Thermococcus coalescens sp. nov., a cell-fusing hyperthermophilic archaeon from Suiyo Seamount

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A cell-fusing hyperthermophilic archaeon was isolated from hydrothermal fluid obtained from Suiyo Seamount of the Izu-Bonin Arc. The isolate, TS1T, is an irregular coccus, usually 0.5–2 μm in diameter and motile with a polar tuft of flagella. Cells in the exponential phase of growth fused at room temperature in the presence of DNA-intercalating dye to become as large as 5 μm in diameter. Fused cells showed dark spots that moved along in the cytoplasm. Large cells with a similar appearance were also observed upon culture at 87 °C, suggesting the occurrence of similar cell fusions during growth. Transmission electron microscopy revealed that cells in the exponential phase possessed a thin and electron-lucent cell envelope that could be lost subsequently during culture. The fragile cell envelope must be related to cell fusion. The cells grew at 57–90 °C, pH 5.2–8.7 and at NaCl concentrations of 1.5–4.5%, with the optima being 87 °C, pH 6.5 and 2.5% NaCl. The isolate was an anaerobic chemo-organotroph that grew on either yeast extract or tryptone as the sole growth substrate. The genomic DNA G+C content was 53.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the isolate was closely related to Thermococcus species. However, no significant DNA–DNA hybridization was observed between genomic DNA of strain TS1T and phylogenetically related Thermococcus species. We propose that isolate TS1T represents a novel species, Thermococcus coalescens sp. nov., with the name reflecting the cell fusion activity observed in the strain. The type strain is TS1T (=JCM 12540T = DSM 16538T).

Micro-organisms establish individuality by acquiring cell surface structures, such as the cell envelope, peptidoglycan, outer membrane and/or surface layer (Sleytr & Messner, 1988). Before the acquisition of such structures, it is thought that micro-organisms may have frequently fused to exchange genetic materials (Wächtershäuser, 2003). Such ancient pre-cells are expected to have had no surface structures. The only known contemporary cell wall-less
micro-organisms are the members of *Mycoplasma* in *Bacteria* and *Thermoplasmata* in *Archaea*. The genus *Thermoplasmata* currently has only two species, *Thermoplasma acidophilum* (Darland *et al.*, 1970) and *Thermoplasma volcanium* (Segerer *et al.*, 1988). *Thermoplasma acidophilum* is devoid of a cell envelope but has a cytoplasmic membrane, while *Thermoplasma volcanium* has a thin and electron-lucent cell envelope surrounding a cytoplasmic membrane (two-layered) which together are called the triple-layered membrane (Segerer *et al.*, 1988). Cell fusion has been suggested in *Thermoplasma acidophilum* isolates, but without concrete evidence (Yasuda *et al.*, 1995).

*Thermococcus* is a genus belonging to the order *Thermococcales*, the deepest branch of the phylum *Euryarchaeota* in the domain *Archaea* (Zillig, 1992). The two other genera of *Thermococcales*, *Pyrococcus* (Fiala & Stetter, 1986) and *Palaeococcus* (Takai *et al.*, 2000), are distinguished from *Thermococcus* by 16S rRNA gene sequences, genomic DNA G+C content and/or the optimum temperature for growth. *Thermococcus* species are usually spherical, 0.3–2 μm in diameter and obligately anaerobic chemo-organotrophs feeding on peptide substrates with optimal growth temperatures ranging from 75 to 88 °C. As far as has been reported, *Thermococcus* species have thick and/or electron-dense cell envelopes (Zillig *et al.*, 1983; Miroshnichenko *et al.*, 1989, 1998, 2001; Kobayashi *et al.*, 1994; González *et al.*, 1995, 1999; Huber *et al.*, 1995; Godfroy *et al.*, 1996; Dirmeier *et al.*, 1998). *Thermoplasma*-like cell surface structures have not been reported previously in *Thermococcus* species.

In the present study, we report the isolation and characterization of strain TS1T which has a thin and electron-lucent cell envelope. Cells of the isolate fuse at room temperature in the presence of DNA-intercalating dye.

In June 2001, we drilled holes and inserted casing pipes in to the caldera of Suiyo Seamount (28° 34′ N 140° 38′ E). Suiyo Seamount is located in the Izu-Bonin Arc in the Pacific Ocean, 830 km south of Tokyo, and has a floor that is 1380 m deep (Urabe *et al.*, 2001). In October 2001, hydrothermal fluids emitted from the casing pipes were collected by diving using the manned submersible *Shinkai 2000* of the Japan Marine Science and Technology Center. Particulate materials in several litres of the fluid venting from the casing pipe APSK 07 (28° 34′ 17″ N 140° 38′ 38″ E) were collected in situ on a Supor 200 filter (diameter 142 mm, pore size 0.2 μm; Pall) (Nakagawa *et al.*, 2004). The maximum temperature of the fluid was about 100 °C. The filter was frozen at −20 °C on the mother ship R/V *Natsushima*.

The filter was inoculated into Tc medium containing (l−1)
25 g NaCl, 0.33 g KCl, 2.8 g MgCl2.6H2O, 3.4 g MgSO4.7H2O, 10 mg NaBr, 0.3 g K2HPO4, 0.25 g NH4Cl, 0.025 g FeSO4.7H2O, 10 ml each of trace minerals and vitamins solution (Balch *et al.*, 1979), 3 g Bacto-yeast extract, 3 g Bacto-tryptone, 10 g elemental sulfur, 0.5 g Na2S.9H2O and 1 mg resazurin, at pH 6.5 in an anaerobic workstation (Concept Plus; Ruskin Technology) in which the gas phase was N2:H2:CO2 (80:10:10). The enrichment culture was incubated at 77 °C for 16 h. Purification of micro-organisms was performed by the dilution-to-extinction method and then by single colony isolation with a plate of 0.8% Gelrite (Wako) containing Pc medium that contained (l−1) 13.8 g NaCl, 0.352 g KCl, 2.75 g MgCl2.6H2O, 3.5 g MgSO4.7H2O, 0.75 g CaCl2, 7.5 g SrCl2.6H2O, 15 mg H2BO3, 0.05 g NaBr, 0.05 mg Ki, 0.5 g KH2PO4, 2 mg (NH4)2Ni(SO4)2.6H2O, 10 ml trace minerals (Balch *et al.*, 1979), 5 mg citric acid, 1 g Bacto-yeast extract, 5 g Bacto-tryptone, 30 g elemental sulfur, 0.5 g Na2S.9H2O and 1 mg resazurin at pH 6.5. Plates with Pc medium were firmer and easier to streak on than those prepared with Tc medium. The plates were incubated at 70 °C under anaerobic conditions using AnaeroPouch (A-17; Mitsubishi Gas Chemical). The single colony isolation technique was repeated and a single colony was isolated as strain TS1T. Isolate TS1T was grown at 87 °C for 8–10 h in Tc medium for successive cultures. Such cultures remained effective as inocula for at least 3 months. For longer storage, cultures were frozen with 15% (v/v) glycerol in a freezer at −80 °C or in liquid nitrogen.

*Thermococcus celer* JCM 8558T, *Thermococcus profundus* JCM 9378T, *Thermococcus gorgonarius* JCM 10552T, *Thermococcus guaymasensis* JCM 10136T, *Thermococcus fumicolans* JCM 10128T, *Thermococcus stetteri* JCM 8559T, *Thermococcus gammatolerans* JCM 11827T, *Thermococcus peptonophilus* JCM 9653T and *Thermotoga mariitima* JCM 10099T were obtained from the Japan Collection of Micro-organisms (JCM). *Thermococcus siciuli* DSM 12349T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Thermococcus hydrothermalis* CNCMI 1319T was a gift from Dr Anne Godfroy of IFREMER.

An optical microscope (Eclipse E600; Nikon) was used for phase-contrast and epifluorescence microscopy with the Live/Dead BacLight Bacterial Viability kit (L-7007; Molecular Probes; hereafter termed Live/Dead). Solutions A and B from the kit were diluted fivefold with dimethylsulfoxide and 0.15 μl of each solution was added to each 20 μl sample.

For transmission electron microscopy (TEM), cells were centrifuged and suspended in a small volume of Tc medium devoid of elemental sulfur. They were fixed with glutaraldehyde, platinum-shadowed and observed for flagella (Fiala & Stetter, 1986). For observation of thin sections, a similarly prepared cell suspension was mixed with an equal volume of a fixative containing 5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) and fixed at 4 °C for 30 min. Thin sections were prepared and observed as described previously (Honda & Inouye, 2002).

Growth substrates utilizable by isolate TS1T were examined by replacing yeast extract and tryptone in Tc medium by one
of the following nutrients at 0-2 %: yeast extract, tryptone, Casamino acids (containing glutamine, asparagine and tryptophan, each at 0-1%), starch, sucrose, maltose, glucose, citrate, lactate and acetate. The medium was inoculated with 10^6 cells ml^-1 and incubated at 87 °C for 8 h.

Genomic DNA was prepared by a standard protocol using cetyl trimethyl ammonium bromide (Wilson, 2002). A fragment of the 16S rRNA gene was amplified from genomic DNA by PCR with LA Taq polymerase (Takara Bio) according to the manufacturer’s protocol, using forward (5’-ATTCCGTTGTGCTGCGG-3’) and reverse (5’-AGGAGGTGATCGAGCCGTTAAC-3’) primers. PCR was performed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. The product was blunted by T4 DNA polymerase and ligated into the Smal site of pUC119. The ligation mixture was transformed into Escherichia coli JM109. Four recombinant plasmids were purified and sequenced with standard protocols using BcaBEST sequencing primers M13-20 and RV-M (Takara Bio). An oligonucleotide of an internal sequence 5’-ATTGGGCTAAAGCGTCCGTAG-3’ was also used as a sequencing primer. The sequences of the clones were determined and the consensus sequence was generated using MacVector 4.0 (Accelrys).

For analyses of DNA G+C content and DNA–DNA hybridization, genomic DNAs prepared as described above were treated with RNase A (0.1 mg ml^-1) and RNase T1 (0.014 U ml^-1) overnight at 37 °C, followed by extraction with phenol/chloroform and precipitation with ethanol by standard protocols. The ratios of A260/A230 and A280/A260 of the obtained preparation, which are indicative of contamination by sugars and proteins, were less than 0.45 and 0.55, respectively. The G+C content of DNA was determined by HPLC analysis of deoxyribonucleotides after nuclease P1 digestion (Katayama-Fujimura et al., 1984). Microplate DNA–DNA hybridizations were performed in the presence of 50 % formamide (Ezaki et al., 1989). The temperature of hybridization was set at Tm = 45 °C (Goris et al., 1998), where the Tm was calculated from the DNA G+C content (Marmur & Doty, 1962).

Isolate TS1^T was an irregular coccus and motile with a polar tuft of flagella (Fig. 1). The cells were mostly 0.5–2 μm in diameter, but cells as large as 5 μm in diameter were sometimes observed. Some cells had an intracellular spot that appeared bright on phase-contrast and dark on epifluorescence microscopy with Live/Dead (see Supplementary Fig. S1 in IJSEM Online). Cells with a transparent appearance became dominant after overnight culture. These cells, which we named see-through cells (STCs), showed an intracellular spot that appeared dark on phase-contrast and bright on epifluorescence microscopy (see Supplementary Fig. S1 in IJSEM Online). The spots in cocci and STCs appeared to be different based on the optical microscopic observations, but electron micrographs suggested that they are both likely to be a cluster of dense particles (Fig. 2d, f).

The dense particles present in the STCs may have bound nucleic acids leading to epifluorescence when treated with the intercalating dye. Green epifluorescence of STCs with Live/Dead suggested that they might be living, but STCs were likely to be dead for reasons described below. The mode of multiplication of isolate TS1^T remains unknown. Although a diplococcal form of cells, suggestive of binary fission, was frequently observed, we cannot distinguish from static images whether the cells were dividing or fusing. Microscopic observations during growth will be required to settle this issue (Horn et al., 1999).

TEM revealed that the cytoplasm of cells grown for 8 h was heterogeneous, with electron-lucent inner and electron-dense outer regions (Fig. 2a). There were dense particles scattered in the outer region. The cells had a 5 nm-thick cytoplasmic membrane. Most cells (94 %) had a 4 nm-thick cell envelope that was separated by about 8 nm from the cytoplasmic membrane (Fig. 2b). The envelope was broken in some cells, suggesting that it is fragile. The remainder (6 % of 8 h-grown cells) lacked a cell envelope (Fig. 2c). These observations suggest that the cells were formed with the envelope but it could be lost during subsequent culture. The cell surface structures, with and without cell envelope, appeared similar to those of Thermoplasma volcanium (Segerer et al., 1988) and Thermoplasma acidophilum (Darland et al., 1970), respectively. The cytoplasm of 16 h-grown cells was rather homogeneous and the dense particles appeared to have been gathered to form a cluster (Fig. 2d). These cells had no apparent envelope, but possessed a 4 nm-thick electron-dense surface layer (Fig. 2e), which adhered more to the cytoplasmic membrane than the envelope of 8 h-grown cells. The cell surface of 16 h-grown cells appears similar to that of the 8 h-grown envelope-less cells except for the thickness of the surface layer. The cytoplasmic space of STCs was almost vacant apart from the dense particles and cytoplasmic substances surrounding them (Fig. 2f), consistent with their transparent

Fig. 1. Electron micrograph of a platinum-shadowed cell of Thermococcus coalescens TS1^T. Bar, 200 nm.
appearance on optical microscopy. Analysis of the dense particles by energy dispersive X-ray fluorescence spectrometry indicated that the dense particles contain Fe and S (Supplementary Fig. S2 in IJSEM Online). Similar dense particles were also observed in *Thermococcus celer* (Zillig *et al.*, 1988).

When we observed cells grown in Tc medium with epifluorescence microscopy, we occasionally observed cell fusion. Further investigation indicated that, in order to detect cell fusion, cells in the exponential phase of growth should be observed with epifluorescent dyes such as Live/Dead and acridine orange immediately after sampling from culture. However, probably due to lack of synchronization of growth, non-fusing diplococci were also found. Growth conditions were optimized to improve growth synchronization. The cells were first grown at 75°C overnight in Tc medium devoid of elemental sulfur and then the culture was inoculated into Pc medium, which has a lower NaCl concentration (1·4 % NaCl) than Tc medium (2·5 % NaCl). When examined microscopically after 7·5 h of culture at 87°C, the large diplococci were found to fuse (Fig. 3). The green epifluorescence of Live/Dead suggested that the fused cells were alive. TEM revealed that all the cells in the Pc medium had a thin and electron-lucent envelope, suggesting that the cells with the envelope are involved in fusion.

However, some cells had broken envelopes and so the possibility that only cells with broken envelopes can fuse cannot be discounted. Some fused cells showed dark spots that moved along in the cytoplasm (Fig. 3). Cells as large as 5 μm in diameter with a similar appearance were also observed during culture at 87°C, suggesting that they were likely to have fused during growth.

Why was the DNA-intercalating dye necessary to observe fusion? We speculate that there may be some interaction between DNA and the cytoplasmic membrane, similar to that found in rapidly growing *E. coli* (Slater *et al.*, 1995). It is possible that the intercalation of DNA may release the cytoplasmic membrane from the DNA to allow fusion.

In batch cultures of isolate TS1 in Tc medium, the density of cocci suddenly decreased after reaching a peak at a cell density greater than 10^8 cells ml^{-1} (Supplementary Fig. S3 in IJSEM Online). In contrast, the density of STCs increased from just before the peak, suggesting the transformation from coccus to STC. The ATP concentration in the culture followed the density of cocci, suggesting that only cocci produced ATP. Therefore, STCs are likely to represent dead cells. The sudden death seems to be related to the clustering of dense particles. Similar sudden death has also been reported in *Thermococcus celer* (Zillig *et al.*, 1983).
Isolate TS1T grew at 57–90 °C, pH 5–2-8.7 and NaCl concentrations of 1.5–4.5 %, with the optima at 87 °C, pH 6.5 and 2.5 % NaCl (Supplementary Fig. S4 in IJSEM Online). Under the best conditions, the apparent growth rate was as high as 2.5 h⁻¹. Among the growth substrates tested, only yeast extract supported the growth of isolate TS1T at 87 °C. At 77 °C, tryptone also supported rapid growth. We cannot explain why tryptone failed to support growth at 87 °C. Other growth substrates, such as Casamino acids, starch, sucrose, maltose, glucose, citrate, lactate and acetate, did not support the growth of isolate TS1T. H₂/CO₂ (80:20) headspace gas did not support autotrophic growth in Tc medium devoid of yeast extract and tryptone. Elemental sulfur was stimulatory, but not required for growth of isolate TS1T.

Isolate TS1T was sensitive to rifampicin and novobiocin but resistant to chloramphenicol, streptomycin, tetracycline and

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ampicillin at 0·1 mg ml$^{-1}$ at 80 °C. The effectiveness of the antibiotics was verified by using *Thermotoga maritima* as a control at the same temperature.

The core lipids of rapidly growing cultures of TSI$^T$ were analysed by TLC and the hydrocarbon chains were analysed by GLC (Sugai et al., 2000). The core lipids consisted of archaeol (34% by weight), triacyl-calarchaeol (5%) and calarchaeol (63%). No cyclopentane ring-containing core lipids were found, similar to other *Thermococcus* species (Sugai et al., 2004).

The genomic DNA G + C of isolate TSI$^T$ was 53·9 mol%. An almost-complete sequence (1489 bp) of the 16S rRNA gene was determined and was deposited in the DDBJ database. A BLAST search (Altschul et al., 1997) for similar sequences revealed that isolate TSI$^T$ belongs to the genus *Thermococcus*. The similarities between isolate TSI$^T$ and closely related *Thermococcus* species range from 98·8 to 99·6%. A phylogenetic tree indicating the position of isolate TSI$^T$ is shown in Fig. 4.

The status of isolate TSI$^T$ with respect to other *Thermococcus* species was assessed by DNA–DNA hybridization. When the DNA of isolate TSI$^T$ was labelled, the levels of DNA–DNA hybridization to related species ranged from 6 to 57%. When the DNAs of related species were labelled, the DNA–DNA hybridization value to isolate TSI$^T$ was 7–49% (Supplementary Table S1 in IJSEM Online). These values, being less than 70%, strongly suggest that isolate TSI$^T$ represents a novel species (Wayne et al., 1987).

Isolate TSI$^T$ is compared with phylogenetically related *Thermococcus* species in Table 1. Isolate TSI$^T$ is unique in that it has a thin and electron-lucent cell envelope which may be related to the observed proclivity of the cells to fuse. It is distinguishable from *Thermococcus celer* by sensitivity to rifampicin as well as the optimal pH and NaCl concentration for growth, although they are very similar to each other in other characteristics such as sudden death during batch culture. Isolate TSI$^T$ is distinguishable from the other species by the occurrence of sudden death. On the basis of its proclivity for cell-fusion, 16S rRNA gene sequence and low DNA–DNA hybridization values with its closest relatives, we propose that isolate TSI$^T$ represents a novel species of *Thermococcus*. We name this species *Thermococcus coalescens* in recognition of the ability of the cells to fuse.

**Table 1.** Characteristics that distinguish *Thermococcus coalescens* TSI$^T$ from phylogenetically related *Thermococcus* species

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<td>–</td>
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*Inner envelope/outer envelope, averaged values.
†All *Thermococcus* species grow on peptides.
chemo-organotroph. Grows on yeast extract and tryptone. Core lipids consist of archaeol, trialkyl-caldarchaeol and caldarchaeol, without cyclopentane ring. The DNA G+C content is 53-9 mol%.

The type strain, TS1T (=JCM 12540T = DSM 16538T), was isolated from hot water emitted from a casing pipe driven into a hydrothermal site at Suiyo Seamount (28°34'17"N 140°38'38"E) at a depth of 1380 m.

Acknowledgements

We thank the Shinkai 2000 team and the crew of R/V Natsushima for their help with the sampling. We also thank Dr K. Takai (JAMSTEC) for suggesting elemental analysis for dense particles, Mr T. Sakiyama for observation of flagella, Professor M. Akiyama for Latin nomenclature, Professor R. Weisburd for revision of the English of the manuscript and Dr A. Godfroy (IFREMER) for providing T. hydrothermalis. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan through the Special Coordination Fund ‘Archaean Park Project’.

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