB027017, AB039768 and AB039769. The accession numbers for the *gyrB* gene sequences of *T. petrophila* RKU-1^T, *T. naphthophila* RKU-10^T, *Thermotoga maritima* DSM 3109^T and *T. neapolitana* DSM 4359^T are AB039770–AB039773.

Archaea, some hyperthermophiles belong to the genera *Aquifex* and *Thermotoga* in the domain *Bacteria* (Blöchl *et al.*, 1995). According to 16S rRNA gene sequencing, they represent the deepest phylogenetic branches within the domain *Bacteria* (Winker & Woese, 1991). The members of the genus *Thermotoga*, in the order *Thermotogales*, include *Thermotoga maritima* (Huber *et al.*, 1986), *Thermotoga neapolitana* (Belkin *et al.*, 1986; Jannasch *et al.*, 1988), *Thermotoga thermarum* (Windberger *et al.*, 1989), *Thermotoga elfii* (Ravot *et al.*, 1995a), *Thermotoga subterranea* (Jenthon *et al.*, 1995) and *Thermotoga hypogea* (Fardeau *et al.*, 1997). *T. maritima* and *T. neapolitana* grow optimally at 80 °C and are hyperthermophilic species. In the last decade, hyperthermophilic archaea (Beeder *et al.*, 1994; Grassia *et al.*, 1996; L'Haridon *et al.*, 1995; Orphan *et al.*, 2000; Stetter *et al.*, 1993; Takahata *et al.*, 2000) and thermophilic bacteria with an optimal temperature below 80 °C (Beeder *et al.*, 1995; Cayol *et al.*, 1995; Greene *et al.*, 1997; Magot *et al.*, 1997; Nilsen *et al.*, 1996; Rees *et al.*, 1995), including *T. elfii*, *T. subterranea* and *T. hypogea*, have been isolated from the production fluids of oil reservoirs. *Thermotoga*-like bacteria with togas, growing at 65–85 °C, have also been reported from oil reservoirs in Alaska and California (Orphan *et al.*, 2000; Stetter *et al.*, 1993). We describe two novel hyperthermophilic *Thermotoga* species, *Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., isolated from the Kubiki oil reservoir.

METHODS

Reference micro-organisms. *T. maritima* DSM 3109^T, *T. neapolitana* DSM 4359^T and *T. thermarum* DSM 5069^T were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany.

Collection of the sample. The production fluid was taken from the no. 3 storage tank of the Kubiki oil reservoir in Niigata, Japan. The geological structure and physical properties of the Kubiki oil reservoir have been described previously (Takahata *et al.*, 2000). The production fluid was collected in sterile glass bottles, which were then sealed with sterile silicon stoppers and plastic screw caps. They were chilled in a cooler box with ice and transported to the laboratory.

Isolation and cultivation. Anaerobic, heterotrophic hyperthermophiles were grown on YE medium (Takahata *et al.*, 2000) containing 0.2% (w/v) yeast extract in artificial sea water (ASW; Jannasch *et al.*, 1995). One litre of ASW contained 20 g NaCl, 3 g MgCl₂·6H₂O, 6 g MgSO₄·7H₂O, 1 g (NH₄)₂SO₄, 0.3 g CaCl₂·2H₂O, 0.2 g KH₂PO₄, 0.5 g KCl, 0.05 g NaBr, 0.025 g H₃BO₃, 0.02 g SrCl₂·6H₂O, 0.01 g ferric ammonium citrate, 2.25 g bis-tris propane, 10 ml of a trace mineral solution (Wolin *et al.*, 1963), 10 ml of a vitamin solution (Bazylinski *et al.*, 1989) and 0.6 mg resazurin as a redox indicator. The pH was adjusted to 7.0 with 6 M HCl after supplementing the medium with 0.2% yeast extract at room temperature. Aliquots (9 ml) of the liquid medium were dispensed into 30 ml Hungate tubes (Sanshin Kogyo) and autoclaved at 121 °C for 20 min. The tubes were flushed with oxygen-free nitrogen through a heated, H₂-reduced, copper furnace (Sanshin Kogyo). The tubes were then sealed

tightly with sterile butyl-rubber stoppers. Sodium sulfide, which had been sterilized by filtration (disk capsule of 0.2 µm pore size; Fuji), was added to a final concentration of approximately 400 µM. After autoclaving, flushing with nitrogen and adding sodium sulfide, the final pH in the medium was between 6.9 and 7.1 at room temperature. One millilitre of the formation water in the production fluid was inoculated into 9 ml of YE medium in a Hungate tube and incubated at 85 °C for 1–7 d. Hyperthermophiles were purified by the Gelrite plating method as described previously (Takahata *et al.*, 2000).

Electron microscopy. Cell morphology and flagellation were observed with a transmission electron microscope (H-7000; Hitachi) as described previously (Takahata *et al.*, 2000). The cells were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. Ultrathin sections of the cells embedded in Epon 812 resin were cut with a Reichelt-Nissei Ultracut-N ultramicrotome and doubly stained with uranyl acetate and lead citrate (Reynolds, 1963). After fixation with 2.5% glutaraldehyde, whole mounted cells were negatively stained with 4% (w/v) uranyl acetate (Kurr *et al.*, 1991).

Determination of cell numbers. Cell growth was monitored by the acridine orange direct-counting method (Hobbie *et al.*, 1977) under an epifluorescence microscope (model BX-60; Olympus).

Growth characteristics. The effect of temperature on microbial growth was examined in YE medium with a mineral oil bath (model OH-16; Taitec). The effects of pH and NaCl on growth were examined in a YE-based medium incubated at 80 °C. The pH dependency of growth was determined with various buffer systems, as described previously (Hoaki *et al.*, 1994). The pH of the medium was checked and readjusted at room temperature after autoclaving, flushing with nitrogen and adding sodium sulfide. The effects of various gaseous phases on microbial growth were examined by using ASW for autotrophic growth or YE medium for heterotrophic growth. The gaseous phase in a Hungate tube was filled with CO₂, H₂, air or a mixture of H₂ and CO₂ (4:1). To determine the nutritional requirements of the isolates, their growth was examined in 10 ml ASW medium (pH 7.0) without shaking at 80 °C with one of the following carbon sources at a final concentration of 0.1% (w/v): yeast extract, peptone, casein, albumin, Casamino acids, a mixture of 20 amino acids, glucose, galactose, fructose, mannitol, ribose, arabinose, xylose, sucrose, lactose, maltose, starch, acetate, lactate, formate, propionate, maleic and citric acids, methanol and ethanol. Growth was also examined in the presence of 1% (v/v) cellulose, chitin, kerosene, light oil, A-heavy oil (Nippon Mitsubishi Oil) or crude oil in 30 ml ASW medium (pH 7.0) with shaking. Crude oil was obtained from the Kubiki oil reservoir. To confirm growth on any of the substrates, the isolate was subcultured twice in the same medium.

Metabolic products. Metabolic products [H₂, CO₂ and low-molecular-mass (C₂–C₃) organic acids] were analysed by HPLC and GC as described previously (Takahata *et al.*, 2000). D-Glucose was analysed with a glucose analysis kit (no. 716251; Boehringer Mannheim).

Effect of electron acceptors. The effects of sulfate (20 mM), thiosulfate (20 mM) and elemental sulfur (2%) as electron acceptors on growth of the isolates were examined in YE-based medium in which MgSO₄·7H₂O and (NH₄)₂SO₄ had been replaced with MgCl₂·6H₂O and NH₄Cl. Hydrogen sulfide production was analysed with a gas analysis kit (Gastec).

Fig. 1. Neighbour-joining phylogenetic tree of the members of *Thermotoga*, including strains RKU-1^T and RKU-10^T, based on 16S rDNA sequences. The 16S rDNA sequences of *T. maritima*, *T. hypogea*, *T. subterranea*, *T. elfii* were obtained from the GenBank/EMBL/DBJ database. The corresponding sequences of *T. neapolitana* and *T. thermarum* were determined in this study. Outgroup: *Thermosipho melanesiensis* DSM 12029^T (Z70248). Bar, 1 base substitution per 100 nucleotide positions.

Sensitivity to antibiotics. The sensitivity of the isolates to rifampicin, streptomycin, vancomycin and chloramphenicol was examined at a concentration of 100 µg ml⁻¹ in YE medium at 70 °C for 7 d.

Isolation of DNA. Genomic DNA was extracted from each isolate by using a procedure described elsewhere (Ausubel *et al.*, 1987) with a slight modification. RNA was digested with 20 µg DNase-free RNase ml⁻¹ at 37 °C for 1 h after extracting with chloroform/isoamyl alcohol.

DNA base composition. The nucleotide composition was determined by HPLC with a Develosil ODS-HG-5 column (4.6 × 250 mm) and a UV detector (model UV 8010; Tosoh) at 270 nm after digesting the DNA with nuclease P1 (Zillig *et al.*, 1980).

16S rDNA sequence analysis. PCR was used to amplify 16S rDNA from the genomic DNA of each isolate using primers B0R (*Escherichia coli* positions 8–27; 5'-AGAGTTTGAT-CCTGGCTCAG-3') and B9 (1491–1512; 5'-TACGGCTA-CCTTGTTACGACTT-3') (Takahata *et al.*, 2000). A total of 35 cycles of amplification was performed with template DNA denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1 min and primer extension at 72 °C for 2 min. After purifying the PCR products with a QIAEX II gel extraction kit (QIAGEN), the 16S rDNA sequence was determined with an ABI Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer) and an ABI 377 DNA sequencer (Perkin-Elmer). The sequences determined were then aligned with reference 16S rDNA sequences obtained from the GenBank/EMBL/DBJ database. A multiple alignment was obtained by using CLUSTAL W version 1.7 (Higgins *et al.*, 1996; Thompson *et al.*, 1994). A phylogenetic tree was constructed by using the same software using the neighbour-joining method (Saitou & Nei, 1987). The reliability of cluster formation was analysed by bootstrap resampling 1000 times.

gyrB sequence analysis. PCR was used to amplify partial *gyrB* sequences from the genomic DNA of the isolates by using the pair of primers TTG-UF (*T. maritima* positions 841–860; 5'-CAGGAAACAGCTATGACCGAYGGNG-GNACNCAYGTNAC-3') and TTG-UR (*T. maritima* positions 1219–1238; 5'-TGTAACGACGGCCAGT-CARTCNGCNARYTTNCCNGG-3') (Guipaud *et al.*, 1996). A total of 35 cycles of amplification was performed with denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min and primer extension at 72 °C for 2 min. The methods for purification of the PCR products and determination of the *gyrB* sequences were the same as those used for 16S rDNA. The amplified fragments were sequenced with primers M13Rev (5'-CAGGAAACAGC-

TATGACC-3') and –21M13 (5'-TGTAACGACGG-CCAGT-3') (Messing *et al.*, 1977). The similarity of each determined sequence was calculated by GENETYX-MAC 8.0 (Software Development).

DNA–DNA hybridization. DNA–DNA hybridization between the novel isolates, *T. maritima* and *T. neapolitana* was determined by the fluorometric hybridization method in microdilution wells (Ezaki *et al.*, 1989). The DNA probes were labelled with photobiotin and hybridization was performed at 43 °C for 20 h. The optimal temperature for renaturation (T_{or}) was calculated from the following equation: T_{or} (°C) = 0.41 × G + C content (mol %) + 24.3.

RESULTS AND DISCUSSION

Isolation and 16S rDNA sequence analysis

Two hyperthermophilic bacterial strains, RKU-1^T and RKU-10^T, were isolated from a deep subterranean oil reservoir in Niigata, Japan. They were obligately anaerobic heterotrophic rods with a 'toga', a sheath-like structure with ballooning at both ends, which is characteristic of members of the order *Thermotogales*. Phylogenetic analysis using 16S rDNA sequences showed that the novel isolates belong to the genus *Thermotoga* and they were most closely related to the hyperthermophilic species *T. maritima* and *T. neapolitana* (Fig. 1), which were isolated from marine hydrothermal fields (Belkin *et al.*, 1986; Huber *et al.*, 1986). Three previously described *Thermotoga* species, *T. subterranea*, *T. elfii* and *T. hypogea*, have also been isolated from oil reservoirs (Fardeau *et al.*, 1997; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a). However, the phylogenetic tree of 16S rDNA sequences revealed that these three species formed a clade distinct from the hyperthermophilic *Thermotoga* species (Fig. 1).

Morphological and phenotypic properties

Cells of strain RKU-1^T were 2–7 µm long and 0.7–1.0 µm wide. Cells of strain RKU-10^T were 2–7 µm long and 0.8–1.2 µm wide. In the stationary growth phase, the cell shape became spherical, with a diameter of 1–2 µm. While *T. maritima* has a single subpolar flagellum (Huber *et al.*, 1986) and *T. neapolitana* lacks flagella (Belkin *et al.*, 1986), strains RKU-1^T and RKU-10^T possessed several subpolar and lateral flagella (Fig. 2a, b). Similar flagellation has been

Fig. 2. (a)–(b) Electron micrographs of negatively stained cells of strains RKU-1^T (a) and RKU-10^T (b). (c)–(d) Electron micrographs of doubly stained ultrathin sections of cells of strains RKU-1^T (c) and RKU-10^T (d). Arrows indicate the toga (t) and cell wall (cw). Bars, 1 µm.

shown for cells of *T. elfii* and *T. hypogea*. Electron microscopy of thin sections of the isolates showed a cell wall approximately 50 nm thick (Fig. 2c, d).

The temperature ranges for growth were 47–88 °C with an optimum at 80 °C for strain RKU-1^T and 48–86 °C with an optimum at 80 °C for RKU-10^T (Fig. 3a). No growth was apparent at 46 or 89 °C (RKU-1^T) and at 47 or 87 °C (RKU-10^T). At 80 °C, the respective doubling times of strains RKU-1^T and RKU-10^T were 54 and 59 min. While *T. maritima* and *T. neapolitana* also grow optimally at 80 °C, they are not able to grow at temperatures below 55 °C (Belkin *et al.*, 1986; Huber *et al.*, 1986). The optimum temperatures of other *Thermotoga* species isolated from oil reservoirs have been reported to be 66–70 °C (Fardeau *et al.*, 1997; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a). The pH ranges for growth were 5.2–9.0 (RKU-1^T) and 5.4–9.0 (RKU-10^T), with the optimum at 7.0 for both (Fig. 3b). No growth was apparent at pH 5.0 or 9.5 (RKU-1^T) and at pH 5.2 or 9.5 (RKU-10^T). These ranges are about the same as those of *T. maritima* and *T. neapolitana* (Belkin *et al.*, 1986; Huber *et al.*, 1986). The NaCl concentration ranges for growth were 0.1–5.5% (RKU-1^T) and 0.1–6.0% (RKU-10^T), with the optimum at 1.0% for both

(Fig. 3c). No growth was apparent at concentrations of 0 or 6% (RKU-1^T) and at 0 or 6.5% (RKU-10^T). In contrast, thermophilic *Thermotoga* species from oil reservoirs required lower levels of salinity (NaCl concentration range for growth 0–2.8%) (Fardeau *et al.*, 1997; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a).

The isolates did not grow aerobically or autotrophically with a mixture of H₂ and CO₂ (4:1). Carbon dioxide did not affect heterotrophic growth of the isolates in YE medium. They could grow in YE medium with a hydrogen atmosphere, but their final cell yields were only about 10% of those with nitrogen. They grew on yeast extract, peptone and various sugars. Growth of the novel isolates, *T. maritima* and *T. neapolitana* on various sugars is shown in Table 1. All of the strains could grow on all the sugars tested except mannitol or xylose as the sole carbon and energy source, although the final cell densities after incubation for 48 h at 80 °C were different (Table 1). Strain RKU-1^T grew weakly on cellulose, but strain RKU-10^T did not. Neither strain RKU-1^T nor RKU-10^T grew on proteins (casein or albumin), amino acids (Casamino acids or a mixture of 20 amino acids), organic acids (acetate, lactate, formate, propionate, maleic or citric acid), alcohols (methanol or ethanol),

Fig. 3. Effects of temperature (a), pH (b) and NaCl concentration (c) on the specific growth rates of strains RKU-1^T (○) and RKU-10^T (●). Growth rates were calculated from a linear regression analysis along the logarithmic part of the growth curves.

Table 1. Growth of hyperthermophilic *Thermotoga* strains on various sugars

The initial cell density was adjusted to approximately 10^4 cells ml⁻¹. Growth was evaluated from the final cell density after incubation for 48 h at 80 °C, and is scored as: + + +, > 10^8 cells ml⁻¹; + +, 10^7 – 10^8 cells ml⁻¹; +, 10^6 – 10^7 cells ml⁻¹; –, no growth. All four taxa were scored as + for growth on arabinose.

Sugar	RKU-1 ^T	RKU-10 ^T	<i>T. maritima</i>	<i>T. neapolitana</i>
Glucose	+	++	+++	++
Galactose	++	++	+++	+++
Fructose	++	+++	++	++
Mannitol	–	++	–	–
Ribose	+++	++	++	+++
Xylose	–	–	+++	++
Sucrose	++	++	+++	+++
Lactose	+++	++	++	+++
Maltose	+++	++	+++	+++
Starch	+++	++	+++	+++

Table 2. Fermentation of glucose by strains RKU-1^T and RKU-10^T

Glucose consumed was calculated by subtracting the glucose concentration after cultivation from that before cultivation. Metabolites produced were measured after incubation for 100 h in ASW containing 0.1 % glucose at 80 °C.

Strain	Cell density (cells ml ⁻¹)		Glucose consumed (mM)	Production of (mM):				Total carbon recovery (%)
	Initial	Final		Lactate	Acetate	CO ₂	H ₂	
RKU-1 ^T	5.2×10^4	7.3×10^6	0.27	0.07	0.48	0.11	1.01	79.0
RKU-10 ^T	6.3×10^4	1.2×10^7	1.36	1.05	1.72	0.56	5.39	87.6

Fig. 4. Effects of electron acceptors on the growth of strain RKU-10^T. Production of hydrogen sulfide (a) and growth (b) are shown with no additions (●) or with the addition of 20 mM sulfate (○), 20 mM thiosulfate (△) or 2% elemental sulfur (□).

chitin or hydrocarbons (kerosene, light oil, A-heavy oil or crude oil) as the sole carbon and energy source.

Lactate, acetate, CO₂ and H₂ were produced by strains RKU-1^T and RKU-10^T during growth on glucose. The concentration of glucose consumed and those of the final fermentation products after cultivation at 80 °C for 100 h are shown in Table 2. The ratio of acetate/lactate in strain RKU-10^T (2.8) was higher than those in strain RKU-1^T (1.64) and *T. maritima* (0.40; Huber *et al.*, 1986).

Sulfur utilization was reported to be one of the discriminatory characteristics between hyperthermophilic *Thermotoga* species from hydrothermal fields and thermophilic *Thermotoga* species from oil reservoirs (Fardeau *et al.*, 1997). While elemental sulfur has been reported to enhance the final cell yields and specific growth rates of *T. maritima* and *T. neapolitana* by removing growth-inhibitory H₂ (Ravot *et al.*, 1995b), it inhibited the growth of *T. elfii* and *T. subterranea* (Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a). Strains RKU-1^T (data not shown) and RKU-10^T (Fig. 4a) did not reduce sulfate to hydrogen sulfide but did reduce sulfur and thiosulfate. The final yields

of H₂S production from elemental sulfur by these two strains were almost the same (about 400 μg ml⁻¹; Fig. 4a). While RKU-1^T (data not shown) and RKU-10^T (Fig. 4b) reduced elemental sulfur to H₂S, their final cell yields and specific growth rates were reduced. Growth of six *Thermotoga* reference strains was stimulated by thiosulfate reduction (Ravot *et al.*, 1996). Strain RKU-1^T also reduced thiosulfate to H₂S (120 μg ml⁻¹; data not shown), but its final cell yield and specific growth rate were reduced. The final yield of H₂S from thiosulfate by strain RKU-10^T was much less than that of strain RKU-1^T (approx. 10 μg ml⁻¹; Fig. 4a). The final cell yield and specific growth rate were not affected significantly by the presence of thiosulfate (Fig. 4b). These results indicate that the utilization of sulfur or thiosulfate by these isolates from Japanese oil reservoirs was quite different from that of *Thermotoga* reference species.

Strains RKU-1^T and RKU-10^T did not grow in YE medium containing 100 μg rifampicin, streptomycin, vancomycin or chloramphenicol ml⁻¹ at 70 °C within 7 d. In contrast, *T. maritima* and *T. neapolitana* were not sensitive to rifampicin at the same concentration (Belkin *et al.*, 1986; Huber *et al.*, 1986).

These results indicated that the morphological and phenotypic properties of strains RKU-1^T and RKU-10^T, such as type of flagellum, minimum growth temperature, sugar utilization, effects of sulfur and thiosulfate on growth and rifampicin sensitivity, were clearly different from those of *T. maritima* and *T. neapolitana*. They also differed from other *Thermotoga* species isolated from oil reservoirs in their optimum growth temperature, range of NaCl concentrations for growth and effects of sulfur and thiosulfate on growth. However, the morphological and phenotypic properties of these two isolates were quite similar, except in mannitol and cellulose utilization and thiosulfate reduction.

Genotypic analysis

The DNA G+C contents of strains RKU-1^T and RKU-10^T were 46.6 and 46.1 mol%. These G+C contents are similar to that of *T. maritima* (46 mol%). DNA reassociation values between any two of the hyperthermophilic *Thermotoga* strains were 11.0–30.8% (Table 3). According to the criteria of Wayne *et al.* (1987), these results indicate that each of the new isolates should be classified as a distinct species.

The 16S rDNA sequence evolves so slowly that it is not suitable for distinguishing closely related species. The *gyrB* gene, encoding the subunit B protein of DNA gyrase (topoisomerase type II), has been proposed to be more suitable to distinguish closely related species (Yamamoto *et al.*, 1999). Amplified *gyrB* gene fragments from strains RKU-1^T and RKU-10^T, *T. maritima* and *T. neapolitana* were sequenced directly and their partial *gyrB* sequences (358 bp) were determined. The similarity (77.8–94.7%; Table 3) of *gyrB* sequences between any two of the four hyper-

Table 3. Nucleotide sequence similarity of *gyrB* and DNA–DNA hybridization between hyperthermophilic *Thermotoga* strains

Values above the diagonal are percentage sequence similarities of *gyrB* gene sequences. Values below the diagonal are mean percentages of DNA reassociation, and the minimum and maximum values are given in parentheses ($n = 3$).

Strain	1	2	3	4
1. RKU-1 ^T	–	86.0	94.7	77.8
2. RKU-10 ^T	15.0 (10.3–17.4)	–	85.7	77.9
3. <i>Thermotoga maritima</i> DSM 3109 ^T	16.9 (10.0–28.1)	25.7 (21.1–28.3)	–	78.4
4. <i>Thermotoga neapolitana</i> DSM 4359 ^T	11.0 (8.3–15.9)	11.1 (5.8–14.9)	30.8 (25.2–35.3)	–

thermophilic *Thermotoga* strains was much less than that in their 16S rDNA sequences (99.1–99.6%). These results support the genotypic difference among them.

On the basis of their phenotypic characteristics and phylogenetic relationships, the two isolates from a Japanese oil reservoir are thought to be novel species. They are designated *Thermotoga petrophila* sp. nov., with the type strain RKU-1^T, and *Thermotoga naphthophila* sp. nov., with the type strain RKU-10^T.

Description of *Thermotoga petrophila* sp. nov.

Thermotoga petrophila (pe.tro'phi.la. Gr. n. *petros* rock; Gr. adj. *philos* loving; N.L. fem. adj. *petrophila* rock-loving).

Cells are rods, 2–7 µm long by 0.7–1 µm wide. Cells possess an outer sheath-like structure (toga) and several subpolar and lateral flagella. Heterotrophic and obligate anaerobe of the domain *Bacteria*. The temperature for growth is 47–88 °C, with the optimum at 80 °C. The pH range for growth is 5.2–9.0, with the optimum at 7.0. The NaCl concentration range for growth is 0.1–5.5%, with the optimum at 1.0%. The doubling time at the optimum temperature is 54 min. Requires yeast extract, peptone, glucose, galactose, fructose, ribose, arabinose, sucrose, lactose, maltose, starch or cellulose as the sole carbon and energy source. The end products from glucose fermentation are acetate, lactate, H₂ and CO₂. While the cells reduce elemental sulfur or thiosulfate to H₂S, their growth rate and cellular yield decrease in the presence of these electron acceptors. Sensitive to 100 µg rifampicin, streptomycin, vancomycin or chloramphenicol ml⁻¹. The DNA G + C content is 46.6 mol%. Isolated from the formation water of production fluid in the Kubiki oil reservoir.

The type strain is RKU-1^T (= DSM 13995^T = JCM 10881^T).

Description of *Thermotoga naphthophila* sp. nov.

Thermotoga naphthophila (naph.tho'phi.la. Gr. n. *naphtha* bitumen; Gr. adj. *philos* loving; N.L. fem. adj. *naphthophila* bitumen-loving).

Cells are rods, 2–7 µm long by 0.8–1.2 µm wide. Cells possess an outer sheath-like structure (toga) and several subpolar and lateral flagella. Heterotrophic and obligate anaerobe of the domain *Bacteria*. The temperature for growth is 48–86 °C, with the optimum at 80 °C. The pH range for growth is 5.4–9.0, with the optimum at 7.0. The NaCl concentration range for growth is 0.1–6.0%, with the optimum at 1.0%. The doubling time at the optimum temperature is 59 min. Requires yeast extract, peptone, glucose, galactose, fructose, mannitol, ribose, arabinose, sucrose, lactose, maltose or starch as the sole carbon and energy source. The end products from glucose fermentation are acetate, lactate, H₂ and CO₂. While the cells reduce elemental sulfur to H₂S, their growth rate and cellular yield decrease in the presence of elemental sulfur. While the species reduces thiosulfate weakly to H₂S, thiosulfate shows no effect on the growth rate or cellular yield. Sensitive to 100 µg rifampicin, streptomycin, vancomycin or chloramphenicol ml⁻¹. The DNA G + C content is 46.1 mol%. Isolated from the formation water of production fluid in the Kubiki oil reservoir.

The type strain is RKU-10^T (= DSM 13996^T = JCM 10882^T).

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