Thermococcus celericrescens sp. nov., a fast-growing and cell-fusing hyperthermophilic archaeon from a deep-sea hydrothermal vent

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A fast-growing and cell-fusing hyperthermophilic archaeon was isolated from a hydrothermal vent at Suiyo Seamount, Izu-Bonin Arc, Western Pacific Ocean. Strain TS2^T is an irregular, motile coccus that is generally 0.7–1.5 µm in diameter and possesses a polar tuft of flagella. In the mid-exponential phase of growth, cells that appeared black under phase-contrast microscopy fused at room temperature in the presence of a DNA-intercalating dye, as previously observed in *Thermococcus coalescens*. Cell fusion was not observed in later growth phases. Transmission electron microscopy revealed that the cells in the mid-exponential phase had a 5 nm-thick, electron-dense cell envelope that appeared to associate loosely with the cytoplasmic membrane. As the growth stage progressed, a surface layer developed on the membrane under the envelope and the envelope eventually peeled off. These observations suggest that the surface layer prevents the fusion of cells. Cells of strain TS2^T grew at 50–85 °C, pH 5.6–8.3 and at NaCl concentrations of 1.0 to 4.5 %, with optimal growth occurring at 80 °C, pH 7.0 and 3.0 % NaCl. Under optimal growth conditions, strain TS2^T grew very fast with an apparent doubling time of 20 min. It is suggested that the biosynthesis of the surface layer cannot catch up with cell multiplication in the mid-exponential phase and thus cells without the surface layer are generated. Strain TS2^T 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 54.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the isolate belongs to the genus *Thermococcus*. However, no significant DNA–DNA hybridization was observed between the genomic DNA of strain TS2^T and phylogenetically related *Thermococcus* species. On the basis of this evidence, strain TS2^T is proposed to represent a novel species, *Thermococcus celericrescens* sp. nov., a name chosen to reflect the fast growth of the strain. The type strain is TS2^T (=NBRC 101555^T=JCM 13640^T=DSM 17994^T).

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Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Thermococcus celericrescens* sp. nov. TS2^T is AB107768.

Phase-contrast microscope images of cells of strain TS2^T, a movie showing cell fusion, graphs showing the effects of temperature, pH and NaCl concentration on the growth of strain TS2^T and a growth curve are available as supplementary material in IJSEM Online. A supplementary table detailing DNA–DNA hybridization between genomic DNA of strain TS2^T and phylogenetically related *Thermococcus* species is also available.

In the early stages of the evolution of life, micro-organisms are thought to have undergone both cell division and fusion, thus sharing genetic material among them (Woese, 1998; Wächtershäuser, 2003). Cell fusion is also hypothesized to have played a role in the evolution of eukaryotes (Gupta & Golding, 1996; Margulis, 1996; Martin & Müller, 1998; Moreira & López-García, 1998; Horiike *et al.*, 2004; Lake *et al.*, 2005). However, contemporary prokaryotes have established their uniqueness by producing cell-surface structures, such as the cell envelope, peptidoglycan, the outer membrane and/or a surface layer, and cell fusion has not been demonstrated in free-living micro-organisms. We recently described a cell-fusing archaeon, *Thermococcus coalescens*, that was isolated from a deep-sea hydrothermal

vent (Kuwabara *et al.*, 2005). The presence of DNA-intercalating dye is necessary for the cells to fuse at room temperature. The artificially fused cells show a peculiar feature; epifluorescent granules moving in the cytoplasm. Cells with a similar appearance are also observed during normal cultivation, suggesting the occurrence of similar cell fusion events during growth.

Fusion is rather common in eukaryotic cells as they do not have such firm cell-surface structures as those observed in free-living prokaryotes. Eukaryotic cell fusion occurs during normal developmental processes, where fusing cells recognize each other through the interaction of proteins on the surfaces of the fusing membranes. It is postulated that such an interaction triggers the reorganization of the actin cytoskeleton (Chen & Olson, 2005).

In contrast to eukaryotic fusion, prokaryotic fusion is poorly understood, especially whether it occurs randomly as postulated in the pre-cell hypothesis (Wächtershäuser, 2003) or whether it involves the recognition of a partner for fusion as observed in eukaryotic systems. It is still not known whether cell fusion is restricted to cells of *Thermococcus coalescens* that show signs of morbidity; cell density declines steeply during batch cultures (Kuwabara *et al.*, 2005). If cell fusion occurs in other species and also between species, its impact on lateral gene transfer must be significant, a situation actually observed among hyperthermophiles (Nelson *et al.*, 1999; Inagaki *et al.*, 2006). In the present study, we report the isolation of a novel strain, strain TS2^T, which shows similar cell fusion to that observed in *Thermococcus coalescens*. However, in contrast to *Thermococcus coalescens*, the novel strain grows normally, without a steep decline in cell density during batch cultures.

Samples were collected from a hydrothermal vent at Suiyo Seamount (28° 34' N 140° 38' E) at a depth of 1380 m, which is located in the Izu-Bonin Arc, Western Pacific Ocean. Samples were collected using the manned submersible *Shinkai 2000* (Japan Marine Science and Technology Center; referred to as JAMSTEC) during the NT01-09 cruise (28 September to 26 October, 2001) of R/V *Natsushima* (JAMSTEC). Several litres of a hydrothermal fluid were filtrated *in situ* through a filter with a pore size of 0.2 µm (Kuwabara *et al.*, 2005). The filter was divided into pieces, frozen at -20 °C and transported to the laboratory. Anaerobic cultivation was performed as described previously (Kuwabara *et al.*, 2005). A piece of the filter was inoculated into 12 ml of Tt medium (pH 6.5) in a 100 ml serum bottle that contained (l⁻¹) 27 g NaCl, 0.16 g KCl, 1.4 g MgCl₂·6H₂O, 1.8 g MgSO₄·7H₂O, 0.56 g CaCl₂·2H₂O, 3.8 mg SrCl₂·6H₂O, 2.0 mg NiCl₂·6H₂O, 7.5 mg H₃BO₃, 25 mg NaBr, 12.5 µg KI, 0.5 g KH₂PO₄, 15 ml trace minerals (Balch *et al.*, 1979), 2.5 mg citric acid, 0.5 g Bacto yeast extract, 5.0 g starch, 0.5 g Na₂S₉H₂O and 1.0 mg resazurin. The serum bottle was incubated at 60 °C for 16 h. Successive cultures were performed at 80 °C. A coccus was purified by the dilution-to-extinction method and then by a repeat of the single colony isolation,

performed at 60 °C by using a plate containing 0.8% Gelrite (Wako Pure Chemical Industries) in Tt medium. Single colonies were obtained after 3 days. A colony was liquid-cultured and the purified strain was named TS2^T. After identification of strain TS2^T as a species of the genus *Thermococcus*, the growth medium was changed to TcP medium (pH 7.0) that contained (l⁻¹) 30 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 a vitamin solution (Balch *et al.*, 1979), 3.0 g Bacto yeast extract, 3.0 g Bacto tryptone, 6.0 g PIPES buffer, 10.0 g elemental sulfur, 0.5 g Na₂S₉H₂O and 1.0 mg resazurin. For successive cultures, the isolate was grown overnight in TcP medium at 80 °C. Such cultures remained effective as inocula for at least 3 months. For longer storage, cultures were frozen with 15% (v/v) glycerol at -80 °C or in liquid nitrogen.

Thermococcus coalescens JCM 12540^T was isolated in our laboratory (Kuwabara *et al.*, 2005). *Thermococcus siculi* DSM 12349^T, *Thermococcus celer* JCM 8558^T, *Thermococcus hydrothermalis* CNCMI 1319^T, *Thermococcus profundus* JCM 9378^T, *Thermococcus guaymasensis* JCM 10136^T, *Thermococcus gorgonarius* JCM 10552^T, *Thermococcus fumicolans* JCM 10128^T, *Thermococcus stetteri* JCM 8559^T, *Thermococcus peptonophilus* JCM 9653^T, *Thermococcus gammatolerans* JCM 11827^T and *Thermotoga maritima* JCM 10099^T were used as reference strains (Kuwabara *et al.*, 2005).

An optical microscope (Eclipse E600; Nikon) was used for phase-contrast and epifluorescence microscopy which was performed using the Live/Dead BacLight Bacterial Viability kit (L7007; Molecular Probes, hereafter termed Live/Dead), as described previously (Kuwabara *et al.*, 2005).

For transmission electron microscopy (TEM) to observe flagella, a 5 µl sample of the culture was placed on a Formvar-coated grid. After standing for 3 min, the liquid was absorbed with a filter paper and the cells were fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) for 1 min. The sample was rinsed twice with a drop of pure water, dried at room temperature and shadow-cast with platinum-palladium alloy at an angle of 7°. To observe thin sections, cells were suspended in an elemental sulfur-free culture medium. The suspensions were mixed with an equal volume of a fixative containing 2% glutaraldehyde in cacodylate buffer and incubated at 4 °C for 30 min. Cells grown for 1.5 h were fixed for 90 min using the same fixative. Samples were washed with cacodylate buffer by centrifugation at 1670 g and 25 °C for 20 min and post-fixed with 1% osmium tetroxide in cacodylate buffer at 4 °C for 30 min. After washing with cacodylate buffer, the samples were dehydrated with ethanol, embedded in Spurr's resin and thin-sectioned as described previously (Honda & Inouye, 2002). Transmission electron microscopy was performed using a JEM1010 instrument (JEOL) at 80 kV.

Growth substrates utilizable by strain TS2^T were examined by replacing the yeast extract and tryptone in the TcP

medium by one of the following nutrients at 0.2 %: yeast extract, tryptone, Casamino acids (supplemented with glutamine, asparagine and tryptophan, each at 0.1 %), starch, sucrose, maltose, glucose, citrate, lactate or acetate. The medium was inoculated with 10^6 cells ml^{-1} and incubated at 80 °C for 8 h.

The 16S rRNA gene sequence of strain TS2^T was determined by cloning the gene using plasmid pUC109 and *Escherichia coli* JM109, as described previously (Kuwabara *et al.*, 2005). Genomic DNA, which was free of sugars and proteins, was prepared as described previously (Kuwabara *et al.*, 2005). DNA G+C content 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 ($T_m - 45$) °C (Goris *et al.*, 1998), where the T_m was calculated from the DNA G+C content (Marmur & Doty, 1962).

Cells of strain TS2^T were irregular, motile cocci with a polar tuft of flagella (Fig. 1). Cells were generally 0.7–1.5 µm in diameter; however, cells as large as 3–4 µm in diameter were frequently observed in the mid-exponential phase. Cells exhibited different colours under phase-contrast microscopy, namely, bluish cells with contour, whitish cells with contour and black cells without contour (see Supplementary Fig. S1 in IJSEM Online). The occurrence of symmetric diplococci suggested that strain TS2^T multiplies by binary fission. Asymmetric diplococci were also observed (Fig. 2a), suggesting the occurrence of natural cell fusion in the growth medium (see below).

TEM observations revealed that strain TS2^T had a 5 nm-thick cytoplasmic membrane (Fig. 2b–e). The outer leaflet of the membrane stained more densely than the inner one, indicating that the membrane is vertically asymmetric. Cells in the mid-exponential phase (1.5 h growth) had a 5 nm-thick cell envelope that was densely stained; however, the envelope appeared to be loosely associated with the

Fig. 1. Shadowing of cells of strain TS2^T with platinum–palladium alloy. Cells grown for 6 h were observed. Bar, 500 nm.

membrane (Fig. 2b). The membrane and the envelope were separated by a space of about 12 nm. In the late exponential phase (4 h growth), a surface layer was obvious on the membrane under the envelope and the envelope was being removed in some cells (Fig. 2c). Thus, the envelope of strain TS2^T, although electron-dense, resembled that of *Thermococcus coalescens* in that it is removed during cultivation (Kuwabara *et al.*, 2005). Cells in the early stationary phase (8 h growth) possessed a thicker surface layer (Fig. 2d). In the late stationary phase (16 h growth), cells had a thicker surface layer on which an electron-dense layer was present. In the present study, it was not determined whether this electron-dense layer is the envelope or another newly formed structure. Nevertheless, these observations suggest that the surface structure of strain TS2^T changes dynamically during the course of growth.

Fig. 2. Thin sections of cells of strain TS2^T. (a) An asymmetric cell of strain TS2^T grown for 1.5 h. (b–e) Surface structures of cells grown for 1.5 h (b), 4 h (c), 8 h (d) and 16 h (e). CM, cytoplasmic membrane; CE, cell envelope; SL, surface layer. Bars, 500 nm (a), 50 nm (b–e).

Fig. 3. Fusion of cells of strain TS2^T. An overnight culture of strain TS2^T was inoculated into TcP medium at 10⁶ cells ml⁻¹ and incubated at 80 °C for 1.5 h. A portion of the culture was withdrawn and loaded with Live/Dead. A movie of the phase-contrast microscopy was video recorded (see Supplementary Fig. S2 in IJSEM Online) as described previously (Kuwabara *et al.*, 2005). The photographs shown are snapshots from the movie at designated times after the start of the recording. That the fused cell was living was confirmed by epifluorescence microscopy performed with Live/Dead. The material neighbouring the fusing cells is a precipitate present in the culture medium. Bar, 3 µm.

Among the cells in the mid-exponential phase, only those that appeared black under phase-contrast microscopy (Supplementary Fig. S1 in IJSEM Online) fused at room

temperature in the presence of Live/Dead (Fig. 3 and Supplementary Fig. S2 in IJSEM Online), as observed in the case of *Thermococcus coalescens* (Kuwabara *et al.*, 2005). Thus, the large cells observed in cultures appear to have fused during cultivation, although we were unable to observe natural cell fusion at growth temperature. Cells in other growth phases have never fused at room temperature; this suggests that the surface layer-less surface structure (Fig. 2b) is related to fusion. However, there must be other factors involved, since all the cells in the mid-exponential phase had such a surface structure, but only the black cells fused. Which structure is associated with which cell colour (as observed by phase-contrast microscopy) and how the structure relates to the ability of the cells to fuse remains to be elucidated.

Strain TS2^T grew at 50–85 °C, pH 5.6–8.3 and at an NaCl concentration of 1.0–4.5 % (see Supplementary Fig. S3 in IJSEM Online). Optimal growth occurred at 80 °C, pH 7.0 and 3.0 % NaCl (Table 1). Under the optimal growth conditions, strain TS2^T grew very fast with a doubling time of 20 min without a lag phase (see Supplementary Fig. S4 in IJSEM Online). There was no sudden decline in cell density during batch cultures up to 25 h. Among the growth substrates tested, only yeast extract and tryptone supported growth. Other growth substrates, such as Casamino acids (supplemented with glutamine, asparagine and tryptophan), starch, sucrose, maltose, glucose, citrate, lactate and acetate, did not support the growth of strain TS2^T. A headspace gas mixture of H₂/CO₂ (80:20) did not support autotrophic growth in TcP medium that was devoid of yeast extract and

Table 1. Characteristics that distinguish strain TS2^T from phylogenetically related *Thermococcus* species

Taxa: 1, strain TS2^T (data from this study); 2, *T. siculi* (Grote *et al.*, 1999); 3, *T. celer* (Zillig *et al.*, 1983); 4, *T. coalescens* (Kuwabara *et al.*, 2005); 5, *T. barossii* (Duffaud *et al.*, 1998); 6, *T. hydrothermalis* (Godfroy *et al.*, 1997); 7, *T. profundus* (Kobayashi *et al.*, 1994); 8, *T. kodakaraensis* (Atomi *et al.*, 2004); 9, *T. peptonophilus* (González *et al.*, 1995); 10, *T. gorgonarius* (Miroshnichenko *et al.*, 1998); 11, *T. guaymasensis* (Canganella & Jones, 1994; Canganella *et al.*, 1998); 12, *T. gammatolerans* (Jolivet *et al.*, 2003); 13, *T. stetteri* (Miroshnichenko *et al.*, 1989); 14, *T. fumicolans* (Godfroy *et al.*, 1996). +, Positive; –, negative; NR, not reported; S, stimulatory; R, required.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DNA G+C content (mol%)	54.6	55.8	56.6	53.9	60	58	52.5	52	52	50.6	46	51.3	50.2	54–55
Motility	+	–	+	+	–	+	+	+	+	+	+	+	+/–	+
Growth optimum														
Temperature (°C)	80	85	88	87	82.5	85	80	85	85	80–88	88	88	75–88	85
pH	7	7	5.8	6.5	6.5–7.5	6	7.5	6.5	6	6.5–7.2	7.2	6	6.5	8
NaCl (%)	3	2	4	2.5	1–4	3–4	2–4	3	3	2–3.5	3	2	2.5	2–4
Doubling time at optimum (min)	20	130	50	24	33	90	50	60	25	NR	15	95	72	NR
Growth substrate:*														
Starch	–	–	NR	–	NR	–	+	+	–	–	+	–	+	–
Maltose	–	–	NR	–	–	+	+	NR	–	–	+	–	–	+
Sulfur requirement	S	S	S	S	R	S	R	S	S	R	S	R	R	S
Sudden death	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Rifampicin resistance	–	–	+	–	NR	+	–	NR	–	NR	+	–	–	–

*All *Thermococcus* species grow on peptides.

tryptone. Elemental sulfur was stimulatory; however, it was not required for growth.

Strain TS2^T was sensitive to rifampicin, but resistant to novobiocin, chloramphenicol, streptomycin, tetracycline and ampicillin at 0.1 mg ml⁻¹ and 80 °C. The effectiveness of the antibiotics was verified by using *Thermotoga maritima* as a control bacterium at the same temperature.

The core lipids of strain TS2^T were analysed by TLC and the hydrocarbon chains were analysed by GLC (Sugai *et al.*, 2000). The core lipids consisted of archaeol (41 % by weight), trialkyl-caldarchaeol (1 %) and caldarchaeol (58 %). As with other *Thermococcus* species, no cyclopentane ring-containing core lipids were found (Sugai *et al.*, 2004).

The genomic DNA G+C content of strain TS2^T was 54.6 mol%. An almost complete sequence (1486 bp) of the

16S rRNA gene was determined and deposited in the DNA DataBank of Japan (DDBJ). A BLAST search (Altschul *et al.*, 1997) for similar sequences revealed that strain TS2^T belongs to the genus *Thermococcus*. The sequence similarities between strain TS2^T and closely related *Thermococcus* species range from 98.1 % to 99.2 % (the highest value belonging to *Thermococcus siculi*). A phylogenetic tree indicating the position of strain TS2^T is shown in Fig. 4.

The status of strain TS2^T with respect to other *Thermococcus* species was assessed by DNA–DNA hybridization. When DNA of strain TS2^T was labelled, the levels of DNA–DNA hybridization to related species ranged from 7 % to 56 % (see Supplementary Table S1 in IJSEM Online). When the DNA of related species was labelled, the levels of DNA–DNA hybridization to strain TS2^T ranged from 12 % to 49 %. These values, being less than 70 %, strongly suggest that strain TS2^T represents a novel species (Wayne *et al.*, 1987).

Fig. 4. Phylogenetic tree showing the position of *Thermococcus celericrescens* sp. nov. TS2^T. The 16S rRNA gene sequences (1288 bp) of *Thermococcus* species were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). The tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values greater than 70 % in 1000 resamplings are shown. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per sequence position.

Strain TS2^T and *Thermococcus coalescens* are similar in that they are both fast-growing (Table 1) and have a proclivity to fuse. We found recently that *Thermococcus guaymasensis*, with a doubling time as short as 15 min (Canganella & Jones, 1994), also underwent a similar cell fusion process (unpublished data). From these data, we speculate that fast growth and cell fusion may be related; namely, multiplication is so fast in the mid-exponential phase that biosynthesis of the surface layer can not keep pace with the rate of multiplication. This situation must generate cells without a surface layer (Fig. 2b) and thus they are capable of fusion.

In comparison with phylogenetically related species of the genus *Thermococcus* (Table 1), strain TS2^T is distinguishable from *Thermococcus coalescens* by the absence of sudden death during batch cultures and resistance to novobiocin. Strain TS2^T can be distinguished from *Thermococcus guaymasensis* by a high genomic DNA G+C content, inability to use starch and maltose for growth and sensitivity to rifampicin. It is distinguishable from the other phylogenetically related *Thermococcus* species by the occurrence of surface layer-less cells and/or cell fusion at room temperature. On the basis of the fast growth, proclivity for cell fusion, 16S rRNA gene sequence and the low DNA–DNA hybridization values with its closest relatives, we propose that strain TS2^T represents a novel species of the genus *Thermococcus*. The name *Thermococcus celericrescens* sp. nov. is proposed for strain TS2^T in recognition of its fast-growing nature.

Description of *Thermococcus celericrescens* sp. nov.

Thermococcus celericrescens (ce.le.ri.cres'cens. L. adj. *celer* -eris -ere quick, speedy; L. part. adj. *crescens* growing; N.L. part. adj. *celericrescens* fast-growing).

Cells are irregular cocci, variable in size (0.7–4.0 µm in diameter, depending on the level of cell fusion) and motile with a polar tuft of flagella. Sulfur is not necessary for growth, but enhances it. Cells grow in the temperature range of 50 to 85 °C (optimum at 80 °C), pH range of pH 5.6 to 8.3 (optimum at pH 7.0) and with 1.0–4.5 % NaCl (optimum 3.0 %). The shortest doubling time is 20 min. Strict anaerobe. Obligate chemo-organotroph. Grows on yeast extract and tryptone. Core lipids consist of archaeol, trialkyl-caldarchaeol and caldarchaeol, without a cyclopentane ring. The DNA G+C content is 54.6 mol%.

The type strain, TS2^T (=NBRC 101555^T=JCM 13640^T=DSM 17994^T), was isolated from hot water emitted from a casing pipe driven into a hydrothermal site at Suiyo Seamount (28° 34' N 140° 38' E) at a depth of 1380 m.

Acknowledgements

We thank the captain and crew of R/V *Natsushima*, the *Shinkai 2000* team and members of "Archaean Park Project" for their help with the

sampling. We also thank Professor Akihiko Sugai, Kitasato University, for determination of the lipids. 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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