Thermococcus fumicolans sp. nov., a New Hyperthermophilic Archaeon Isolated from a Deep-Sea Hydrothermal Vent in the North Fiji Basin

ANNE GODFROY,^{1*} JEAN-ROCH MEUNIER,^{1,2} JEAN GUEZENNEC,¹ FRANÇOISE LESONGEUR,¹
GÉRARD RAGUÉNÈS,¹ ALAIN RIMBAULT,³ AND GEORGES BARBIER¹

Laboratoire de Biotechnologie, Département Environnement Profond IFREMER, Centre de Brest, 29280 Plouzané,¹
Unité des Enterobactéries, Institut Pasteur, 75724 Paris cedex 15,² and Laboratoire de Microbiologie, Unité
Microbiologie Anaérobie, Faculté des Sciences Pharmaceutiques et Biologiques de Paris,
Université René Descartes, 75270 Paris cedex 06,³ France

An extremely thermophilic archaeon, strain ST557^T (T = type strain), was isolated from a deep-sea hydrothermal vent in the North Fiji Basin. This strain is a strictly anaerobic coccus whose cells are about 0.8 to 2 μ m in diameter. The optimum temperature, pH and sea salt concentration for growth are 85°C, 8.5, and 20 to 40 g/liter, respectively. Strain ST557^T grows preferentially in the presence of elemental sulfur on proteinaceous substrates and on a mixture of 20 amino acids. It grows slowly on pyruvate and maltose. Growth is inhibited by rifampin. The DNA G+C content is 54 to 55 mol%. Sequencing of the 16S rRNA gene revealed that strain ST557^T belongs to the genus *Thermococcus*. We propose that this organism should be placed in a new species, *Thermococcus fumicolans*.

Since the discovery of deep-sea hydrothermal vents in 1977, a number of hyperthermophiles have been isolated from various sites in the deep sea, including sites on the East Pacific Rise, in the southwest Pacific Ocean and on the Mid-Atlantic Ridge (for a review see reference 39). The deep-sea hyperthermophiles that have been described previously belong to the archaeal domain. These organisms are methanogens belonging to the genera *Methanococcus* (23, 24) and *Methanopyrus* (28); sulfate reducers, such as *Archaeoglobus profundus* (7); and sulfur metabolizers, such as members of the genera *Staphylothermus* (14), *Desulfurococcus* (21), *Pyrococcus* (12, 22), *Pyrodictium* (37), and *Thermococcus* (15, 20, 27) and isolated ES1 and ES4 (35, 36). Most of the genera mentioned above are not restricted to deep-sea vents, and members of these genera also occur in shallow-water or terrestrial hot spring systems.

During the French-Japanese "Starmar II" cruise in July 1989 in the North Fiji Basin, new hydrothermal sites were explored by the manned submersible "Nautil" (10). At these sites, temperatures up to 288°C were measured. During this cruise, many samples (including samples of chimney walls, rock debris, hydrothermal fluid, and animals) were recovered and used to inoculate enrichment cultures for hyperthermophilic sulfur metabolizers that were incubated at 80 and 95°C (1, 12). In this paper, we describe a new hyperthermophilic heterotrophic archaeal species that was isolated from chimney wall fragments.

MATERIALS AND METHODS

Reference strains. *Thermococcus celer* DSM 2476^T (T = type strain), "*Thermococcus litoralis*" DSM 5474^T, *Thermococcus stetteri* DSM 5262^T, *Pyrococcus furiosus* DSM 3638^T, *Pyrococcus woesei* DSM 3773^T, *Sulfolobus acidocaldarius* DSM 639^T, and *Thermotoga maritima* DSM 3109^T were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig-Stöckcim, Germany. *Thermococcus profundus* DT5432^T was provided by Tetsuo Kobayashi (RIKEN, Wako, Japan), *Thermococcus* sp. strain ES1 was provided by John Baross (University of Washington, Seattle), and *Pyrococcus abyssi* GES was provided by Gaël Erauso (Centre National de la Recherche Scientifique, Station Biologique de Roscoff, Roscoff, France).

Culture conditions. 2216-S medium (3) contains (per liter) 2 g of peptone, 0.5 g of yeast extract, 30 g of Sea Salt (Sigma Chemical Co., St. Louis, Mo.), 6.05 g of PIPES [piperazine-*N,N'*-bis(ethanesulfonic acid)] buffer (Sigma), 10 g of sulfur, and 1 mg of resazurin. BHI-S medium contains (per liter) 9 g of brain heart infusion, 23 g of NaCl (instead of Sea Salt, to prevent formation of a mineral precipitate), 6.05 g of PIPES buffer, 10 g of sulfur, and 1 mg of resazurin. 20AA-S medium contains (per liter) 30 g of Sea Salt, 6.05 g of PIPES buffer, 10 g of sulfur, 1 mg of resazurin, 10 ml of a mineral solution (2), and 10 ml of a vitamin solution (2) and each of the 20 classical amino acids at a concentration of 0.2 mM. A stock solution containing each of the amino acids at a concentration of 2 mM was sterilized separately by filtration (pore size, 0.22 μ m; Nalgene filterware). Unless indicated otherwise, the pH was adjusted to 7.5 with 5 N NaOH. In each case the medium was sterilized by steaming it at 100°C for 30 min on 2 successive days, transferred into an anaerobic chamber containing N₂, H₂, and CO₂ (90:5:5), reduced by adding sodium sulfide to a final concentration of 0.5 g/liter, and distributed into Hungate tubes or into 50-ml serum vials with butyl rubber stoppers. Unless indicated otherwise, cultures were incubated at atmospheric pressure under the anaerobic chamber gas mixture at 80°C.

Enrichment cultures and purification. After samples (rock suspensions) were collected, they were stored at room temperature in serum vials filled with sterile seawater under anaerobic conditions (N₂ headspace gas, 0.5 g of sodium sulfide per liter). Enrichment cultures were grown on 2216-S medium that was incubated at 80°C for 2 or 3 days. Cultures were purified by streaking samples onto 2216-S medium that was solidified with Gelrite (Scott Laboratories, Long Island, N.Y.) and incubated in anaerobic jars at 80°C (11). Purified isolates were stored at 4°C and could be used as inocula for at least 1 year. For long-term storage, pure cultures were stored anaerobically at –70°C in the presence of 5% (wt/vol) dimethyl sulfoxide (Sigma) as a cryoprotectant.

Determination of cells number. The numbers of cells in liquid cultures were determined by direct visual counting. Samples were diluted in sterile water containing Sea Salt (30 g/liter), formalin (2.5%), and acridine orange (0.01%), filtered with black polycarbonate membrane filters (pore size, 0.2 μ m; Nucleopore), and examined with an Olympus model BH-2 microscope (19).

Determination of growth parameters. To determine the optimum temperature, cells were grown in Hungate tubes containing 6 ml of 2216-S medium. The headspace gas was N₂-CO₂-H₂ (90:5:5) at a pressure of 100 kPa for temperatures under 100°C and at a pressure of 200 kPa for temperatures of 100°C or more. Temperatures were maintained with aluminum heating blocks (Bioblock, Illkirch, France) and were monitored with temperature probes placed in control tubes. To determine growth rates at different pHs, 2216-S medium was modified by using the following buffers (Sigma), each at a concentration of 1 g/liter: for pH 2.5, 3.5, and 4.5, no buffer; for pH 5.5, MES (morpholineethanesulfonic acid) buffer; for pH 6.5, PIPES buffer; for pH 7.5, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer; and for pH 8.5 and 9.5, AMPSO {[3-(1,1-Dimethyl-2-hydroxyethylamino)-2-hydroxypropanesulfonic acid] buffer. After sodium sulfide was added, the pH was adjusted with 1 M HCl. To determine the salt requirement, 2216-S medium was prepared with different dilutions of Sea Salt. The effects of pH and salinity were determined at 80°C. Three replicates were studied simultaneously at each temperature, pH, or level of salinity.

Determination of growth requirements. The ability of isolate ST557^T to utilize

* Corresponding author. Mailing address: Laboratoire de Biotechnologie, Département Environnement Profond IFREMER Centre de Brest, BP70, 29280 Plouzané, France.

various carbon sources was determined in media in which the yeast extract and peptone of 2216-S medium were replaced by 0.4 g of ammonium chloride per liter, 10 ml of a mineral solution (2) per liter, 10 ml of a vitamin solution (2) per liter, and carbon sources. The tests were performed by using tubes containing no nitrogen and carbon sources as controls for growth on the inoculum. Most of the carbon sources were tested at a concentration of 5 g/liter; the exceptions were starch, cellulose, and chitin, which were tested at a concentration of 10 g/liter, and ethanol, which was tested at a concentration of 5 ml/liter. Growth on BHI-S medium and growth on 20AA-S medium were also tested. In addition, autotrophic growth under H_2 - CO_2 (80:20) headspace gas as the sole carbon source was tested. To determine the ability of the isolate to grow in the absence of elemental sulfur, cells were cultured on 2216-S medium from which sulfur compounds had been omitted; in these experiments the headspace gas was N_2 , N_2 - CO_2 (80:20), or H_2 - CO_2 (80:20) at a pressure of 100 kPa, and the medium was reduced by titanium nitrilotriacetate (32) at a concentration of 200 μ M. To determine H_2S production, an uninoculated control was grown, and samples were immediately analyzed for H_2S production by the colorimetric method of Cline (8). To determine the ability of the isolate to use a range of electron acceptors, elemental sulfur was replaced by potential electron acceptors at a concentration of 1 mM. Growth on cystine (10 g/liter) and growth on polysulfide (10 mM) (6) were also investigated.

Antibiotic susceptibility. Susceptibility to the antibiotics vancomycin, penicillin, kanamycin, streptomycin, rifampin, and chloramphenicol at final concentrations of 50, 100, and 150 μ g/ml was determined under the standard growth conditions. The bacterium *Thermotoga maritima* was used as a control to establish the effectiveness of antibiotics at 80°C.

Electron microscopy. Cells were harvested at the end of the log phase. For transmission electron microscopy, pellets were resuspended and fixed for 1 h at room temperature in 0.4 M cacodylate buffer (pH 7.4)–5.5% (wt/vol) NaCl–8% (vol/vol) glutaraldehyde (2:1:1) and then postfixed for 1 h at 4°C in 0.4 M cacodylate buffer (pH 7.4)–10% (wt/vol) NaCl–2% (wt/vol) OsO_4 (1:1:2). After dehydration with increasing ethanol concentrations, cells were embedded in Spurr's resin, thin sectioned, contrasted with 1% (wt/vol) uranyl acetate and 1% (wt/vol) lead citrate (Bio-Rad S.A., Ivry, France), and examined with a model EM201 transmission microscope (Philips, Eindhoven, The Netherlands).

For scanning electron microscopy, cells were fixed with 7% (wt/vol) glutaraldehyde and then filtered with a 0.22- μ m-pore-size Nuclepore filter. After salt was eliminated and the preparation was dehydrated with increasing ethanol concentrations, samples were critical point dried, coated with gold (catalog no. SCD 040; Balzers, Balzers, Liechtenstein), and examined with a Cambridge Stereoscan S100 microscope.

Lipid analysis. (i) **Lipid extraction.** Lipids were extracted from lyophilized cells (50 to 100 mg) by using a modified Bligh-Dyer method (4, 45). The extracted lipids were fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography by using chloroform, acetone, and methanol, respectively. The methanol fraction containing polar ethers was dried under a vacuum and stored under N_2 until further analysis.

(ii) **Glycerol ether analysis.** The polar phase of the Bligh-Dyer extraction preparation was subjected to strong acid hydrolysis, and the resulting core ether lipids were digested with 55% HI for 18 h at 100°C. The resulting alkyl iodide were extracted with hexane and successively washed with $Na_2S_2O_3$ and Na_2CO_3 solutions (25, 29, 34). After extraction with hexane, the iodide derivatives were analyzed with a gas chromatograph equipped with an electron capture detector by using 1,2-di-*o*-hexadecyl-rac-glycerol as the internal standard. Authentic glycerol diethers and diglycerol tetraethers were purchased from Sigma or were isolated from *Sulfolobus acidocaldarius*.

Organic acid production. The isolate was grown on BHI-S medium and on 20AA-S medium at 80°C for 15 h. Organic acids were analyzed by gas chromatography and by gas chromatography-mass spectrometry as *tert*-butyl dimethylsilyl derivatives (40).

DNA base composition. Cells were cultured in 4.5 liters of 2216-S medium at 80°C and were harvested at the end of the exponential phase of growth. After centrifugation, the cell pellet was suspended in 5 ml of lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 0.50 mM EDTA; pH 8), 1% (wt/vol) Sarkosyl was added, and then 1% (wt/vol) sodium dodecyl sulfate and proteinase K (final concentration, 0.4 mg/liter) were added for cell lysis. After 3 h of incubation at 40°C, three phenol-chloroform-isoamyl alcohol (24:24:1) extraction and one chloroform extraction were performed. The DNA was precipitated by adding 2 volumes of ethanol at –20°C. The DNA pellet was resuspended in TE (10 mM Tris-HCl, 2 mM EDTA [pH 7.4]) buffer and treated with RNase (5 μ g/liter) for 1 h at 60°C. The DNA base composition was determined by high-performance liquid chromatography (HPLC) at the Deutsche Sammlung von Mikroorganismen Identification Service and by the thermal denaturation method (30).

Qualitative DNA-DNA hybridization (dot blots). DNAs (0.15 μ g) extracted from reference strains and strain ST557^T were applied to a nylon membrane (Hybond N; Amersham, Amersham Place, United Kingdom) by using a manifold attached to a suction device. The DNAs had been denatured previously by treating them with NaOH (final concentration, 0.1 M) for 5 min and had been neutralized with Na_2HPO_4 (final concentration, 0.1 M). The DNAs were immobilized on the nylon membrane by using UV irradiation and then hybridized with peroxidase-labelled ST557^T DNA as the probe (ECL direct labelling kit; Amersham).

FIG. 1. Scanning electron micrograph of strain ST557^T. Bar = 2 μ m.

16S rRNA gene sequence analysis. The *rs* gene was amplified by the PCR (41) as described previously (9) except that "archaeal"-specific primers were used. The PCR products were cloned into pUC18 vectors by using a SureClone ligation kit (Pharmacia, Uppsala, Sweden) and were subcloned as *Hind*III-*Eco*RI insertions into M13mp18 and M13mp19 vectors. Both strands of one clone were sequenced by the dideoxy chain termination method (43), except that we used a HotTub DNA sequencing kit to resolve numerous compressions observed when genes of hyperthermophilic microorganisms were sequenced. PCR products were also directly sequenced by using a cycle sequencing kit (type CSDS; Gibco BRL, Gaithersburg, Md.) and 32 P-labelled primers (Amersham). A total of 1,309 bp were sequenced. The sequences which we determined were then aligned with a representative collection of reference 16S rRNA sequences. A multiple alignment, a phylogenetic tree, and levels of similarity were obtained by using the MegAlign program of the DNASTAR package (Promega, Madison, Wis.) (Clustal method with weighted residue, weight table PAM 250) and the neighbor-joining method (42).

Quantitative DNA-DNA hybridization. The DNAs of the *Thermococcus celer* and "*Thermococcus litoralis*" reference strains were used as labelled probes. Up to 20 liters of a culture were needed to get enough DNA for the experiment. A 4- to 5- μ g portion of each reference strain DNA was labelled by incorporating both [3 H]dATP and [3 H]dGTP with a Megaprime kit (Amersham). The nuclease S1 method of quantitative DNA-DNA hybridization was performed as described by Popoff and Coynault (38) on type DE81 filters (Whatman International, Maidstone, United Kingdom) by using a β matic IV scintillation counter (Kontron Instruments, Montigny le Bretonneux, France).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA of strain ST557^T has been deposited in the GenBank database under accession number Z70250.

RESULTS

Collection of samples. Small pieces of rocks and chimney walls were obtained by using the manned submersible "Nautile" at the White Lady hydrothermal site in the North Fiji Basin (16°59'S, 173°55'W; depth, 2,000 m) (10) during the French-Japanese "Starmer II" cruise in July 1989. At this site, active chimneys are composed mainly of anhydrite (16), and fluid temperatures up to 288°C were measured.

Enrichment and purification. Enrichment cultures were

FIG. 2. Transmission electron micrographs of strain ST557^T. Bars = 1 μ m.

grown on 2216-S medium at 80°C. After 2 or 3 days increases in turbidity and microscopic observations indicated that growth had occurred. Purification was performed by plating samples on solid sulfur-containing medium. A total of 16 isolates were obtained. The G+C contents of these organisms were determined by the thermal denaturation method and ranged from 52 to 59 mol%. A preliminary study of growth temperatures (data not shown) revealed that one isolate, strain ST557^T, exhibited rapid growth at temperatures of 90°C or more. This isolate was chosen for further study.

Morphology. Scanning electron microscopy (Fig. 1), transmission electron microscopy (Fig. 2), and phase-contrast microscopy (data not shown) revealed that isolate ST557^T cells were coccoid and occurred singly, in pairs, or in small aggregates. The cell size ranged from 0.8 to 2 μ m. The cells appeared to be motile, and polar flagella were observed by phase-contrast microscopy after specific coloration with Spot Test flagellum stain (Difco Laboratories, Detroit, Mich.). Cells most likely divided by constriction, as indicated by electron micrographs (Fig. 2a). Thin sections revealed a two-layer cell envelope (Fig. 2b).

Antibiotic susceptibility. The control bacterium *Thermotoga*

maritima exhibited the expected pattern of antibiotic susceptibility at a high temperature, demonstrating that the antibiotics utilized in this study were not deactivated by heating. Isolate ST557^T was resistant to vancomycin, penicillin, kanamycin, streptomycin, and chloramphenicol at concentrations up to 150 μ g/ml but was susceptible to rifampin at concentration of 100 and 150 μ g/ml.

Lipid analysis. The polar lipid fraction of strain ST557^T consisted of glycerol ether lipids, with diphytanyl glycerol diether (C₂₀) accounting for 70% of the total lipids. In the residual phase of the Bligh-Dyer extraction preparation the glycerol tetraethers having various proportions of cyclopentane rings predominated along with small amount of diethers. The proportions of the acyclic, monocyclic, and bicyclic tetraethers were estimated to be 60, 20, and 20% of the total ethers, respectively.

Determination of growth parameters. Isolate ST557^T grew at 73 to 103°C, and the optimum temperature for growth was 85 to 90°C (Fig. 3a). Growth was observed at pH 4.5 to 9.5, and the optimum pH was near 8; no growth was observed at pH 3.5 (Fig. 3b). Growth was observed at salt concentrations ranging from 10 to 80 g/liter, and the optimum salt concentration was between 20 and 40 g/liter (corresponding to 13 to 26 g of NaCl per liter); no growth was detected at salt concentrations of 5 and 100 g/liter (Fig. 3c).

FIG. 3. Temperature, pH, and Sea Salt concentration optima for growth of isolate ST557^T on 2216-S medium. Specific growth rates are plotted as a function of temperature (in the presence of 30 g of Sea Salt per liter at pH 7.5) (a), pH (in the presence of 30 g of Sea Salt per liter at 80°C) (b), and salinity (at 80°C and pH 7.5) (c). Growth rates were calculated by performing a linear regression analysis along the logarithmic part of the growth curves. If enough data were available, information about the precision of the growth rate is given (regression coefficient $\pm$ 2 standard errors).

TABLE 1. Growth requirements of strain ST557^T: carbon sources and electron acceptors

Compound(s)	Growth ^a
Carbon sources	
Yeast extract + peptone (2216-S medium).....	+++
Meat extract.....	+++
Yeast extract.....	++
Malt extract.....	++
Peptone.....	+
Tryptone.....	+
Brain heart infusion (BHI-S medium).....	+++
Casein.....	+
Casamino Acids.....	-
Maltose.....	++
Glucose.....	-
Sucrose.....	-
Fructose.....	-
Lactose.....	-
Galactose.....	-
Cellulose.....	-
Starch.....	-
Chitin.....	-
Ethanol.....	-
Sorbitol.....	-
Mannitol.....	-
Formate.....	-
Acetate.....	-
Pyruvate.....	+
Glutamate.....	-
Lysine.....	-
Glycine.....	-
20 Amino acids.....	++
None (under H ₂ -CO ₂).....	-
Brain heart infusion (under H ₂ -CO ₂).....	++
Electron acceptors	
Sulfur.....	+++
None (under N ₂).....	+
None (under N ₂ -CO ₂).....	+
None (under H ₂ -CO ₂).....	-
Cystine.....	++
Polysulfide.....	++
Sodium thiosulfate.....	-
Sodium thioglycolate.....	-
Sodium dithionite.....	-
Sodium sulfite.....	-
Sodium formate.....	-
Sodium malate.....	-

^a +++, >2 × 10⁸ cells per ml of culture (final concentration); ++, 5 × 10⁷ to 2 × 10⁸ cells per ml of culture (final concentration); +, 1.5 × 10⁷ to 5 × 10⁷ cells per ml of culture (final concentration); -, no growth.

Determination of growth requirements. 2216-S medium supported rapid growth of isolate ST557^T. Yeast extract-peptone could be replaced by several complex substrates, including tryptone, yeast extract, meat extract, peptone, brain heart infusion, malt extract, and casein. Significant growth was also observed when the 20 amino acids were used as a carbon source. Maltose and pyruvate supported slow growth, but none of the other sugars, alcohols, carboxylic acids, individual amino acids, or polysaccharides tested supported growth of ST557^T. H₂-CO₂ (80:20) headspace gas did not support autotrophic growth and decreased the growth rate of ST557^T on BHI-S medium (Table 1). Very slow growth occurred in the absence of elemental sulfur, and growth was rapidly inhibited by 80% H₂ (Fig. 4). Cystine and polysulfide supported rapid growth of ST557^T, but none of the other electron acceptors tested supported growth of ST557^T (Table 1). When elemental sulfur was

FIG. 4. Growth of strain ST557^T in the absence of elemental sulfur. Symbols: ●, growth under N₂; ■, growth under N₂-CO₂ (80:20); ○, growth under H₂-CO₂ (80:20); □, growth in the presence of sulfur under N₂.

present in the culture medium, growth of strain ST557^T was accompanied by the production of large amounts of H₂S (Fig. 5). Under these conditions H₂S was not produced in the uninoculated control.

Organic acid production. In BHI-S and 20AA-S media, acetate (5.4 and 4.7 mM, respectively), phenylacetate (3.03 and 0.87 mM), lactate (1.13 and 1.47 mM), and 2 methylpropionate (2.97 and 0.61 mM) were the major fermentation products; propanoate (1.14 and 0.67 mM), indole acetate (0.31 and 0.4 mM), 3-methylthiopropionate (1.0 and 0.43 mM), and *p*-hydroxyphenylacetate (0.58 and 0.25 mM) were also present.

DNA bases composition. The G+C contents of the DNA of isolate ST557^T determined by the thermal denaturation method and by direct HPLC analysis were 55 and 54 mol%, respectively.

DNA-DNA homology. Strain ST557^T exhibited only low levels of similarity to previously described members of the *Thermococcales* and appeared to be more closely related to the genus *Thermococcus* than to the genus *Pyrococcus* (data not shown). On the phylogenetic tree based on 16S rRNA genes sequences (see below) the levels of similarity were quantified by using *Thermococcus celer* and "*Thermococcus litoralis*," which exhibited average levels of homology of 22 and 15% with strain ST557^T, respectively.

16S rRNA sequence analysis. The sequence of the 16S rRNA-encoding gene of strain ST557^T was determined and aligned with the other available archaeal 16S rRNA sequences. A comparison of the 16S rRNA sequences in which a phylo-

FIG. 5. Growth of and sulfide production by strain ST557^T cultivated at 80°C on 2216-S medium. Symbols: ■, growth; □, sulfide production; ○, uninoculated control.

FIG. 6. Phylogenetic relationships based on 16S rRNA sequences. Most 16S rRNA sequences were obtained from the GenBank and EMBL databases. The exceptions were the sequences of "*Thermococcus litoralis*," and *Thermococcus profundus*, which were determined in this study. The units for the scale are the number of substitution events inferred. The accession numbers of the sequences of the organisms used in this analysis are as follows: *Thermococcus celer*, M215291; "*Thermococcus peptonophilus*," D37982; *Pyrococcus abyssi*, L19921; *Pyrococcus shinkai*, D45214; *Pyrococcus furiosus*, U20163; *Pyrodicticum occultum*, M21087; *Sulfolobus solfataricus*, X03235; *Sulfolobus acidocaldarius*, D14876; "*Thermococcus litoralis*," Z70251; and *Thermococcus profundus*, 275233.

genetic tree was constructed (Fig. 6) revealed that strain ST557^T belongs to the order *Thermococcales*, which includes two genera, the genera *Pyrococcus* and *Thermococcus* (5). The levels of similarity between the 16S rRNA of ST557^T and the 16S rRNAs of other *Thermococcus* species are as follows: *Thermococcus celer*, 89.2%; "*Thermococcus litoralis*," 90.8%; *Thermococcus profundus*, 88.5%; and "*Thermococcus peptonophilus*," 86.4%. The results of this analysis clearly indicate that strain ST557^T belongs to the genus *Thermococcus* and represents a new species of this genus.

DISCUSSION

Novel marine hyperthermophilic strain ST557^T belongs to the archaeal domain on the basis of its resistance to antibiotics, the presence of di- and tetraether lipids, and its 16S rRNA sequence. On the basis of its G+C content, DNA-DNA hybridization data, and its 16S rRNA sequence, strain ST557^T clearly belongs to the genus *Thermococcus*. This classification is consistent with the morphological and physiological characteristics of strain ST557^T, which are similar to the characteristics of this genus. Strain ST557^T resembles "*Thermococcus litoralis*," *Thermococcus stetteri*, *Thermococcus profundus*, and "*Thermococcus peptonophilus*" in its susceptibility to 100 µg of rifampin per ml. Like "*Thermococcus litoralis*," strain ST557^T grows slowly on pyruvate but clearly differs from this species and from the recently described species "*Thermococcus alcaliphilus*" (26) and "*Thermococcus chitonophagus*" (20) in its DNA G+C content. Strain ST557^T resembles "*Thermococcus peptonophilus*" in its temperature range for growth and *Thermococcus profundus* in its physiological characteristics (pH and salinity ranges for growth and growth on pyruvate and maltose) but clearly differs from these organisms in its ability to grow at temperatures above 100°C. ST557^T differs from the other previously described *Thermococcus* species in its optimum pH (around pH 8) and temperature range for growth. Characteristics of *Thermococcus* species are summarized in Table 2. Optimal growth occurs in the presence of proteinaceous substrates, and ST557^T is able to grow on a mixture of 20 amino acids as a sole carbon and energy source. The ability to grow under these conditions has been reported previously for *Pyrococcus abyssi* (12, 44), for *Pyrococcus* sp. strain ES4 (36), for *Desulfurococcus* sp. strain SY, for *Pyrococcus* sp. strain GB-D

TABLE 2. Characteristics of *Thermococcus* species and isolate ST557^T

Strain	Reference	G+C content (mol%)	Growth temp (°C)		pH		NaCl concn (g/liter)		Carbon sources used	Growth on amino acids	Sulfur effect ^a	Rifampin resistance ^b
			Range	Optimum	Range	Optimum	Range	Optimum				
<i>Thermococcus celer</i> DSM 2476 ^T	46	57	≥93	88		5.8		40	Peptides (stimulated by sucrose)	+	E	R
" <i>Thermococcus litoralis</i> " DSM 5474 ^T	33	38	65–95	88	6.2–8.5	7.2	18–65	25	Peptides, pyruvate	–	E	S
<i>Thermococcus stetteri</i> DSM 5262 ^T	31	50	60–85	75	5.7–7.2	6.5	10–40	25	Peptides, starch, pectin	–	R	S
<i>Thermococcus profundus</i> DT 5432 ^T	27	52.2	50–90	80	4.5–8.5	7.5	10–60	20	Peptides, pyruvate, starch, maltose	ND ^c	R	S
" <i>Thermococcus peptonophilus</i> " JCM 9653 ^T	15	52	60–100	85	4–8	6	10–50	30	Peptides	ND	E	S
ES1	35	58.6	54–91	82	5–8.3	6.5	6–40	6–20	Peptides	ND	E	ND
" <i>Thermococcus alcaliphilus</i> " DSM 10322	26	42.4	56–90	85	6.5–10.5	9	10–60	20–30	Peptides	ND	E	ND
" <i>Thermococcus chitonophagus</i> " DSM 10152	20	46.5	60–93	85	3.5–9	6.7	8–80	20	Choline, peptides	ND	E	ND
ST557 ^T (= CIP 104680)		54–55	73–103	85	4.5–9.5	8	6–40	13–26	Peptides, pyruvate	+	E	S

^a E, elemental sulfur stimulates growth; R, elemental sulfur is required for growth.

^b R, resistant; S, susceptible.

^c ND, not determined.

(18), and for *Thermococcus* sp. isolates KS1, KS2, and KS8 (17) and has been observed for other members of the *Thermococcales* isolated from deep-sea hydrothermal ecosystems (14a). The ability to utilize amino acids could be related to the organic matter available in the hydrothermal ecosystems. Growth occurs on maltose and pyruvate, but small amounts of complex organic compounds (such as the brain heart infusion added with the inoculum) seem to be required as no growth was observed after successive cultures on media containing maltose and pyruvate (data not shown). Growth of strain ST557^T is not strictly dependent on elemental sulfur, but elemental sulfur greatly enhances the growth of strain ST557^T; elemental sulfur can be replaced by cystine or polysulfide, but growth is slower. In the absence of elemental sulfur, the growth yields are low; this is apparently due to inhibition by H₂ that evolves as an end product, as reported previously for *Pyrococcus furiosus* (13) and *Pyrococcus abyssi* (12).

On the basis of its physiological characteristics, its 16S rRNA sequence, and DNA-DNA hybridization data, new isolate ST557^T represents a new species of the genus *Thermococcus*; this species is named *Thermococcus fumicolans* because it was isolated from smoker wall pieces collected at deep-sea hydrothermal vents. *Thermococcus fumicolans* is the most thermophilic species in the genus *Thermococcus* (temperature range for growth, 73 to 103°C).

Description of *Thermococcus fumicolans* sp. nov. *Thermococcus fumicolans* Godfroy and Meunier (fu.mi.co.lans. L. m. n. *fumus*, smoke, referring to the smokers or chimneys of deep-sea vents; L. partic. pres. *colans*, living on; M. L. adj. *fumicolans*, living on smoke). Cells are spherical cocci (diameter, 0.8 to 2 µm) that are motile by means of polar tufts of flagella. Cell division occurs by constriction. Obligately anaerobic. Grows optimally in the presence of 20 to 40 g of Sea Salt per liter and at a pH around 8. The temperature range for growth is 73 to 103°C, and the optimum temperature is around 85°C. Obligately chemoorganotrophic. Grows on proteolysis products and a mixture of amino acids. Sulfur is not necessary for growth but greatly enhances growth. The results of 16S rRNA sequence comparisons place *Thermococcus fumicolans* in the *Thermococcales*. Type strain ST557 (= CIP 104680 [Collection de l'Institut Pasteur, Paris, France]) was isolated from an active chimney wall fragment obtained at a hydrothermal site in the North Fiji Basin in the southwest Pacific Ocean.

ACKNOWLEDGMENTS

We thank the cochief scientists of the French-Japanese "Starmer II" cruise, Daniel Desbruyères and Suguru Otha, for inviting one of us to participate in the cruise. Anne-Lise Tredan is gratefully acknowledged for her important contribution to this work. We thank Philippe Cras-sous for performing scanning electron microscopy, Françoise Gaill and Lahcen Hamraoui for performing transmission electron microscopy, and Joël Querellou for performing the numerical analysis of the 16S rRNA sequence data. We thank the captain and crew of N.O. "Le Nadir" and the D.S.V. "Nautilé" pilots and support crew.

This work was supported by IFREMER, and Région Bretagne, STA was co-organizer of the French-Japanese Starmer program.

REFERENCES

- Antoine, E., G. Barbier, J. C. Caprais, G. Erauso, A. Godfroy, J. Guezennec, D. Prieur, and G. Raguene. 1991. Isolation and identification of anaerobic sulfur dependent thermophilic bacteria from two new hydrothermal sites in SW Pacific (Lau Basin and North Fiji Basin). *Kiel. Meeresforsch.* **8**:178–181.
- Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* **43**:260–296.
- Belkin, S., and H. W. Jannasch. 1985. A new extremely thermophilic, sulfur-reducing heterotrophic marine bacteria. *Arch. Microbiol.* **141**:181–186.
- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917.
- Blöchl, E., S. Burggraf, G. Fiala, G. Lauerer, G. Huber, R. Huber, R. Rachel, A. Segerer, K. O. Stetter, and P. Völkl. 1995. Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms. *World J. Microbiol. Biotechnol.* **11**:9–16.
- Blumentals, I. I., M. Itoh, G. J. Olson, and R. M. Kelly. 1990. Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **56**:1255–1262.
- Burggraf, S., H. W. Jannasch, B. Nicolaus, and K. O. Stetter. 1990. *Archaeoglobus profundus* sp. nov., represents a new species within the sulfate-reducing archaeobacteria. *Syst. Appl. Microbiol.* **13**:24–28.
- Cline, J. D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **14**:454–458.
- Dauga, C., and P. A. D. Grimont. 1991. Nucleotide sequence of 16S rRNA from ten *Serratia* species. *Res. Microbiol.* **141**:1139–1149.
- Desbruyères, D., A. M. Alayse-Danet, S. Otha, and the Scientific Parties of Biolau and Starmer Cruises. 1994. Deep-sea hydrothermal communities in southwestern Pacific back-arc basins (the North-Fiji and Lau basins): composition, microdistribution and food web. *Mar. Geol.* **116**:227–242.
- Erauso, G., A. Godfroy, G. Raguene, and D. Prieur. Plate cultivation techniques for strictly anaerobic, thermophilic, sulfur-metabolizing Archae. Protocols for archaeobacterial research.
- Erauso, G., A. L. Reysenbach, A. Godfroy, J. R. Meunier, B. Crump, F. Partensky, J. A. Baross, V. Marteinsson, G. Barbier, N. R. Pace, and D. Prieur. 1993. *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Arch. Microbiol.* **160**:338–349.
- Fiala, G., and K. O. Stetter. 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100°C. *Arch. Microbiol.* **145**:56–61.
- Fiala, G., K. O. Stetter, H. W. Jannasch, T. A. Langworthy, and J. Madon. 1986. *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic archaeobacteria growing up to 98°C. *Syst. Appl. Microbiol.* **8**:106–113.
- Godfroy, A. Unpublished data.
- Gonzalez, J. M., C. Kato, and K. Horikoshi. 1995. *Thermococcus peptonophilus* sp. nov., a fast-growing, extremely thermophilic archaeobacterium isolated from deep-sea hydrothermal vents. *Arch. Microbiol.* **164**:159–164.
- Grimaud, D., J. I. Ishibashi, Y. Lagabrielle, J. M. Auzende, and T. Urabe. 1991. Chemistry of hydrothermal fluids from the 17°S active site in the North Fiji Basin ridge (SW Pacific). *Chem. Geol.* **93**:209–218.
- Hoaki, T., M. Nishijima, M. Kato, K. Adachi, S. Mizobuchi, N. Hanzawa, and T. Maruyama. 1994. Growth requirements of hyperthermophilic sulfur-dependent heterotrophic archaea isolated from a shallow submarine geothermal system with reference to their essential amino acids. *Appl. Environ. Microbiol.* **60**:2898–2904.
- Hoaki, T., C. O. Wirsén, S. Hanzawa, T. Maruyama, and H. W. Jannasch. 1993. Amino acid requirements of two hyperthermophilic archaeal isolates from deep-sea vents, *Desulfurococcus* sp. strain SY and *Pyrococcus* sp. strain GB-D. *Appl. Environ. Microbiol.* **59**:610–613.
- Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**:1225–1228.
- Huber, R., J. Stöhr, S. Honenhaus, R. Rachel, S. Burggraf, H. W. Jannasch, and K. O. Stetter. 1995. *Thermococcus chitonophagus* sp. nov., a novel, chitin-degrading, hyperthermophilic archaeum from a deep-sea hydrothermal environment. *Arch. Microbiol.* **164**:255–264.
- Jannasch, H. W., C. O. Wirsén, S. J. Molyneux, and T. A. Langworthy. 1988. Extremely thermophilic fermentative archaeobacteria of the genus *Desulfurococcus* from deep-sea hydrothermal vents. *Appl. Environ. Microbiol.* **54**:1203–1209.
- Jannasch, H. W., C. O. Wirsén, S. J. Molyneux, and T. A. Langworthy. 1992. Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* sp. strain GB-D. *Appl. Environ. Microbiol.* **58**:3472–3481.
- Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe. 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Arch. Microbiol.* **136**:254–261.
- Jones, W. J., C. E. Stugard, and H. W. Jannasch. 1989. Comparison of thermophilic methanogens from submarine hydrothermal vents. *Arch. Microbiol.* **151**:314–318.
- Kates, M. 1964. Bacterial lipids. *Adv. Lipid Res.* **2**:17–90.
- Keller, M., F.-J. Braun, R. Dirmeyer, D. Hafenbradl, S. Burggraf, R. Rachel, and K. O. Stetter. 1995. *Thermococcus alcaliphilus* sp. nov., a new hyperthermophilic archaeum growing on polysulfide at alkaline pH. *Arch. Microbiol.* **164**:390–395.
- Kobayashi, T., Y. S. Kwak, T. Akiba, T. Kudo, and K. Horikoshi. 1994. *Thermococcus profundus* sp. nov. a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Syst. Appl. Microbiol.* **17**:232–236.
- Kurr, M., R. Huber, H. König, H. W. Jannasch, H. Fricke, A. Trincone, J. K. Kristjansson, and K. O. Stetter. 1991. *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Arch. Microbiol.* **156**:239–247.

29. Langworthy, T. A., G. Holzer, J. G. Zeikus, and T. G. Tornabene. 1983. Iso- and anteiso-branched glycerol diethers of the thermophilic anaerobe *Thermodesulfobacterium commune*. Syst. Appl. Microbiol. 4:1-17.
30. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.
31. Miroshnichenko, M. L., E. A. Bonch-Osmolovskaya, A. Neuner, N. A. Kostrikin, N. A. Chernych, and V. A. Alekseev. 1989. *Thermococcus stetteri* sp. nov., a new extremely thermophilic marine sulfur-metabolizing archaeobacterium. Syst. Appl. Microbiol. 12:257-262.
32. Moensch, T. T., and J. G. Zeikus. 1983. An improved preparation method for a titanium(III) media reductant. J. Microbiol. Methods 1:199-202.
33. Neuner, A., H. W. Jannasch, S. Belkin, and K. O. Stetter. 1990. *Thermococcus litoralis* sp. nov.: a new species of extremely thermophilic marine archaeobacterium. Arch. Microbiol. 153:205-207.
34. Pauly, G. G., and E. S. Van Vleet. 1986. Acyclic archaeobacterial ether lipids in swamp sediments. Geochim. Cosmochim. Acta 50:1117-1125.
35. Pledger, R. J., and J. A. Baross. 1989. Characterization of an extremely thermophilic archaeobacterium isolated from a black smoker polychaete (*Paralvinella* sp.) at the Juan de Fuca Ridge. Syst. Appl. Microbiol. 12:249-256.
36. Pledger, R. J., and J. A. Baross. 1991. Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures up to 110°C isolated from a submarine hydrothermal vent environment. J. Gen. Microbiol. 137:203-211.
37. Pley, U., J. Schipka, A. Gambacorta, H. W. Jannasch, H. Fricke, R. Rachel, and K. O. Stetter. 1991. *Pyrodicticum abyssi* sp. nov. represents a novel heterotrophic marine archaeal hyperthermophile growing at 110°C. Syst. Appl. Microbiol. 14:245-253.
38. Popoff, M. Y., and C. Coynault. 1980. Use of DEAE cellulose filters in the S1 nuclease method for bacterial deoxyribonucleic acid hybridization. Ann. Inst. Pasteur/Microbiol. (Paris) 131A:151-155.
39. Prieur, D., G. Erauso, and C. Jeanthon. 1995. Hyperthermophilic life at deep-sea hydrothermal vents. Planet. Space Sci. 43:115-121.
40. Rimbault, A., J. Guezennec, M. Fromage, P. Niel, A. Godfroy, and F. Rocchiccioli. 1993. Organic acids and stable isotopes: metabolic studies of a thermophilic sulfur-dependent anaerobic archaeon. J. Microbiol. Methods 18:329-338.
41. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.
42. Saitou, M., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 44:406-425.
43. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5473-5467.
44. Watrin, L., V. Martin-Jezequel, and D. Prieur. 1995. Minimal amino acid requirements of the hyperthermophilic archaeon *Pyrococcus abyssi* isolated from deep-sea hydrothermal vents. Appl. Environ. Microbiol. 61:1138-1140.
45. White, D. C., J. D. Nickels, J. D. King, and R. J. Bobbie. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia (Berlin) 40:51-62.
46. Zillig, W., I. Holtz, D. Janekovic, W. Schäfer, and W. D. Reiter. 1983. The archaeobacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaeobacteria. Syst. Appl. Microbiol. 4:88-94.