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Thermococcus marinus sp. nov. and *Thermococcus radiotolerans* sp. nov., two hyperthermophilic archaea from deep-sea hydrothermal vents that resist ionizing radiation

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Abstract Enrichments for anaerobic, organotrophic hyperthermophiles were performed with hydrothermal chimney samples collected from the Mid-Atlantic Ridge at a depth of 3,550 m (23°22′N, 44°57′W) and the Guaymas Basin (27°01′N, 111°24′W) at a depth of 2,616 m. Positive enrichments were submitted to γ-irradiation at doses of 20 and 30 kGy. Two hyperthermophilic, anaerobic, sulfur-metabolizing archaea were isolated. Strain EJ1^T was isolated from chimney samples collected from the Mid-Atlantic Ridge after γ-irradiation at 20 kGy, and strain EJ2^T was isolated from the Guaymas Basin after γ-irradiation at 30 kGy. Only strain EJ2^T was motile, and both formed regular cocci. These new strains grew between 55 and 95 °C with the optimal temperature being 88 °C. The optimal pH for growth was 6.0, and the optimal NaCl concentration for growth was around 20 g l^{-1}. These strains were obligate anaerobic heterotrophs that utilized yeast extract, tryptone, and peptone as a carbon source for growth. Ten amino acids were essential for the growth of strain EJ1^T, such as arginine, aspartic acid, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tyrosine, and valine, while strain EJ2^T was unable to grow on a mixture of amino acids. Elemental sulfur or cystine was required for EJ2^T growth and was reduced to hydrogen sulfide. Rifampicin inhibited growth for both strains EJ1^T and EJ2^T. The G+C contents of the genomic DNA were 52.3 and 54.5 mol% for EJ1^T and EJ2^T, respectively. As determined by 16S rRNA gene sequence analysis, these strains were more closely related to *Thermococcus gorgonarius*, *T. celer*, *T. guaymasensis*, *T. profundus*, and *T. hydrothermalis*. However, no significant homology was observed between them with DNA–DNA hybridization. These novel organisms also possess phenotypic traits that differ from those of its closest phylogenetic relatives. Therefore, it is proposed that these isolates, which are amongst the most radioresistant hyperthermophilic archaea known to date with *T. gammatolerans* (Jolivet et al. 2003a), should be described as novel species *T. marinus* sp. nov. and *T. radiotolerans* sp. nov. The type strain of *T. marinus* is strain EJ1^T (=DSM 15227^T=JCM 11825^T) and the type strain of *T. radiotolerans* is strain EJ2^T (=DSM 15228^T=JCM 11826^T).

Keywords Archaea · Deep-sea hydrothermal vent · Hyperthermophile · Radioresistance · *Thermococcus*

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The GenBank accession numbers for the 16S rRNA sequence of *Thermococcus marinus* strain EJ1^T and *Thermococcus radiotolerans* EJ2^T are AF479012 and AF479013, respectively.

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Introduction

Since the discovery of the first hyperthermophile isolated from a deep-sea hydrothermal system, a methanogen, *Methanococcus jannashii*, growing optimally at 86 °C (Jones et al. 1983), more than 20 different genera of hyperthermophilic archaea have been isolated. Numerous hyperthermophilic strains, belonging to the *Thermococcus* genus, have been isolated and described the last

10 years from various deep-sea hydrothermal vents, shallow marine hydrothermal areas, and more recently, in high-temperature oil reservoirs (Kobayashi 2001; Miroshnichenko et al. 2001; Jolivet et al. 2003a). *Thermococcus* (Zillig et al. 1983), along with *Pyrococcus* (Fiala and Stetter 1986) and *Palaeococcus* (Takai et al. 2000), represent the three genera of the family Thermococcaceae, the single family of the order Thermococcales. These three genera can be distinguished by their optimal growth temperature (between 75 and 88 °C for *Thermococcus* and *Palaeococcus* species and between 96 and 100 °C for *Pyrococcus* members) and by the clustering of their 16S rRNA sequences as separate clades within the Thermococcales (Takai et al. 2000). The genus *Thermococcus* includes at present 22 species that share similar physiological characteristics and can be divided into two groups on the basis of their respective G + C content. One group of strains with high G + C content (from 50 to 58 mol%) includes 12 species: *T. celer* (Zillig et al. 1983), *T. stetteri* (Miroshnichenko et al. 1989), *T. profundus* (Kobayashi et al. 1994), *T. waiotapuensis* (Gonzalez et al. 1999), *T. peptonophilus* (Gonzales et al. 1995), *T. fumicolans* (Godfroy et al. 1996), *T. hydrothermalis* (Godfroy et al. 1997), *T. gorgonarius* (Miroshnichenko et al. 1998), *T. pacificus* (Miroshnichenko et al. 1998), *T. barossii* (Duffaud et al. 1998), *T. siculi* (Grote et al. 1999), and *T. gammatolerans* (Jolivet et al. 2003a). Another group of strains with low G + C content (from 38 to 47 mol%) includes ten species: *T. litoralis* (Neuner et al. 1990), *T. chitonophagus* (Huber et al. 1995), *T. alcaliphilus* (Keller et al. 1995), *T. Zilligii* (Rominus et al. 1997), *T. guaymasensis* (Canganella et al. 1998), *T. aggregans* (Canganella et al. 1998), *T. acidaminovorans* (Dirmeier et al. 1998), *T. barophilus* (Marteinson et al. 1999), *T. aegaeicus* (Arab et al. 2000), and *T. sibiricus* (Miroshnichenko et al. 2001). All these species belonging to the *Thermococcus* genus grow optimally at salt concentrations of 20–40 g l^{-1} NaCl. These microorganisms are organotrophic, thermophilic anaerobes growing at temperatures ranging from 50 to over 100 °C, preferentially on proteinaceous compounds such as yeast extract or peptone in the absence or in presence of sulfur. Some of these strains are able to grow in the absence of elemental sulfur, but elemental sulfur significantly stimulates growth of many strains, and hydrogen sulfide is produced. For some species of the Thermococcales order, molecular hydrogen inhibits growth, and the production of hydrogen sulfide in the presence of elemental sulfur is a type of detoxification reaction.

In the deep-sea hydrothermal environments of the East Pacific Rise, the polychaetes *Alvinella* colonize the walls of active chimneys and are exposed to natural radioactivity levels (^{210}Pb, ^{210}Po, ^{222}Rn) a hundred times higher than what humans are normally exposed to (Cherry et al. 1992). Deep-sea hydrothermal vents could therefore represent an attractive milieu for studying the effects of ionizing radiation on thermophilic microorganisms.

In this paper, we describe two novel strains that were isolated following γ-irradiation of enrichment cultures from deep-sea hydrothermal vents on the Mid-Atlantic Ridge and in the Guaymas Basin. Based on their 16S rRNA sequences and on DNA–DNA hybridizations, we propose strains EJ1^T and EJ2^T as two novel species to be named *T. marinus* sp. nov. and *T. radiotolerans* sp. nov., respectively. These isolates, EJ1^T and EJ2^T, can support high levels of γ-irradiation such as 20 and 30 kGy, respectively. They represent with *T. gammatolerans* the first hyperthermophilic archaea isolated following γ-irradiation of enrichment cultures, and they constitute the most radioresistant hyperthermophilic archaea isolated to date.

Materials and methods

Reference strains

Thermococcus gorgonarius DSM 10395^T (T = type strain), *T. celer* DSM 2476^T, *T. guaymasensis* DSM 11113^T, *T. profundus* DSM 5432^T, *T. gammatolerans* DSM 15229^T, and *Thermotoga maritima* DSM 3109^T were obtained from the Deutshe Sammlung von Mikroorganismen, Braunscweig-Stockeim, Germany. *T. hydrothermalis* AL662^T was kindly provided by Georges Barbier (IFREMER, Plouzané, France).

Culture conditions

The medium referred to as YPS was used to cultivate reference strains and the new isolates. The YPS medium contained per liter of distilled water: 35 g sea salt (Sigma), 3.46 g PIPES, 1 g yeast extract, 4 g peptone, 5 g elemental sulfur, 0.5 g NH_4Cl, 0.35 g KH_2PO_4, 0.2 g $CaCl_2$, 6.7 mg $FeCl_3$, 2.9 mg Na_2WO_4, and 0.1 mg resazurin. The pH was adjusted to 6.8 before autoclaving. Final anaerobiosis was achieved by adding sterile $Na_2S{\cdot}9H_2O$ 5% (w/v) to a final concentration of 0.025%. Cultures in exponential growth phase were stored at 4 °C after the gas phase had been briefly flushed with N_2 in order to remove the H_2S produced during growth.

Collection of samples, enrichments, and isolation

Chimney samples were collected by the submersible *Nautile* during the cruise *Microsmoke* in 1995 in the Mid-Atlantic Ridge from the Snakepit vent field (23°22′N, 44°57′W) at a depth of 3,500 m and during the cruise *Guaynaut* in 1991 in the Guaymas Basin [Gulf of California (27°01′N, 111°24′W)] at a depth of 2,616 m. Samples were immediately transferred into flasks filled with sterile, reduced artificial seawater. The vials were then closed tightly with butyl rubber stoppers and stored at 4 °C until further experiments.

Anaerobic procedures were performed as described by Balch and Wolfe (1976). Enrichment cultures were produced anaerobically in Hungate tubes containing 10 ml of YPS medium and incubated at 85 °C. Aliquots of the positive cultures were irradiated at 20 and 30 kGy on ice with a γ-ray source (^{137}Cs) at a rate of 60 Gy min^{-1} (Institut Curie, Orsay, France). After irradiation, cultures were transferred to YPS medium and incubated at 85 °C. Isolations were performed by plating on YPS medium solidified by 1% of Gelrite (w/v) (Erauso et al. 1995). Plates were incubated in an anaerobic jar at 80 °C (gas phase N_2/CO_2, 80:20, 1 bar). The strains resistant to ionizing radiation that formed colonies were purified after four series of plating and one serial dilution.

Determination of the survival rates following γ-irradiation

The strains were irradiated at the end of the exponential growth phase in YPS medium under anaerobic conditions in Hungate tubes. Air was removed and replaced by N_2 by alternatively applying a vacuum and saturating gas, and Na_2S was added at 0.025% to complete the anaerobiosis. Irradiation was performed on ice using a ^{137}Cs-γ-ray source (Institut Curie) at a rate of 60 Gy min^{-1}. The number of viable cells was estimated with the most probable number technique, based on serial dilutions in anaerobic conditions in their growth medium.

Determination of growth parameters

YPS medium was used unless otherwise stated. To determine the temperature for growth, cells were cultivated in Hungate tubes using N_2 (100%, 100 kPa) as headspace. The optimal pH and NaCl concentration for growth were determined at 85 °C. To determine growth rates at different pHs, YPS medium was modified by using the following buffers (Sigma), each at a concentration of 10 mM: pH 3.5–4.5, no buffer; pH 5.0–6.0, MES buffer; pH 6.5–7.0, PIPES buffer; pH 7.5–8.5, HEPES buffer; and pH 9.0–10.0, no buffer. To determine the optimal NaCl concentration for growth, increasing concentrations of NaCl were added to a medium that contained per liter of distilled water: 10.77 g $MgCl_2.6H_2O$, 3.97 g Na_2SO_4, 0.20 g $NaHCO_3$, 0.09 g KBr, 0.025 $SrCl_2{\cdot}6H_2O$, 0.671 g KCl, 0.26 g H_3BO_3 and 0.003 g NaF, 3.46 g PIPES, 1 g yeast extract, 4 g peptone, 5 g elemental sulfur, 0.5 g NH_4Cl, 0.35 g KH_2PO_4, 0.2 g $CaCl_2$, 6.7 mg $FeCl_3$, 2.9 mg Na_2WO_4, and 0.1 mg resazurin. The pH was adjusted to 6.8 before autoclaving. Final anaerobiosis was achieved by adding sterile $Na_2S{\cdot}9H_2O$ 5% (w/v) to a final concentration of 0.025%. All the experiments were performed in duplicate.

Determination of substrate utilization

The ability of these isolates to use single carbon sources for growth was tested at optimal growth temperature on YPS medium in which yeast extract and peptone were omitted. A filter-sterilized solution of vitamins (10 ml l^{-1}) (Widdel and Bak 1992) was added, and N_2 was used as headspace. This medium was supplemented with one of the following carbon sources: starch, maltose, gelatin, glycogen, sucrose, cellobiose, xylose, or lactose to a final concentration of 0.5% (w/v), while yeast extract, peptone, tryptone, casein hydrolysate, beef extract, casamino acids, succinate, propionate, pyruvate, and acetate were added at a final concentration of 0.2% (w/v).

Possible autotrophic growth was tested in the basal medium using H_2/CO_2 (80:20, 200 kPa) as headspace. The requirement for elemental sulfur and cystine (10 g l^{-1}) was studied by comparing growth yield in the standard YPS and in the same medium lacking these compounds. The possibility for these isolates to use alternative electron acceptors was studied by replacing them with sodium thiosulfate (10 mM), sodium sulfate (20 mM), or sodium sulfite (3 mM). Growth was determined by direct cell counts in a Petroff-Hausser chamber (depth, 0.02 mm) with a phase contrast microscope after incubation at 85 °C.

Antibiotic susceptibility

Sensitivity of strains EJ1^T and EJ2^T to chloramphenicol, ampicillin, penicillin, kanamycin, vancomycin, streptomycin, and rifampicin was tested at 150 µg ml^{-1} in YPS medium at 80 °C. Simultaneous experiments were performed with an antibiotic-sensitive bacterium, *Thermotoga maritma* (Huber et al. 1986). This strain was cultivated under the same conditions to demonstrate the efficiency of the antibiotic at 80 °C.

Optical and electron microscopy

An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe and count the cells. For negative staining, 20-µl bacterial suspension fixed with 2% glutaraldehyde (w/v) was dropped on Formavar/carbon-coated grids (400 mesh) and stained with the 4% uranyl acetate (w/v). Micrographs were taken with a model CM 100 electron microscope (Philips) at an acceleration voltage of 80 kV.

Isolation of DNA and DNA base composition

Genomic DNA of the strains EJ1^T and EJ2^T was isolated by using the procedure described by Charbonnier and Forterre (1994). The DNA was purified on cesium chloride gradient (Sambrook et al. 1989), and purity was checked spectrophotometrically with GeneQuant II RNA/DNA Calculator (Pharmacia Biotech). The G + C content of DNA was determined from the melting point according to Marmur and Doty (1962) using DNA (Sigma) from *Escherichia coli* (52 mol% G + C), *Clostridium perfringens* (24 mol% G + C), and *Micrococcus luteus* (73 mol% G + C) as references.

PCR amplification and cloning of the 16S rDNA

16S rDNA was amplified by PCR with *Taq* polymerase (Promega), using the genomic DNA from strain EJ1^T or EJ2^T as the template and two primers, one specific for archaea (4F, 5′-TCCGGTTGATCCTGCCGG-3′) and one universal (1492R, 5′-GGTTACCTT GTTACGACTT-3′). PCR reactions were typically carried out in a volume of 50 µl containing 50–100 ng template, 100 ng of each of the two specific primers, 250 µM of dNTP, 1.5 mM $MgCl_2$, 1× buffer (Promega), and 2.5 U polymerase. The different steps of PCR were as follows: 5 min at 95 °C; then 25 cycles of 1.5 min at 95 °C, 1.5 min at 53 °C and 2.5 min at 72 °C; and then finally, a polymerization step of 8 min at 72 °C. PCR products were cloned in vector PCRII2.1 (Invitrogen), and several clones were sequenced to ensure the sequence quality using Texas Red-labeled primers, a Thermosequenase kit (RPN 2444, Amersham), and a Vistra 725 automated sequencer. Eight primers labeled with Texas Red were used for sequencing: 4Fa, 5′-TCCGGTTGATCCTGCCGG-3′; Topo 21F: 5′-TTGG TACCGAGCTCGGAT-3′; T7R, 5′-TAATACGACTCACTA TAG G-3′; 515F, 5′-GTGCCAGC(AC)GCCGCGGTA-3′; 906F, 5′-GAAACTTAAA(GT)GAATTG-3′; 1492R, 5′-GGGTTACCT TGTTACGACTT-3′; 906R, 5′-CAATTCC(AC)TTTAAGTTTC-3′; and 515R, 5′-TACCGCGGC(GT)GCTGGCAC-3′. The reactions were run and analyzed with a DNA sequencer 725 (Vistra).

Phylogenetic analysis of the 16S rDNA gene sequences

16S rDNA sequences of strains EJ1^T and EJ2^T were manually aligned with a representative set of 16S rRNA sequences obtained from the Ribosomal Database Project (Maidak et al. 1996) and from recent GenBank releases (Benson et al. 1999). The secondary structure was used as a guide to ensure that only homologous regions were compared. For the phylogenetic analysis, 1,320 nucleotides were used. Distance matrix, maximum-parsimony (Fitch 1971), and maximum-likelihood (Felsenstein 1981) methods were applied, as implemented in the Arb software package (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/) and in the Phylo-Win software package (Galtier et al. 1996). The neighbor-joining method (Saitou and Nei 1987) was performed with the Jukes and Cantor distance correction (Jukes and Cantor 1969), and bootstrap analysis was used to provide confidence estimates for the phylogenetic tree topologies (Felsenstein 1985).

Nucleotide-sequence accession number

The sequence of strains EJ1^T and EJ2^T have been deposited in the EMBL database under accession numbers AF479012 and AF479013, respectively.

DNA homology

Genetic relatedness was investigated by slot-blot DNA-DNA hybridization by using a random-prime labeling and signal amplification system (Amersham Life Sciences) following the procedure described by Kristjansson et al. (1994). The DNAs of reference strains of *T. celer*, *T. profundus*, *T. hydrothermalis*, *T. guaymasensis*, *T. gorgonarius*, and *T. gammatolerans* were first used as labeled probes in a single hybridization experiment with strains EJ1^T and EJ2^T. Strains EJ1^T and EJ2^T were then used as labeled probes in hybridization experiments with the DNA of reference strains. Increasing amounts of target DNA (25, 50, 100 ng) denatured in 0.4 M NaOH were slotted onto a nylon hybridization membrane (Bio-Rad), fixed for 25 s under UV light, and probed with 200 ng of labeled DNA with fluorescine as indicated in the ECL kit (RPN 3040 ECL Kit, Amersham, UK). For each duplicate of DNA-DNA association (15 h in buffer 1× SSC with formamide, 0.5% blocking agent, 5% dextran sulfate, and 100 µg denatured, sheared salmon sperm DNA ml^{-1}), the temperature of DNA-DNA hybridization (~60 °C) was chosen in the optimal range in the hybridization buffer (Johnson 1984; Ivanova et al. 1988). Final high-stringency washes and signal amplification were performed according to the manufacturers' instructions. Hybridization signals were detected with a Storm fluorescent scanner (Molecular Dynamics) and analyzed by using the NIHImage program (http://rsb.info.nih.gov/nih-image/download.html). Signal (maximum peak area) produced by self-hybridization of the probe with homologous target DNA was set as 100%.

Results and discussion

Enrichments and isolations

Samples of chimneys collected in the Guaymas Basin and in the Mid-Atlantic Ridge were used to enrich for organotrophic hyperthermophiles at 85 °C. The positive enrichments obtained after 2 days of incubation consisted of irregular motile and unmotile coccoid cells. They were then exposed to γ-irradiation at doses of 20 and 30 kGy. The subsequent transfers of the irradiated cells yielded abundant growth after 3 days of incubation at 85 °C. Creamy colonies were obtained on YPS medium solidified with 1% Gelrite (w/v). Two colonies were randomly picked and were streaked on YPS-Gelrite plates four times successively. Purity of these isolates (designated EJ1^T and EJ2^T) was checked microscopically after purification by a serial dilution step.

Survival rates to γ-irradiation

The survival rates to γ-irradiation of isolates EJ1^T and EJ2^T were evaluated and compared to that of *Thermococcus gammatolerans* DSM 15229^T, *Pyrococcus abyssi* GE5^T and *Thermococcus stetteri* DSM 5262^T. After irradiation at increasing doses, the surviving fraction was enumerated by the most probable number technique. Strains EJ1^T and EJ2^T were found to resist doses of 2 and 3 kGy without loss of cultivability, respectively (Fig. 1). At higher irradiation doses, they appeared more resistant to ionizing radiation than *P. abyssi* and *T. stetteri*, but less than *T. gammatolerans*. Like *Deinococcus radiodurans* (Battista 1997) and *T. gammatolerans* (Jolivet et al. 2003a), a fraction of an end-exponential culture of the strains EJ1^T and EJ2^T was able to grow after γ-irradiation exposures of 20 and 30 kGy, respectively. When tested for this ability, cells of *P. abyssi* and *T. stetteri* were not able to cultivate after irradiation doses exceeding 11 and 18 kGy, respectively (data not shown).

Phenotypic characteristics

Cells of strains EJ1^T and EJ2^T formed regular cocci occurring singly or in pairs. Strain EJ2^T was motile by three polar flagella (Fig. 2b), while strain EJ1^T was not motile (absence of polar flagella) (Fig. 2a). The diameter of the cells observed for strain EJ1^T ranged from 0.7 to 1.4 µm, and the diameter of cells observed for strain EJ2^T ranged from 0.5 to 1.1 µm. Cells of strains EJ1^T and EJ2^T appeared to divide by constriction. Both isolates grew between 55 and 95 °C, and the optimal temperature for growth was 88 °C. No growth was detected at 50 and 96 °C (Table 1). Optimal pH was between 5.5

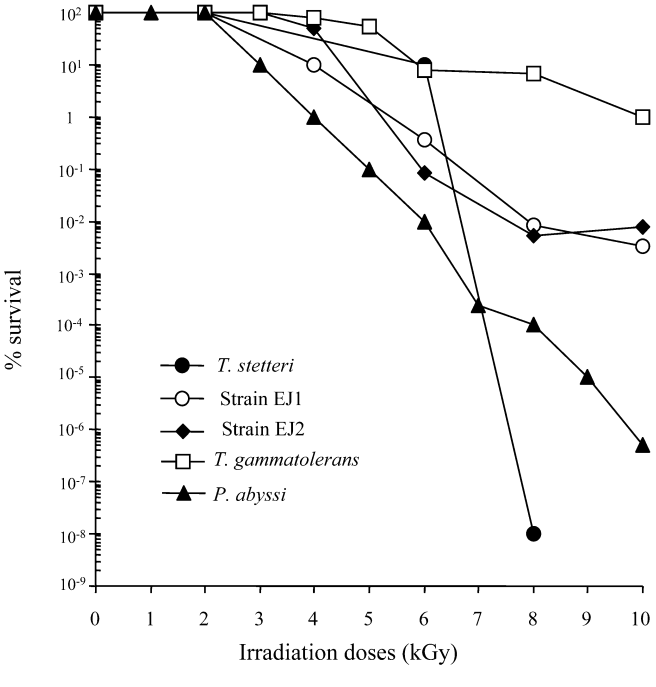

Fig. 1 γ-Radiation survival curves of strains EJ1^T (*white circles*) and EJ2^T (*black diamonds*). The new isolates EJ1^T and EJ2^T were irradiated at the end of the exponential growth phase in YPS growth medium under anaerobic conditions. These values are an average of two independent experiments. Survival curves of *Thermococcus gammatolerans* (*white squares*), *T. stetteri* (*black circles*), and *Pyrococcus abyssi* (*black triangles*) were taken from Jolivet et al. (2003a), Kopylov et al. (1993) and Gérard et al. (2001)

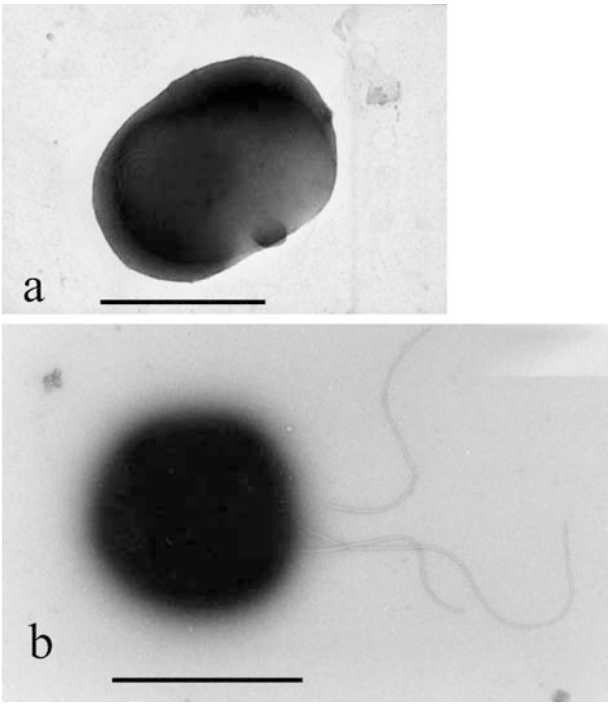

Fig. 2a, b Transmission electron micrograph of strains EJ1^T (**a**) and EJ2^T (**b**). *Scale bar* 1 μm

and 6.5. No growth occurred at pH 3.0 and 9.0. The optimal NaCl concentration was 20 g l^{-1}. No growth was detected at NaCl concentrations of 0 and 40 g l^{-1}. Under optimal growth conditions (temperature, pH, and NaCl), the doubling times of the isolates EJ1^T and EJ2^T were around 62 and 67 min, respectively.

Since no growth was observed in aerated conditions and in mineral medium supplemented with vitamins and an H_2/CO_2 (80:20) headspace, strains EJ1^T and EJ2^T appeared to be obligate anaerobic organotrophs. Under anaerobic conditions, elemental sulfur and cystine were necessary for growth and reduced to hydrogen sulfide. No growth was detected in the presence of thiosulfate, sulfate, and sulfite. Significant growth was observed on yeast extract, beef extract, peptone, and tryptone. Strain EJ2^T was not able to grow on a mixture of 20 amino acids, while strain EJ1^T was able to grow on a mixture of 20 amino acids and on casein hydrolysate. The minimum requirement of individual amino acids was determined for strain EJ1^T. Results showed that ten amino acids were essential for growth: arginine, aspartic acid, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tyrosine, and valine. No growth was observed on casamino acids, acetate, succinate, propionate, pyruvate, gelatin, sucrose, cellobiose, lactose, maltose, glycogen, xylose, and starch.

Antibiotics susceptibility

Isolates EJ1^T and EJ2^T were resistant to chloramphenicol, ampicillin, penicillin, kanamycin, vancomycin,

Table 1 Characteristics that distinguish EJ1^{T}and EJ2^T from their closest phylogenetic relatives (+ positive,– negative, *ND* not determined, *R* required, *S* stimulatory)

Property	*Thermococcus celer*[a]	*T. profundus*[a]	*T. hydrothermalis*[a]	*T. guaymasensis*[a]	*T. gorgonarius*[a]	*T. gammatolerans*[b]	Strain EJ1^T	Strain EJ2^T
Mobility	+	+	+	–	+	+	–	+
Energy substrate								
Casein	+	+	+	+	ND	–	+	–
Amino acids	ND	ND	+	–	–	–	+	–
Starch	ND	+	–	+	–	–	–	–
Maltose	ND	+	+	+	–	–	–	–
Pyruvate	ND	+	+	ND	Weak	–	–	–
Sulfur requirement	S	R	S	S	R	R	R	R
Rifampicin resistance	+	–	+	+	ND	–	–	–
Growth temperature (°C)								
Range	≤ 93	50–90	55–100	56–90	68–95	55–95	55–95	55–95
Optimum	88	80	85	88	80–88	80–88	88	88
NaCl concentration (g·l^{-1})								
Range	ND	10–60	20–80	ND	10–50	10–40	10–38	10–38
Optimum	40	20	30–40	18	20–35	20	20	20
pH								
Range	NR	4.4–8.5	3.5–9.5	5.6–8.1	5.8–8.5	4–8.5	4–8.5	4–8.5
Optimum	5.8	7.5	6.0	7.2	6.5–7.2	6.0	6.0	6.0
G+C content (mol%)	57[c]	52.2[d]	58[c]	46[c]	50.6[c]	51.3[c]	52.3[c]	54.5[c]

[a]Kobayashi (2001)
[b]Jolivet et al. (2003a)
[c]As measured by thermal desaturation method
[d]As measured by HPLC method

and streptomycin at a concentration of 150 $\mu g \cdot ml^{-1}$, but these isolates were sensitive to rifampicin at the same concentration. *Thermotoga maritima*, used as control, exhibited the expected pattern of antibiotic susceptibility at 80 °C (Huber et al. 1986).

DNA base composition

The G + C content of the DNA of isolates $EJ1^T$ and $EJ2^T$ determined by the thermal denaturation method were 52.3 and 54.5 mol%, respectively (Table 1).

The 16S rDNA sequence analysis

The sequence of the 16S rDNA sequence analysis placed strain $EJ1^T$ and $EJ2^T$ within the genus *Thermococcus* (Fig. 3). The highest levels of similarity between the 16S rDNA sequence of $EJ1^T$ and those of other *Thermococcus* species were as follows: *T. gorgonarius*, 99.3%; *T. celer*, 98.7%; *T. guaymasensis*, 98.5%; *T. profundus*, 98.4%; *T. hydrothermalis*, 98.3%; *T. gammatolerans*, 98.3%; *Pyrococcus furiosus*, 96.7%; and *Palaeococcus ferrophilus*, 94.2%. The highest levels of similarity between the 16S rDNA of $EJ2^T$ and those of other *Thermococcus* species were as follows: *T. celer*, 99.4%; *T. hydrothermalis*, 99.1%; *T. gorgonarius*, 99.1%; *T. profundus*, 99%; *T. guaymasensis*, 98.6%; *T. gammatolerans*, 98.2%; *Pyrococcus furiosus*, 96.4%; and *Palaeococcus ferrophilus*, 94.5%.

DNA homology

Considering the high levels of similarity (more than 98%) existing between our isolates and the reference strains *T. gorgonarius*, *T. celer*, *T. guaymasensis*, *T. profundus*, *T. hydrothermalis*, and *T. gammatolerans*, quantitative DNA–DNA hybridizations between these newly isolated strains and its closest relatives were performed (Table 1). When the reference strains were used as labeled probes, less than 25% homology was obtained with both strains $EJ1^T$ and $EJ2^T$, except for *T. guaymasensis* and strain $EJ2^T$, for which DNA homology was less than 50%. When strain $EJ1^T$ was used as labeled probe, the levels of DNA reassociation were as follows: $EJ2^T$ strain, 15.0%; *T. gorgonarius*, 18.9%; *T. celer*, 22.5%; *T. guaymasensis*, 43.6%; *T. profundus*, 13.7%; *T. hydrothermalis*, 24.2%; and *T. gammatolerans*, 25.1%. Next, when strain $EJ2^T$ was used as the labeled probe, the levels of DNA reassociation were as follows: *T. celer*, 27.5%; *T. hydrothermalis*, 31.5%; *T. gorgonarius*, 23.8%; *T. profundus*, 29.3%; *T. guaymasensis*, 25.4%; and *T. gammatolerans*, 13.6%.

Taxonomic position

When a number of different taxonomic parameters were compared, strains $EJ1^T$ and $EJ2^T$ differed from their closest phylogenetic relatives (Table 1). Strain $EJ1^T$ differs from strain $EJ2^T$ by the absence of mobility and the ability to grow on casein and amino acids. Strain $EJ2^T$ is phenotypically very close to *T. gammatolerans*; difference is noticed in the G + C% content and in its tolerance to ionizing radiation. The strain $EJ1^T$ resembles *T. hydrothermalis* in its ability to grow on an amino acids mixture, but differs strongly with the other species of *Thermococcus* in several physiological features.

On the basis of their morphological and physiological characteristics, their 16S rRNA sequences, and DNA–DNA hybridization data, new isolates $EJ1^T$ and $EJ2^T$ represent novel species of the genus *Thermococcus*. The novel species $EJ1^T$ is named *T. marinus* because this strain was isolated from the Mid-Atlantic Ridge deep-sea hydrothermal vent; the novel species $EJ2^T$ is named *T. radiotolerans* because this strain was more resistant to ionizing radiation than the strain $EJ1^T$.

Hyperthermophilic archaea grow at temperatures that destabilize the primary structure of DNA and may therefore require highly effective DNA-damage repair. The genome analyses of some archaeal species revealed the presence of either bacterial or eukaryotic homologues. The bacterial homologues are represented mostly by genes involved in metabolism, while the eukaryotic ones are found mostly among the genes responsible for the repair, replication, transcription, and translation machineries (Bult et al. 1996; Olsen and Woese 1996; Edgell and Doolittle 1997; Koonin et al. 1997; Cohen et al. 2003). Up to now, several studies revealed that the radioresistance levels of hyperthermophilic archaea such as *T. stetteri*, *P. furiosus*, and *P. abyssi* (Kopylov et al. 1993; DiRugierro et al. 1997; Gérard et al. 2001; Jolivet et al. 2003b) were much lower than that of the bacterium *Deinococcus radiodurans* (Battista 1997). Recently, we have shown that *T. gammatolerans*, isolated from a deep-sea hydrothermal vent, was more resistant to ionizing radiation than the previously studied hyperthermophilic archaea species, and its survival curve was close to that determined for *D. radiodurans* (Jolivet et al. 2003a). As with *T. gammatolerans*, the two strains $EJ1^T$ and $EJ2^T$ have been also isolated from deep-sea hydrothermal vents after γ-irradiation as selection pressure, but they were less resistant to γ-irradiation than *T. gammatolerans*. However, as shown with *T. gammatolerans*, strains $EJ1^T$ and $EJ2^T$ were able to grow after γ-irradiation at 30 kGy; cells of *P. abyssi* and *T. stetteri* were not viable after irradiation doses exceeding 11 and 18 kGy, respectively (data not shown). These microbes, along with *T. gammatolerans*, are the first hyperthermophilic archaea species isolated from samples following irradiation as a selection pressure and these strains are amongst the most radioresistant hyperthermophilic archaea known to date, which makes them suitable

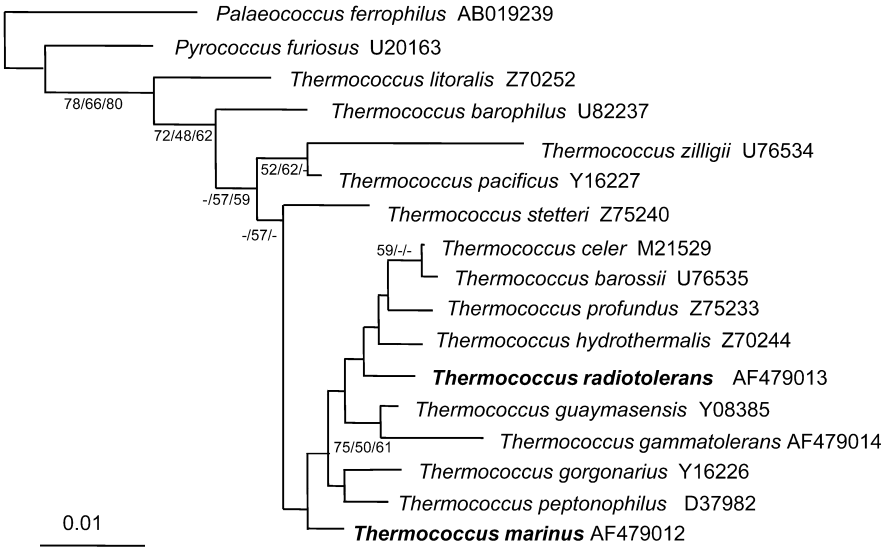

Fig. 3 Phylogenetic position of strains EJ1^T and EJ2^T (*boldface text*) amongst some representatives of the Thermococceae family. For the phylogenetic analysis, 1,320 nucleotides were used. *Numbers after the strain names* are GenBank accession numbers of 16S rDNA sequences. The topology shown is the tree obtained using the neighbor-joining method (Jukes and Cantor distance correction). *Numbers at the nodes* refer to the bootstrap values (100 replicates) in distance, maximum-likelihood, and maximum-parsimony analyses, respectively. Bootstrap values below 50% were not represented or represented by *dashes*. The *scale bar* represents the expected number of changes per sequence position

models to study γ-ray effect on survival and DNA repair of hyperthermophilic archaea.

Description of T. marinus sp. nov.

T. marinus sp. nov. (*marinus*—referring to its marine origin) has cells that are cocci (diameter, 0.7 to 1.4 μm) that are not motile. Cell division occurs by constriction. It is an obligate anaerobe and grows optimally in the presence of 20 g of NaCl per liter at a pH around 6.0. Growth occurs from 55 to 95 °C, with an optimum temperature of around 88 °C. It is an obligate chemorganotroph and grows preferentially on proteolysis products such as yeast, peptone compounds, and casein hydrolysate, and also grows on a mixture of ten essential amino acids (arginine, aspartic acid, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tyrosine, and valine). Sulfur is necessary for growth. The results of 16S rRNA sequence comparisons place *T. marinus* in Thermococcales. Type strain EJ1^T (DSM 15227^T = JCM 11825^T) was isolated from an active chimney-wall fragment recovered from Snakepit hydrothermal site on the Mid-Atlantic Ridge. The isolation was performed after γ-exposure of 20 kGy of the enrichment culture.

Description of T. radiotolerans sp. nov.

T. radiotolerans sp. nov. (*radio*—referring to γ-rays used as selection pressure for isolation; *tolerans*—tolerating; hence, *radiotolerans*—referring to its ability to tolerate high levels of γ-radiation) cells are cocci (diameter, 0.5 to 1.1 μm) that are motile due to the presence of polar flagella. Cell division occurs by constriction. It is an obligate anaerobe and grows optimally in the presence of 20 g of NaCl per liter at a pH around 6.0. Growth occurs from 55 to 95°C, with an optimum temperature at around 88 °C. It is an obligate chemorganotroph and grows preferentially on proteolytic products such as yeast extract and peptone compounds. Sulfur is necessary for growth. The results of 16S rRNA sequence comparisons place *T. radiotolerans* in Thermococcales. Type strain EJ2^T (DSM 15228^T = JCM 11826^T) was isolated from an active chimney-wall fragment recovered from a hydrothermal site in the Guaymas Basin. The isolation was performed after γ-exposure of 30 kGy of the enrichment culture.

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