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# *Thermosipho japonicus* sp. nov., an extremely thermophilic bacterium isolated from a deep-sea hydrothermal vent in Japan

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**Abstract** A novel barophilic, extremely thermophilic bacterium was isolated from a deep-sea hydrothermal vent chimney at the Iheya Basin, in the Okinawa area, Japan. The cells were found to be rod shaped and surrounded by a sheath-like outer structure; the organism did not possess flagella and was not motile. Growth was observed between 45° and 80°C (optimum, 72°C, 45 min doubling time), pH 5.3 and 9.3 (optimum, pH 7.2–7.6), 6.6 and 79 g/l sea salts (optimum, 40 g/l), and 0.1 and 60 MPa (optimum, 20 MPa). Strain IHB1 was found to be a strictly anaerobic chemoorganotroph capable of utilizing yeast extract and proteinaceous substrates such as peptone and tryptone. Elemental sulfur or thiosulfate acted as electron acceptors improving growth. The isolate was able to utilize casein as a sole carbon and energy source in the presence of thiosulfate. The G + C content of the genomic DNA was 31.4 mol%. Phylogenetic analysis based on 16S rDNA sequences and DNA–DNA hybridization analysis indicated that the isolate is closely related to *Thermosipho africanus*; however, it represents a species distinct from the previously described members of the genus *Thermosipho*. On the basis of the physiological and molecular properties, we propose that the new isolate represents a new species, which we name *Thermosipho japonicus* sp. nov. (type strain: IHB1; JCM10495).

**Key words** Extreme thermophile · *Thermosipho* · *Thermotogales* · Deep-sea hydrothermal vent · Bacteria

## Introduction

Hyperthermophilic or extremely thermophilic microorganisms are generally isolated from various geothermal environments such as terrestrial hot springs and solfataric fields, shallow and deep sea hydrothermal vents, and subsurface petroleum reservoirs. Members of the order *Thermotogales* have been isolated from a variety of geothermal environments and are widely distributed in these environments (Huber et al. 1986, 1989, 1990; Stetter et al. 1990; Antoine et al. 1997). The members of the order *Thermotogales* are obligately heterotrophic bacteria and play an important role as decomposers of organic matter. To date, *Thermotogales* are represented by three thermophilic genera containing ten species and two mesophilic genera containing two species (Patel et al. 1985; Huber et al. 1986, 1989, 1990; Jannasch et al. 1988; Windberger et al. 1989; Davey et al. 1993; Jeanthon et al. 1995; Ravot et al. 1995; Andrews and Patel 1996; Antoine et al. 1997). Of these, only *Thermosipho melanesiensis* has been found in a deep-sea hydrothermal vent environment within the order *Thermotogales* (Antoine et al. 1997), and the distribution and diversity of the order *Thermotogales* in the deep-sea hydrothermal vent environments have been little understood.

In this study, we describe a new extremely thermophilic bacterium isolated from a black smoker chimney in the Iheya Basin of the Okinawa area, Japan. Phylogenetic analysis revealed that the new isolate was a member of the order *Thermotogales* and was closely related to *Thermosipho africanus*, which was isolated from a coastal marine hydrothermal vent system in Africa. Based on its physiological properties and the results of DNA–DNA hybridization analysis, the isolate can be described as a new species of *Thermosipho*, which we name *Thermosipho japonicus*.

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## Materials and methods

### Sample collection

A sample from a black smoker chimney was obtained from the hydrothermal field at Iheya Basin in the Okinawa area,

Japan (27°47.220′N, 126°53.900′E) at a depth of 972 m by means of the manned submersible *Shinkai 2000* in a dive (dive 1111) performed in July 1998. The tip of the chimney was brought to the sea surface in a sample box and immediately frozen at −85°C. The sample was stored at −85°C in a plastic bag filled with nitrogen gas before incubation.

## Enrichment and purification

Portions of the thawed and fractured sample were used to inoculate a series of media including MJDYP (described below) medium under a gas phase of 100% nitrogen (2 atm), and the cultures were incubated at 75°, 84°, or 95°C. All tubes of MJDYP medium inoculated with portions of the chimney sample became turbid after 2 days incubation at 75° and 84°C, and most of them contained highly motile, irregular cocci. However, some tubes incubated at 75°C contained straight rods surrounded by a sheath in addition to cocci, and further incubation at 75°C yielded a much larger proportion of rod-shaped cells than coccoid cells. To obtain a pure culture of the rod-shaped cells, enrichment cultures containing a high proportion of the rod-shaped cells were streaked onto solid MJDYP medium plates hardened with 3% (w/v) agar (melting temperature, 80°C; Nacarai Tesque, Kyoto, Japan). After 3 days incubation at 75°C in an anaerobic jar, the plates were examined under a gas phase of 100% nitrogen in an anaerobic chamber (Iuchi, Osaka, Japan). A well-isolated colony of each isolate was chosen and the cells were incubated in fresh liquid MJDYP medium. To ensure a pure culture, the streaking and isolation steps were repeated at least three times for each isolate.

## Sources of organisms

*Thermotoga maritima* (DSM 3109), *Thermosipho africanus* (DSM 5309), and *Thermosipho melanesiensis* (DSM 12029) were purchased from the Deutsche Sammlumg von Mikroorganismen und Zellkulturen GmbH.

## Culture medium and conditions

The new isolate was routinely cultivated in modified MJ medium (Sako et al. 1996; Takai et al. 1999), MJDYP medium consisting of 2 g yeast extract, 2 g trypticase peptone, 0.5 g $Na_2S{\cdot}9H_2O$, and 1 mg resazurin per liter of MJD synthetic seawater. MJD synthetic seawater consists of NaCl, 30.0 g; $K_2HPO_4$, 0.14 g; $CaCl_2{\cdot}2H_2O$, 0.14 g; $MgSO_4{\cdot}7H_2O$, 3.4 g; $MgCl_2{\cdot}6H_2O$, 4.18 g; KCl, 0.33 g; $NiCl_2{\cdot}6H_2O$, 0.5 mg; $Na_2SeO_3{\cdot}5H_2O$, 0.5 mg; $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 0.01 g; trace mineral solution, 10 ml (Balch et al., 1979); and deep-sea hydrothermal vent (DHV) mineral solution, 1 ml. DHV mineral solution consists of $Na_2SiO_3{\cdot}9H_2O$, 2.3 g; $SrCl_2{\cdot}6H_2O$, 1.1 g; $CoSO_4{\cdot}7H_2O$, 1.1 g; $Na_2MoO_4{\cdot}2H_2O$, 0.97 g; $MnSO_4{\cdot}2H_2O$, 0.96 g; $NiCl_2{\cdot}6H_2O$, 0.95 g; $Na_2WO_4{\cdot}2H_2O$, 0.66 g; $ZnSO_4{\cdot}7H_2O$, 0.58 g; $CuSO_4{\cdot}5H_2O$, 0.5 g; $VSO_4{\cdot}xH_2O$, 0.25 g; $Na_2SeO_3$, 0.17 g; and $LiSO_4{\cdot}H_2O$, 0.13 g. To prepare MJDYP medium, 2 g yeast extract, 2 g trypticase peptone, and 1 mg resazurin were dissolved in 1 l of MJD synthetic seawater and the medium was autoclaved. The final medium was prepared by adding separately prepared sodium sulfide (outgassed with 100% $N_2$ and autoclaved; pH 7.5), and the pH of the medium was adjusted to 7.5 with HCl at room temperature, unless otherwise noted. The anaerobic cultivation technique employed was that described by Balch and Wolfe (1976). The medium was dispensed at 20% of the total volume of a bottle or tube, which was then tightly sealed with a butyl rubber stopper, and the gas phase was exchanged with $N_2$ at 200 kPa for routine cultivation or with other gases at 200 kPa for testing the effect of the gas phase.

In an attempt to examine the effect of inorganic substances on growth, elemental sulfur ($S^0$), $Na_2S_2O_3{\cdot}5H_2O$, $FeS_2$, or $Fe_2S_3$ was added to MJDYP medium, each at a final concentration of 5 mM. For testing the effects of pH on growth, MJDYP medium was adjusted with HCl or NaOH at room temperature. The pH was found to be stable during the cultivation period. To test the effect of the concentration of sea salt on growth, dilutions of 2 × MJD synthetic seawater [each dilution contained 1% (v/v) trace mineral solution and 0.1% (v/v) DHV mineral solution] supplemented with 0.2% (w/v) yeast extract and 0.2% (w/v) peptone were used instead of MJDYP medium. To examine the utilization of organic substrates, yeast extract and peptone in MJDYP medium were replaced with several concentrations of other substrates. Autotrophic growth was tested in MJD synthetic seawater containing 0.1% (w/v) $NaHCO_3$, 0.1% (v/v) trace vitamin solution (Balch et al. 1979), and either 20 mM $Na_2S_2O_3{\cdot}5H_2O$ or 3% (w/v) $S^0$ (pH 6.0) in the presence of sodium sulfide under a gas phase of $H_2$–$CO_2$ (80:20; 200 kPa) or $N_2$–$CO_2$ (80:20; 200 kPa) for anaerobic conditions and in the absence of sodium sulfide under a gas phase of $H_2$–$CO_2$–$N_2$–$O_2$ (60:10:25:5; 200 kPa) for microaerobic conditions.

The effect of hydrostatic pressure on growth was examined using the "DEEP-BATH" system, a high-pressure/high-temperature bioreactor, as described previously (Moriya et al. 1995; Takai et al. 1999). Cells precultured in MJDYP medium at 72°C were transferred to a 1.5-l glass vessel containing 1 l of MJDYP medium, preheated at 72°C and prepressurized at 0.1, 10, 20, 30, 40, 50, or 60 MPa under 100% $N_2$, and then weakly stirred.

## Light and electron microscopy

Cells were routinely observed with a phase-contrast microscope (Optishot 2, Nikon). For microscopy at about 70°C, a drop of culture at 75°C was placed on a slide preheated to 75°C and observed immediately. Micrographs were obtained using a Nikon Optishot microscope equipped with a Nikon FX-II camera system. Transmission electron microscopy of negatively stained cells was carried out as described by Zillig et al. (1990). Cells grown in MJDYP medium at 75°C in the midexponential phase of growth were negatively stained with 2% (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV.

### Susceptibility to antibiotics and lysozyme

Susceptibility to vancomycin, streptomycin, chloramphenicol, and rifampicin at final concentrations of 10, 50, and 100 μg/ml was determined in MJDYP medium. Morphological changes in the cells were examined by a phase-contrast microscope after the addition of lysozyme (1 mg/ml) to the culture in the midexponential phase of growth and the incubation at 37°C for 3, 10, or 30 min.

### Measurement of growth

Growth of the new isolate was measured by direct cell count after staining with 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig 1980) using a Nikon Optishot microscope equipped with a Nikon FX-II camera system. Cultures were prepared in duplicate. The cells were grown in 100-ml glass bottles (Schott Glaswerke) each containing 20 ml of medium, in a temperature-controlled dry oven (Taitec). The effect of pH on growth was determined at 75°C. The growth conditions for all other cultivation tests were determined at 75°C and pH 7.5 adjusted at room temperature, unless otherwise noted.

### Organic substrates for growth

In an attempt to find organic substrates that could support the growth of strain IHB1, experiments were conducted in which the yeast extract and peptone in MJDYP medium were replaced with other organic materials as potential substrates in the presence or absence of 20 mM $Na_2S_2O_3 \cdot 5H_2O$. These were added at concentrations of 0.02% or 0.2% (w/v). The cells were precultured in each medium before inoculation of the same medium. These tests were run in duplicate at 75°C.

### Cellular fatty acid composition

Cellular fatty acid composition was analyzed using cells of strain IHB1 cultivated in MJDYP medium at 75°C. Lyophilized cells (300 mg) were placed in a Teflon-lined, screw-capped tube containing 5 ml of anhydrous methanolic HCl and heated at 100°C for 10 h. After cooling, fatty acid methyl esters were extracted with *n*-hexane. The samples were examined by thin-layer chromatography developed in *n*-hexane-diethyl ether-acetic acid (70:30:1, by volume). All compounds were visualized by spraying with 50% (w/v) sulfuric acid, followed by heating at 160°C for 5 min. The intensity of the colored spot corresponding to each compound was measured using a spectrophotoscanner PDI (Toyobo, Osaka, Japan) and expressed as the integral optical density (IOD). Authentic monobasic and dibasic fatty acids extracted from *Thermosipho africanus* grown in MJDYP medium at 75°C were used as a reference (Huber et al. 1989). Then *n*-hexane-soluble methyl esters were also analyzed using a gas-liquid chromatograph/mass spectrometer (Komagata and Suzuki 1987).

### Isolation and base composition of DNA

DNA was prepared as described by Marmur and Doty (1962) and Lauerer et al. (1986). The G + C content of DNA was determined by direct analysis of deoxyribonucleotides by high-performance liquid chromatography (HPLC) (Tamaoka and Komagata 1984). Nonmethylated DNA from bacteriophage λ (49.8 mol% G + C; TaKaRa, Kyoto, Japan) (Sanger et al. 1982) and genomic DNA extracted from *Thermosipho africanus* (30 mol% G + C) (Huber et al. 1989) were used as reference materials.

### Amplification of 16S rRNA gene and sequence determination

The 16S rRNA gene (rDNA) was amplified by the polymerase chain reaction (PCR) method using Eubac 27F and 1492R primers (DeLong 1992). The 1.5-kb PCR product was directly sequenced by the dideoxynucleotide chain termination method using a DNA sequencer (model 373As; Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The rDNA sequence was analyzed using SIMILARITY_RANK and ALIGN_SEQUENCE from the Ribosomal Database Project (RDP) (Larsen et al. 1993) and the gapped-BLAST search algorithm (Altschul et al. 1997; Benson et al. 1998) to estimate the degree of similarity to other archaeal 16S rDNA sequences.

### Data analysis

The almost complete sequence (1503 bp) of the 16S rDNA of IHB1 was manually aligned to 16S rDNA data from the RDP based on primary and secondary structure considerations using the Genetic Data Environment (GDE) multiple sequence editor. Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences (Takai and Sako 1999). Least-squares distance matrix analysis (Olsen 1988), based on evolutionary distances, was performed using the correction of Kimura (1980). Neighbor-joining analysis was accomplished using the ODEN software package (Version 1.1; National Institute of Genetics, Mishima, Japan). Maximum-likelihood analysis was performed using the PHYLIP package (Version 3.5; obtained from J. Felsenstein, University of Washington, Seattle). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

### DNA–DNA hybridization analysis

DNA–DNA hybridization was carried out at 40°C for 3 h with buffers containing various concentrations of formamide (30%, 40%, 50%, and 60%, v/v) and was measured fluorometrically using photobiotin according to the method of Ezaki et al. (1989).

Nucleotide sequence accession number

The 16S rDNA sequence of IHB1 has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB024932.

## Results

Sample characterization, enrichment, and purification

A chimney sample was obtained from a black smoker at Iheya Basin at a depth of 972 m. The temperature of the effluent vent water was more than 300°C, and the black smoker vent water contained a much higher concentration of $CO_2$, $CH_4$, other carbon hydrates, and $H_2S$ than any other black smokers of arc-backarc hydrothermal vent systems as described by Ishibashi and Urabe (1995). The tip of the chimney (~150 g) was brought to the sea surface in a sample box and immediately frozen at −85°C. The frozen sample was fractioned using mortar and pestle under a gas phase of 100% nitrogen in an anaerobic chamber, and the thawed portions were used to inoculate a series of media including MJDYP medium. Growth of rod-shaped cells was observed in the enrichment cultures of MJDYP medium at 75°C. Ten rod-shaped strains were successfully purified by streaking and isolation technique using the solid MJDYP medium plate. Isolate IHB1, which was obtained first in pure culture, was studied in detail.

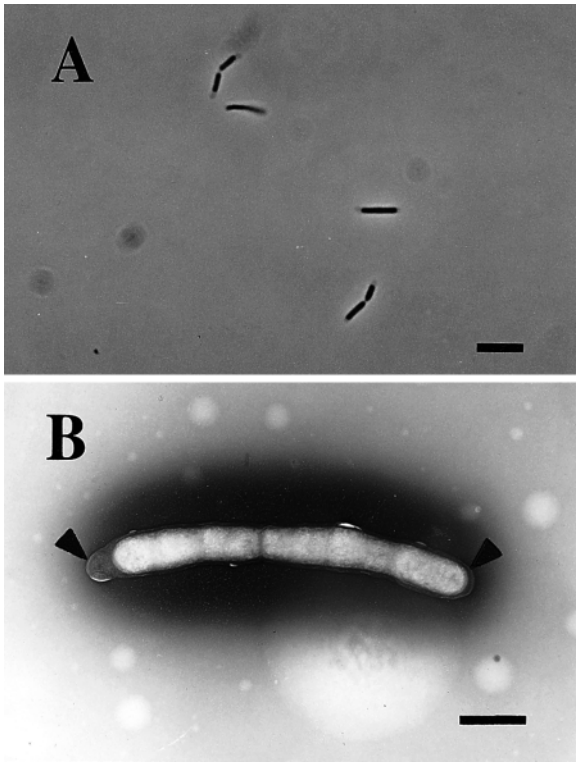


**Fig. 1A,B.** *Thermosipho japonicus* cells. **A** Phase-contrast micrographs of rod-shaped cells in the midexponential phase of growth. *Bar* 5 μm. **B** Electron photomicrograph of negatively stained cells in the midexponential phase of growth. *Closed triangles*, sheath-like structure. *Bar* 1 μm

Morphology

The cells were rod shaped with an average length of 3–4 μm and a width of about 0.5 μm (Fig. 1). The cells were surrounded by a sheath-like structure (Fig. 1A). In the very early exponential phase of growth, rods singly and in pairs were predominantly observed. In the late exponential and stationary phase of growth, the rods tended to form chains of four to six cells and sometimes formed long chains containing as many as ten cells. In addition, some of the rods became large or small spheres in the stationary phase of growth and during growth at temperatures above 75°C, and the proportion of spherical cells increased. As observed by light microscopy at 75°C, the cells were nonmotile, and the results of transmission electron microscopy examining negatively stained cells indicated that the cells had no flagella (Fig. 1B). The cells gave a negative Gram-staining reaction.

Antibiotic and lysozyme susceptibility

Growth of IHB1 was inhibited by vancomycin, streptomycin, or chloramphenicol at a concentration of 10 μg/ml whereas no inhibition of growth was observed in the presence of rifampicin when added at 10 or 50 μg/ml. However, rifampicin was inhibitory at 100 μg/ml.

The addition of lysozyme (1 mg/ml) to the culture in the midexponential phase of growth resulted in the formation of spheres after incubation at 37°C for 3 min. The outer sheath retained its shape after incubation at 37°C for 10 min, indicating the presence of murein in the cell wall.

Physiological characterization of growth

The new isolate grew only under strictly anaerobic conditions (Table 1). The isolate was heterotrophic, as it did not grow under any of the autotrophic culture conditions tested (Table 1). The effect of the gas phase on heterotrophic growth was examined in the absence and presence of potential electron acceptors. In the absence of any added electron acceptor, the presence of $H_2$ resulted in complete inhibition of growth, whereas $H_2$ inhibition was overcome in the presence of elemental sulfur ($S^0$) or thiosulfate ($Na_2S_2O_3$), or both (Table 1). Hydrogen gas was produced during growth in the absence of $S^0$ or $Na_2S_2O_3$, whereas hydrogen sulfide was detected as the major product during growth in the presence of $S^0$ or $Na_2S_2O_3$ (data not shown). Under all conditions examined, the growth of the isolate was strongly improved in the presence of $S^0$ or $Na_2S_2O_3$ (Table 1). These results indicated that $S^0$ and $Na_2S_2O_3$ served as an electron acceptor for the growth of the isolate.

The isolate grew over the temperature range of about 45°–80°C, showing optimum growth at 72°C with a genera-

**Table 1.** The effects of the gas phase and electron acceptor on heterotrophic growth of *Thermosipho japonicus*

| Growth condition | Maximum cell yield (cells/ml)[a] |
|---|---|
| MJDYP<br>+100% $N_2$ (2 atm) | +++ |
| MJDYP<br>+80% $N_2$, 20% $CO_2$ (2 atm) | +++ |
| MJDYP<br>+95% $N_2$, 5% $O_2$ (2 atm) | − |
| MJDYP<br>+80% $H_2$, 20% $CO_2$ (2 atm) | − |
| MJDYP ($S^0$)[b]<br>+100% $N_2$ (2 atm) | ++++ |
| MJDYP ($S^0$)<br>+80% $H_2$, 20% $CO_2$ (2 atm) | ++ |
| MJDYP ($Na_2S_2O_3$)<br>+100% $N_2$ (2 atm) | ++++ |
| MJDYP ($Na_2S_2O_3$)<br>+80% $H_2$, 20% $CO_2$ (2 atm) | +++ |
| MJDYP ($FeS_2$)<br>+100% $N_2$ (2 atm) | ++ |
| MJDYP ($FeS_2$)<br>+80% $H_2$, 20% $CO_2$ (2 atm) | − |
| MJDYP ($Fe_2S_3$)<br>+100% $N_2$ (2 atm) | ++ |
| MJDYP ($Fe_2S_3$)<br>+80% $H_2$, 20% $CO_2$ (2 atm) | − |
| MJDYP (S + $Na_2S_2O_3$)<br>+100% $N_2$ (2 atm) | ++++ |
| MJDYP (S + $FeS_2$)<br>+100% $N_2$ (2 atm) | ++ |
| MJDYP ($Na_2S_2O_3$ + $FeS_2$)<br>+100% $N_2$ (2 atm) | ++ |

MJDYP, media (see text)
Temperature for cultivation, 75°C; pH of all media, 7.5 at room temperature
[a] ++++, $>1.0 \times 10^9$ cells/ml; +++, $>5.0 \times 10^8$, $<1.0 \times 10^9$ cells/ml; ++, $>1.0 \times 10^8$, $<5.0 \times 10^8$ cells/ml; +, $<1.0 \times 10^8$ cells/ml; −, not grown
[b] As an electron acceptor, $S^0$ was added to the medium at 30 g/l, or $Na_2S_2O_3$, $FeS_2$, or $Fe_2S_3$ was added at 20 mM

tion time of about 45 min at pH 7.5 (Fig. 2A). No growth was observed at 85°C. Growth of the new isolate at 75°C occurred between pH 5.3 and 9.3, with optimum growth at about pH 7.2 (Fig. 2B). No growth was detected below pH 5.3 or above pH 9.3.

The new isolate required sea salts for growth. It grew over the concentration range of about 6.6 to 79 g/l of sea salts, with optimum growth at about 40 g/l sea salts at 75°C and pH 7.5 (Fig. 2C). Below 6.6 or above 79 g/l sea salts, growth was inhibited.

The growth rate of the isolate was enhanced at a hydrostatic pressure of 20 MPa (Fig. 2D). The reference strain, *Thermosipho africanus*, was found to be sensitive to elevated hydrostatic pressure. These results indicate that the new isolate is a barophilic microorganism with an optimum pressure for growth similar to the in situ hydrostatic pressure (10 MPa) at the isolation site.

## Nutrition

Organic substrates that support heterotrophic growth were determined in the absence or presence of $Na_2S_2O_3$. In either case, the isolate grew in media containing complex substrates such as yeast extract, peptone, and tryptone (Table 2). In addition, higher growth yields were observed in media containing 0.2% of the substrates than at 0.02% (Table 2). No growth occurred on starch, casamino acids, xylan, pectin, chitin, several sugars, organic acids, or amino acids. Although the new isolate did not grow in the medium containing casein in the absence of $Na_2S_2O_3$, it could utilize casein as a sole carbon and energy source in the presence of $Na_2S_2O_3$ (Table 2).

The fermentation of carbohydrates in the presence of thiosulfate was investigated because Ravot et al. (1996) have detected this ability in *T. africanus*. In media containing yeast extract, peptone, tryptone, or casein, growth of the new isolate was highly improved by the addition of maltose or glucose (Table 2). When the isolate was grown in media containing yeast extract, peptone, or tryptone, galactose weakly enhanced the growth yield (Table 2). However, each of the carbohydrates tested had the effect of increasing the maximum cell yield during growth with casein (Table 2). These results indicated that the new isolate was able to ferment some carbohydrates using $Na_2S_2O_3$ as an electron acceptor.

## Fatty acid composition of cellular lipids and DNA base composition

Analysis of the acid methanolysis products of cellular lipids from the new isolate and *T. africanus* by thin-layer chromatography revealed two components. The two components were believed to represent a monocarboxylic fatty acid methyl ester and a long-chain dicarboxylic fatty acid dimethyl ester based on their migration properties ($R_f$ value, 0.92 and 0.84, respectively). Huber et al. (1989) reported that two components of cellular lipids from *T. africanus* were detected in approximately equal proportions by thin-layer chromatography. In this study, almost equal proportions of monobasic and dibasic fatty acids were also observed; however, the proportions of monobasic and dibasic fatty acids were 27% and 70%, respectively, in the case of strain IHB1. Gas chromatographic analysis of the monobasic fatty acids of the isolate revealed a composition similar to that of *T. africanus*, that is, 9.8% $C_{14:0}$, 0.7% iso-$C_{15:0}$, 7.7% $C_{15:0}$, 1.4% anteiso-$C_{15:0}$, 74.3% $C_{16:0}$, 0.5% iso-$C_{17:0}$, 0.4% $C_{17:0}$, 0.5% anteiso-$C_{17:0}$, 2.9% $C_{18:0}$, and 1.8% anteiso-$C_{18:0}$. The GC content of the genomic DNA of strain IHB1 was found to be 31.4 ± 0.5 mol% (Table 3).

## Phylogenetic analyses

The almost complete sequence (1503 bp) of the 16S rRNA gene from strain IHB1 was determined. In addition, the partial rDNA sequences (corresponding to positions 28–514 of the 16S rDNA sequences of *E. coli*) were determined with respect to nine other isolates. All partial rDNA sequences of the isolates had more than 99.8% similarity with the sequence of the strain IHB1. The rDNA

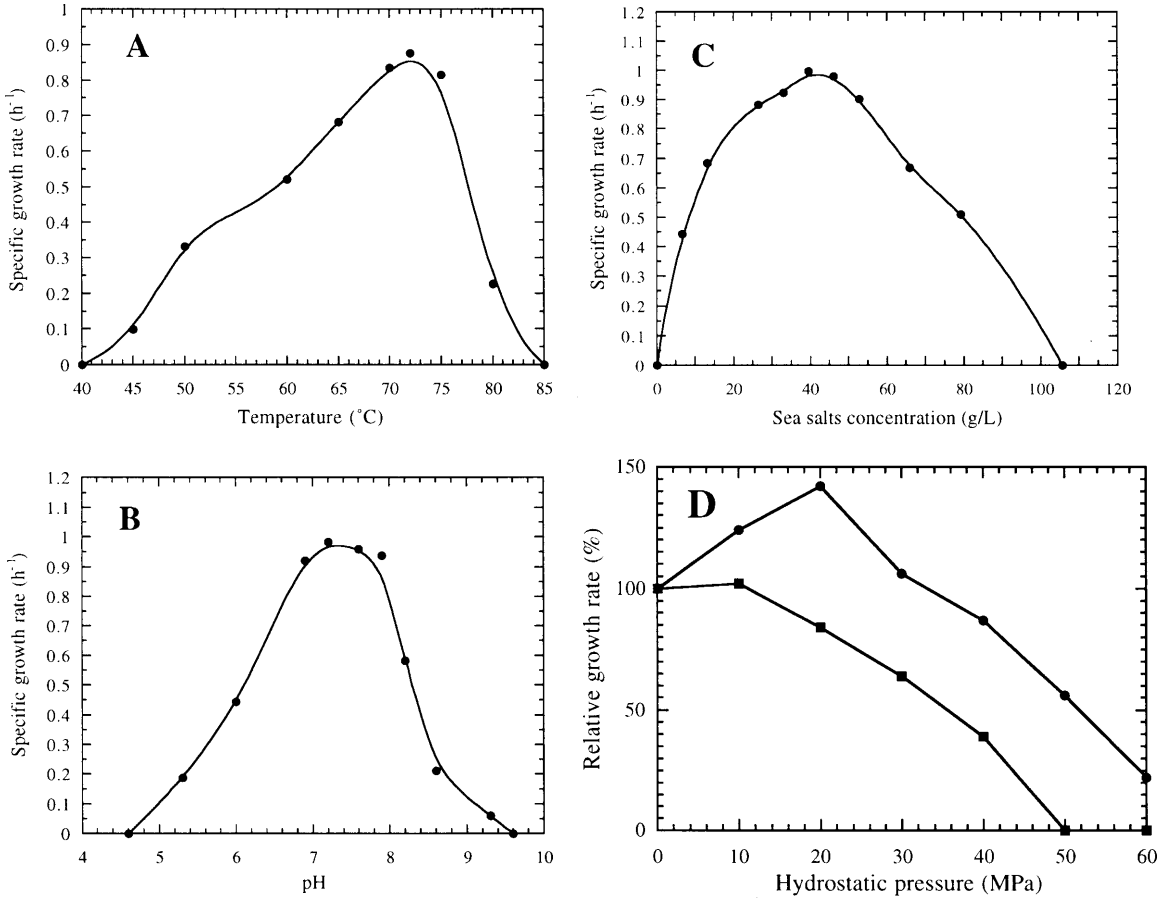


**Fig. 2A–D.** Effects of temperature (**A**), pH (**B**), sea salts concentration (**C**), and hydrostatic pressure (**D**) on growth of *T. japonicus*. **D** The growth rate of *T. japonicus* IHB1 (*circles*) and that of *T. africanus* (*squares*) were determined in MJDYP medium at 73°C and pH 7.5 using the "DEEP-BATH" system

**Table 2.** Substrate utilization by *Thermosipho japonicus*

| Substrate | Maximum cell yield with | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | $Na_2S_2O_3$ (20 mM) | Starch | Sucrose | Maltose | Ribose | Glucose | Galactose |
| Yeast extract (0.2%) Peptone (0.2%) | +++[a] | +++ | ++ | +++ | ++++ | +++ | ++++ | ++++ |
| Yeast extract (0.02%) | + | ++ | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Yeast extract (0.2%) | ++ | +++ | ++ | ++ | ++++ | ++ | +++ | +++ |
| Peptone (0.02%) | − | − | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Peptone (0.2%) | + | ++ | + | + | ++ | + | ++ | ++ |
| Tryptone (0.02%) | − | − | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Tryptone (0.2%) | ++ | +++ | ++ | ++ | ++++ | ++ | ++++ | +++ |
| Casein (0.02%) | − | ++ | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Casein (0.2%) | − | ++ | +++ | +++ | ++++ | +++ | +++ | +++ |

Cells were grown at 75°C under a gas phase of $N_2$ (100%, 200 kPa); pH of all media was adjusted to 7.5 at room temperature
Each carbohydrate was added at a concentration of 20 mM in the medium containing 20 mM $Na_2S_2O_3$
[a] ++++, $>1.0 \times 10^9$ cells/ml; +++, $>5.0 \times 10^8$, $<1.0 \times 10^9$ cells/ml; ++, $>1.0 \times 10^8$, $<5.0 \times 10^8$ cells/ml; +, $<1.0 \times 10^8$ cells/ml; −, not grown; N.D., not determined

**Table 3.** DNA–DNA hybridization among *Thermosipho* members

| Strain | G + C content (mol%) | DNA–DNA hybridization (%) with | | |
|---|---|---|---|---|
| | | IHB1 | *T. africanus* | *T. melanesiensis* |
| Strain IHB1 | 31.4 | 100 | 50 | 27 |
| *T. africanus* | 30.0[a] | 47 | 100 | 21 |
| *T. melanesiensis* | 30.5[b] | 20 | 17 | 100 |

[a] This value was originally reported by Huber et al. (1989) and was confirmed in this study
[b] This value is taken from Antoine et al. (1997)

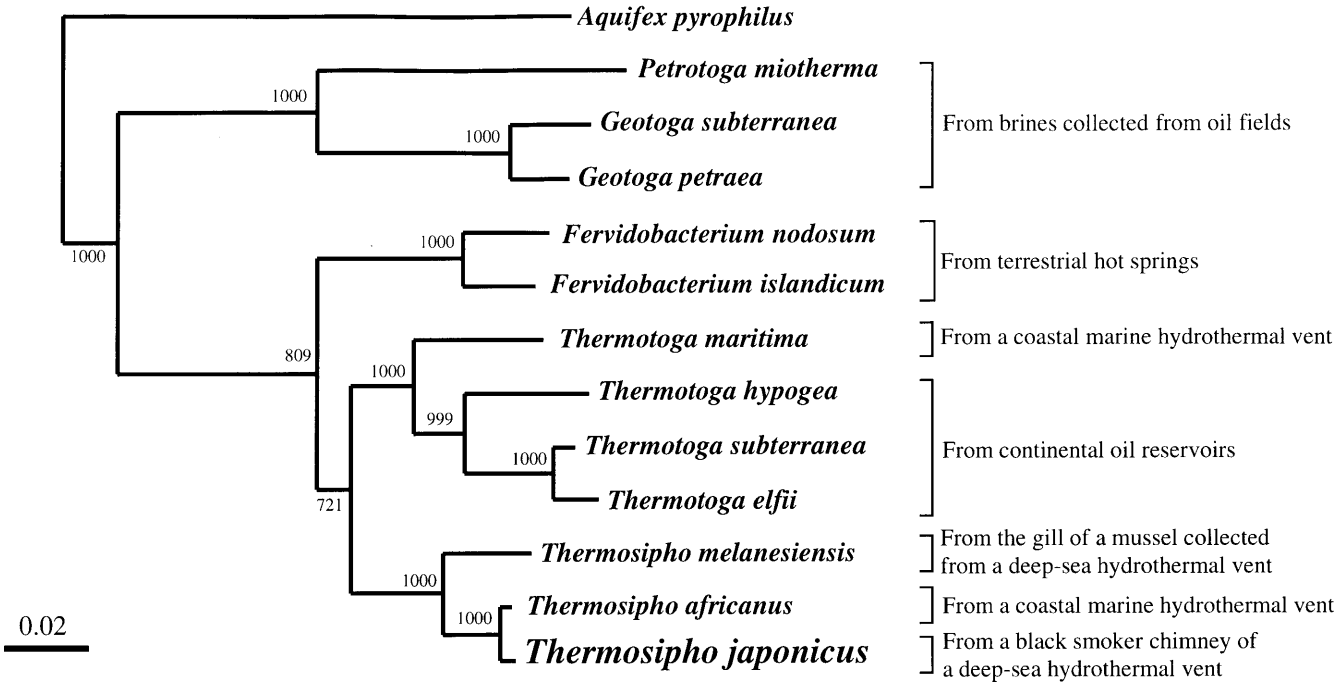


**Fig. 3.** Phylogenetic tree of representative members of the order *Thermotogales* inferred from 16S rDNA sequences by the neighbor-joining method using 1482 homologous sequence positions for each organism. Each number represents the bootstrap value for branching (1000 replicates). The *scale bar* indicates 2 substitutions per 100 nucleotides. The 16S rDNA sequences in this figure are from GenBank with the following accession numbers: *Aquifex pyrophilus*, M83548; *Petrotoga miotherma*, L10657; *Geotoga subterranea*, L10659; *G. petraea*, L10658; *Fervidobacterium nodosum*, M59177; *F. islandicum*, M59176; *Thermotoga maritima*, M21774; *T. hypogea*, U89768; *T. subterranea*, U22664; *T. elfii*, X80790; *Thermosipho melanesiensis*, Z70248; *T. africanus*, M83140; *T. japonicus*, AB024932

sequence of the strain IHB1 was most closely related to those of *T. africanus* (98.7%) and *T. melanesiensis* (95.6%). These values suggest that the new isolate is a member of the genus *Thermosipho* within the order *Thermotogales*. To determine the phylogenetic relationship of the isolate to other *Thermotogales* members, phylogenetic trees were constructed based on the neighbor-joining method and the maximum-likelihood method. Phylogenetic analyses by the neighbor-joining and maximum-likelihood methods indicated exactly identical topology, indicating that the new isolate was most closely related to *T. africanus* (Fig. 3). The bootstrap confidence estimation also revealed high significance in the placement of IHB1 (Fig. 3).

### DNA–DNA hybridization

The results of DNA–DNA hybridization analysis comparing *Thermosipho* strains are shown in Table 3. As expected from the result of phylogenetic analysis, the new isolate was closely related to *T. africanus* isolated from a shallow marine hydrothermal vent system rather than to *T. melanesiensis* obtained from a deep-sea hydrothermal vent system (Table 3). The average hybridization value comparing the new isolate and *T. africanus* was 49%, and the value comparing the new isolate and *T. melanesiensis* was 24%.

## Discussion

A novel barophilic, extremely thermophilic bacterium was isolated from a black smoker chimney at the Iheya Basin in the Okinawa area, Japan. The isolate was found to be a strictly anaerobic, obligately heterotrophic microorganism capable of growth using complex substrates such as yeast extract, peptone, and tryptone. The presence of sulfur or thiosulfate as an electron acceptor strongly improved its growth, and several carbohydrates were fermented with yeast extract, peptone, tryptone, and casein in the presence of thiosulfate. Based on the results of 16S rDNA sequence analysis, the new isolate was found to be a member of the genus *Thermosipho* within the order *Termotogales*. To date, two species have been described within the genus *Thermosipho* (Huber et al. 1989; Antoine et al. 1997). *T. africanus*, the first *Thermosipho* species identified, was isolated from a coastal marine hydrothermal vent system at Obock, Djibouti, Africa (Huber et al. 1989), while *T. melanesiensis* was isolated from the gills of a deep-sea hydrothermal vent mussel, *Bathymodiolus brevior*, inhabiting the bottom of a black smoker at the Lau Basin, in the southwestern Pacific Ocean (Antonine et al. 1997). Although the new isolate was obtained from a deep-sea hydrothermal vent environment, phylogenetic analysis revealed that the new isolate is more closely related to *T. africanus* than to *T. melanesiensis*.

*T. africanus* grows between 35° and 77°C ($T_{opt}$ = 75°C) and is a neutrophilic marine bacterium showing an absolute requirement for NaCl for growth (Huber et al. 1989). It grows only on complex substrates as a sole carbon and energy source; however, it is able to ferment various carbohydrates in conjunction with yeast extract, peptone, and tryptone using sulfur or thiosulfate as a electron acceptor (Huber et al. 1989; Rovot et al. 1996). These features support the phylogenetic relatedness between *T. africanus* and strain IHB1. However, several differences were also found. The new isolate is able to utilize casein to support growth (see Table 2). This is the first example of members of the genus *Thermosipho* growing on casein as a sole carbon and energy source. In addition, fewer types of carbohydrates improved the growth of the new isolate as compared to *T. africanus* (Table 2). Another difference was found in terms of the response to hydrostatic pressure. The growth of the isolate is enhanced by elevated hydrostatic pressure whereas *T. africanus* is sensitive to elevated pressure. In addition, the presence of a relatively high proportion of dibasic fatty acids in strain IHB1 represents a distinctive chemical taxonomic feature. Finally, the results of DNA–DNA hybridization analysis support the view that the new isolate can be differentiated from *T. africanus* at the species level. On the basis of the results presented here, we describe a new species within the genus *Thermosipho*, named *Thermosipho japonicus*. The type strain is *Thermosipho japonicus* IHB1 (JCM 10495).

Description of *Thermosipho japonicus* sp. nov.

*Thermosipho japonicus* (ja.pon'i.cus. M. L. masc. adj. *japonicus*, named after Japan, where this strain was isolated). Straight to slightly curved rods, with an average length of 3–4 μm and a width of about 0.5 μ, surrounded by a sheath-like structure; Gram stain is negative. Nonmotile cells and flagella are not present. Cells occur singly or in pairs in the very early exponential phase of growth, becoming long chains of four to ten cells in the late exponential and stationary phase of growth. Clear round brown-colored colonies are formed on solid medium. Sensitive to vancomycin, streptomycin, or chloramphenicol at 10 μg/ml, and to rifampicin at 100 μg/ml. Sensitive to lysozyme. Strictly anaerobic. The temperature range for growth is 45°–80°C, the optimum being 72°C. The pH range for growth is from 5.3 to 9.3, with optimum growth occurring at pH 7.0–7.5. Growth requires sea salts in the range of 6.6 to 79 g/l with optimum growth occurring at 40 g/l. Growth is inhibited by hydrogen. Growth occurs with yeast extract, peptone, tryptone, or casein as the sole carbon and energy source. Growth on these complex organic materials is enhanced in the presence of sulfur or thiosulfate. The species uses maltose and glucose with yeast extract, peptone, or tryptone; it also uses maltose, glucose, galactose, starch, sacharose, and ribose with casein. The G + C content of the genomic DNA is 31.4%. Cellular lipids show a high proportion of dibasic fatty acids. Major monobasic fatty acids are 9.8% $C_{14:0}$, 0.7% iso-$C_{15:0}$, 7.7% $C_{15:0}$, 1.4% anteiso-$C_{15:0}$, 74.3% $C_{16:0}$, 0.5% iso-$C_{17:0}$, 0.4% $C_{17:0}$, 0.5% anteiso-$C_{17:0}$, 2.9% $C_{18:0}$, and 1.8% anteiso-$C_{18:0}$. On the basis of 16S rDNA analysis, *T. japonicus* is closely related to *T. africanus* with the DNA–DNA hybridization value being 49%.

The type strain is *Thermosipho japonicus* IHB1, JCM 10495 (Japan Collection of Microorganisms, Wako, Japan).

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## References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Andrews KT, Patel BKC (1996) *Fervidobacterium gondwanense* sp. nov., a new thermophilic anaerobic bacterium isolated from nonvolcanically heated geothermal waters of the Great Artesian Basin of Australia. Int J Syst Bacteriol 46:265–269

Antoine E, Cilia V, Meunier JR, Guezennec J, Lesongeur F, Barbier G (1997) *Thermosipho melanesiensis* sp. nov., a new thermophilic anaerobic bacterium belonging to the order *Thermotogales*, isolated from deep-sea hydrothermal vents in the Southwestern Pacific Ocean. Int J Syst Bacteriol 47:1118–1123

Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Benson DA, Boguski MS, Lipman DJ, Ostell J, Ouellette BFF (1998) Genbank. Nucleic Acids Res 26:1–7

Davey MB, Wood WA, Key R, Nakamura K, Stahl D (1993) Isolation of three species of *Geotoga* and *Petrotoga*: two new genera, representing a new lineage in the bacterial line of descent distantly related to the "*Thermotogales*." System Appl Microbiol 16:191–200

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39:224–229

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima*, sp. nov., represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144:324–333

Huber R, Woese CR, Langworthy TA, Fricke H, Stetter KO (1989) *Thermosipho africanus*, gen. nov., represents a new genus of thermophilic eubacteria within the "*Thermotogales*." Syst Appl Microbiol 12:32–37

Huber R, Woese CR, Langworthy TA, Kristjansson JK, Stetter KO (1990) *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the "*Thermotogales*." Arch Microbiol 154:105–111

Ishibashi J, Urabe T (1995) Hydrothermal activity related to arc-backarc magmatism in the Western Pacific. In: Taylor B (ed) Backarc basins: tectonics and magmatism. Plenum Press, New York, pp. 451–495

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov., of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Jeanthon C, Reysenbach AL, L'Haridon S, Gambacorta A, Pace NR, Glenat P, Prieur D (1995) *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch Microbiol 164:91–97

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

Komagata K, Suzuki K (1987) Lipid and cell-wall analysis in bacterial systematics. Methods Microbiol 19:161–207

Larsen N, Olsen GJ, Maidak BL, McCaughey MJ, Overbeek R, Macke TJ, Marsh TL, Woese CR (1993) The ribosomal database project. Nucleic Acids Res 21:3021–3023

Lauerer G, Kristjansson JK, Langworthy TA, König H, Stetter KO (1986) *Methanothermus sociabilis* sp. nov., a second species within the *Methanothermaceae* growing at 97°C. Syst Appl Microbiol 8:100–105

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Moriya K, Inada T, Kyo M, Horikoshi K (1995) Large-scale fermentation under high hydrostatic pressure using a newly developed deep-sea baro/thermophilic collection and cultivation system. J Mar Biotechnol 2:175–177

Olsen GJ (1988) Phylogenetic analysis using ribosonal RNA. Methods Enzymol 164:793–812

Patel BKC, Morgan HW, Daniel RM (1985) *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol 141:63–69

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting microflora. Limnol Oceanogr 25:943–948

Ravot G, Magot M, Fardeau ML, Patel BKC, Prensier G, Egan A, Garcia JL, Ollivier B (1995) *Thermotoga elfii* sp. nov., a novel thermophilic bacterium from an African oil-producing well. Int J Syst Bacteriol 45:308–314

Ravot G, Ollivier B, Patel BKC, Magot M, Garcia JL (1996) Emended description of *Thermosipho africanus* as a carbohydrate-fermenting species using thiosulfate as an electron acceptor. Int J Syst Bacteriol 46:321–323

Sako Y, Takai K, Ishida Y, Uchida A, Katayama Y (1996) *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. Int J Syst Bacteriol 46:1099–1104

Sanger F, Coulson AR, Hong GF, Hill OF, Petersen GB (1982) Nucleotide sequence of bacteriophage λ DNA. J Mol Biol 162:729–773

Stetter KO, Fiala G, Huber G, Huber R, Segerer A (1990) Hyperthermophilic microorganisms. FEMS Microbiol Rev 75:117–124

Takai K, Sako Y (1999) A molecular view of archaeal diversity in marine and terrestrial hot water environments. FEMS Microbiol Lett 28:177–188

Takai K, Inoue A, Horikoshi K (1999) *Thermaerobacter marianensis* gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11 000 m deep Mariana Trench. Int J Syst Bacteriol 49:619–628

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

Windberger E, Huber R, Trincone A, Fricke H, Stetter KO (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Arch Microbiol 151: 506–512

Zillig W, Holz I, Janekovic D, Klenk HP, Imsel E, Trent J, Wunderl S, Forjaz VH, Coutinho R, Ferreira T (1990) *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaebacterium that ferments peptides. J Bacteriol 172:3959–3965