***Thermococcus gorgonarius* sp. nov. and *Thermococcus pacificus* sp. nov.: heterotrophic extremely thermophilic archaea from New Zealand submarine hot vents**

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Two extremely thermophilic archaea, designated W-12 and P-4, were isolated from a geothermal vent in the tidal zone of Whale Island, New Zealand, and from geothermally heated bottom deposits of the Bay of Plenty, New Zealand, respectively. Cells of isolate W-12 are irregular cocci, 0.3–1.2 µm in diameter, motile with polar flagella. The cell envelope consists of one layer of subunits with a major protein of *M*_r 75 000. Cells produce protrusions of different kinds: prostheca-like, chains of bubbles, or network of fimbriae. Cells of isolate P-4 are regular cocci, 0.7–1.0 µm in diameter, motile with polar flagella. The cell envelope consists of two layers of subunits; its major protein has an *M*_r of 56 000. Both organisms are obligate anaerobes, fermenting peptides in the case of strain W-12, or peptides and starch in the case of P-4. Elemental sulfur is required for growth and is reduced to hydrogen sulfide. The optimal growth temperature of the new isolates is in the range 80–88 °C. The optimal growth pH is 6.5–7.2. The G+C content of the DNA of strain W-12 is 50.6 mol %, and of strain P-4 is 53.3 mol %. Based on physiological characteristics, 16S rDNA sequence comparison and DNA base composition, the new isolates were considered to be members of the genus *Thermococcus*. The low level of DNA–DNA hybridization with the type strains of other *Thermococcus* species confirms the novel species status of the new isolates. The new isolates are described as *Thermococcus gorgonarius* sp. nov., with type strain W-12 (= DSM 10395^T), and *Thermococcus pacificus* sp. nov., with type strain P-4 (= DSM 10394^T).

Keywords: *Thermococcus gorgonarius* sp. nov., *Thermococcus pacificus* sp. nov., thermophiles, archaea

INTRODUCTION

During the last decade, a number of extremely thermophilic archaea, among which sulfur-dependent organotrophs are the most abundant, have been isolated from marine sources (16, 25). With the exception of *Pyrodictium abyssi* (20), which has flat cells with a complicated network of filaments, all the extremely thermo-

philic anaerobic organotrophs are coccoid in shape, often with a bundle of flagella. Extremely thermophilic organotrophic cocci belong to the genera *Thermococcus* (7, 9–12, 16–18, 27), *Pyrococcus* (3, 4), *Staphylothermus* (5) and *Hyperthermus* (28). Although only remotely related phylogenetically, they are quite similar in physiological and morphological characteristics. These organisms grow anaerobically on peptides or polysaccharides and sugars. It has been shown for representatives of several genera that elemental sulfur serves as a sink for electrons released in the course of fermentation but not as an electron acceptor (1). Here we describe two new representatives of the genus *Thermococcus* isolated from geothermally heated

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Fig. 1. Electron micrographs of negatively stained preparations of isolate W-12 cells, showing chains of blebs and prostheca-like appendages (a-c), flagella and fimbriae (d-e). F, Flagella, f, fimbriae. Bars, 100 nm.

deposits of shallow-water submarine vents in New Zealand.

METHODS

Sources and sampling. Samples from submarine hot vents in New Zealand were obtained during the 18th scientific cruise of the research vessel 'Academic Nesmeyanov', which took place in 1990. Shallow-water hot vents of the Bay of Plenty and Whale Island were investigated. On Whale Island, there is an area of geothermal activity in the tidal zone of the northern coast. When the tide was low, samples of the hot sand with a temperature of 85 °C were collected in glass vessels, hermetically closed and transported to the ship. Samples of geothermally heated mud from the bottom of the Bay of Plenty were obtained from 40 m depth with a sampling device. The temperature of mud when brought to the surface was 85 °C.

Enrichment and isolation. For enrichment cultures of extreme thermophiles, anaerobically prepared marine medium (17), supplemented with peptone (0.5%, w/v) and elemental sulfur (1%, w/v) was used. The pH was adjusted to 6.5 with 5 M H₂SO₄ and the medium was dispensed into Hungate tubes (15 ml); the head space (5 ml) was filled with a N₂/CO₂ gas mixture. Inoculated tubes were incubated at 88 °C. Pure cultures were obtained by isolating colonies in roll-tubes, prepared with the same medium solidified with 0.8–1% (w/v) Gelrite (Gellangum, Kelco). In this case sulfur was substituted by polysulfide (26).

Reference strains. The type strain of *Thermococcus celer* DSM 2476^T was obtained from K. O. Stetter (University of Regensburg, Germany). The type strain of *Thermococcus stetteri* DSM 5262^T was maintained in the culture collection

of the Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia. The other reference strains were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

Microscopy. Observations of microbial growth in enrichments and pure cultures were made using a light microscope AU-12, equipped with phase contrast. Freeze-etching, negative staining and the preparation of thin sections for electron microscopy were as described previously (8).

Studies of cell wall proteins. Electrophoresis and staining methods have been described previously (8).

Physiological studies. Growth substrates, when tested, were added to the same mineral medium as used for enrichments, in concentrations of 0.5% (w/v). H₂/CO₂ (80:20) was added to the headspace of culture tubes. Possible electron acceptors other than elemental sulfur were added to sulfur-free medium with peptone in concentrations of 0.2% (w/v). These included thiosulfate, sulfate, sulfite, fumarate and nitrate as their sodium salts. For growth pH characteristics, the pH of the medium was adjusted at ambient temperature before inoculation by the addition of 5 M H₂SO₄ or 5 M NaOH. Cell growth was monitored by direct cell counting using a light microscope with phase-contrast optics. Hydrogen sulfide was determined by the colorimetric method (24). Products of metabolism were determined using a gas chromatograph Chrom-5 with a flame-ionization detector on a column packed with Chromosorb 10 (Sigma) at 170 °C, using argon as a carrier gas at a flow rate of 40 ml min⁻¹.

DNA characteristics. DNA was isolated according to the methods of Marmur (15), with additional pronase treatment (50 mg ml⁻¹, for 2 h at 37 °C) before 2-propanol fractionation. The mol% G + C content of the DNA was determined by melting point analysis (19). To determine DNA homology

with type strains of other species, DNA was immobilized on nitrocellulose filters (10 mg per filter) and reassociated under optimal conditions (6). Labelled DNA was obtained using the nick-translation reaction and incorporation of [³H]cytidine (14). Partial 16S rDNA sequences were determined as described previously (2, 21). The partial 16S rDNA sequences were manually aligned using the ae2 editor (13) and sequence similarity values calculated by pairwise sequence comparison (13). The sequences were compared against the 16S rRNA/DNA sequences for *Thermococcus* strains available from the public databases.

RESULTS

Enrichment and isolation

Samples of sand from the shore of Whale Island and bottom sediments from a 40 m depth in the Bay of Plenty were inoculated into anaerobic sulfur- and peptone-containing medium and incubated at 88 °C. Bacterial growth was observed after a 6–20 h incubation. When transferred to solidified medium, colonies appeared after 8–10 h incubation at 88 °C. They were regular, round, semi-transparent, and about 0.5 mm in diameter. Colonies were transferred into liquid medium. The isolate from the shore of Whale Island was designated strain W-12, the isolate from deposits of the Bay of Plenty designated strain P-4.

Morphology and fine structure

Cells of isolate W-12 were of variable size. Most cells were 0.4–0.6 µm in diameter, but sometimes larger cells with diameter up to 1.2 µm were also present. Different kinds of protrusions could be observed (Fig. 1a–e). About 50% of cells contained long prostheca-like protrusions (Fig. 1a, c; Fig. 2c, d). The protrusions had an interior channel and terminal enlargements. Some cells had extracellular structures (Fig. 1b, c) consisting of several chains of bubbles apparently derived from cell envelope material. Polar flagella were also present (Fig. 1d). Flagella were very thin, 5–5.5 nm in width. Besides the flagella, a thin network of fimbriae 3–3.5 nm in width was also observed (Fig. 1e). The cell envelope of isolate W-12 consisted of one layer of subunits 15 nm in width attached to a plasma membrane and regularly packed (Fig. 2a, b). The major protein of the cell envelope had an M_r of 75 000. Alcian blue staining for glycoproteins was positive (Fig. 4).

Cells of isolate P-4 were of more regular coccoid shape (Fig. 3a); no protrusions of any kind were ever observed. Polar flagella were present (Fig. 3b). The cell wall of P-4 consisted of two layers with a total width of 30 nm (Fig. 3c, d). The outer layer looked as if it had been partly destroyed, probably being less stable than the inner one.

One of the most intense bands in the cell protein profile of isolate P-4 represented a protein with M_r 56 000 (data not shown). A band of the same M_r was the major one in the set of proteins from isolated cell envelopes (Fig. 4, lane 4), while the amounts of the

other proteins of this set varied in different preparations. Based on these data it was presumed that the protein of M_r 56 000 is a regularly structured S-layer protein (RS protein).

Physiological characteristics

Strain W-12 was able to grow in the temperature range 68–95 °C with the optimum at 80–88 °C. Strain P-4 grew in the temperature range 70–95 °C with the optimum at 80–88 °C. The pH range of growth was 5.8–8.5 for strain W-12 with the optimum at 6.5–7.2 and 6.0–8.0 for strain P-4 with the optimum at 6.5. Both strains required NaCl for growth: 1.0–5.0% (w/v) for strain W-12 and 1.0–6.0% (w/v) for strain P-4. Optimal growth of both strains occurred at 2–3.5% (w/v) NaCl. The growth substrates utilized in the case of strain W-12 were peptone and yeast extract; only weak growth was observed on pyruvate. For strain P-4, peptone, yeast extract, and starch served as substrates for growth. No growth of either isolate occurred in media supplemented with Casamino acids, glucose, maltose, lactate, acetate, propionate, methanol, ethanol, or under H₂/CO₂ atmosphere. Strain W-12 was also unable to grow on starch, and strain P-4 did not utilize pyruvate. The end products of growth of both strains on medium with peptone and elemental sulfur were H₂S, CO₂, acetate, propionate, isobutyrate and isovalerate. Neither isolate grew in the absence of elemental sulfur. Elemental sulfur could not be substituted by thiosulfate, sulfite, sulfate, cystine, fumarate or nitrate.

DNA characteristics

The G + C contents of the DNA of isolates W-12 and P-4 were 50.6 mol% and 53.3 mol% respectively. Results of DNA–DNA reassociation experiments are presented in Table 1. Low levels of DNA–DNA reassociation were found between some of the previously described *Thermococcus* species and strains P-4 and W-12.

Partial 16S rDNA sequences comprising 1142 and 1143 nucleotides were determined for strains W-12 and P-4 respectively. Since the 16S rDNA sequence of *Thermococcus peptonophilus* (accession no. D37982) comprises only 924 nucleotides, the region of sequence available for comparison between all strains was limited to 700 nucleotides in the regions 101–319 and 556–976 (*Escherichia coli* positions). Strains W-12 and P-2 showed 98.6% 16S rDNA similarity to each other. High 16S rDNA similarity values ranging between 97.7 and 99.4% were found between strains W-12, P-4 and the *Thermococcus* species for which 16S rDNA sequences are available. Strain W-12 showed highest partial 16S rDNA sequence similarity (99.4%) to *T. celer* and *Thermococcus profundus*. Strain P-4 showed highest 16S rDNA sequence similarity (99.2%) to *T. stetteri*. The lowest 16S rDNA sequence similarity values (both 97.8%) found between the isolates and *Thermococcus* species are to *Thermococcus fumicolans*.

Fig. 2. Electron micrographs of thin sections of isolate W-12 cells (a, c) and freeze-etch replica of cells (b, d). CM, Cytoplasmic membrane; SL, surface layer. Bars, 100 nm.

Fig. 3. Electron micrographs of isolate P-4 cells: negatively stained preparations (a, b) and thin sections (c, d). F, Flagella; CM, cytoplasmic membrane; SL, surface layer. Bars, 100 nm.

16S rDNA sequence similarities in the range 96.1–97.4% were found between strains W-12, P-4 and the *Pyrococcus* species.

DISCUSSION

The new isolate from Whale Island shore hot vents, strain W-12, is distinguished primarily by its morphological features. Its cells are rather small in size and

possess characteristic protrusions. The most interesting protrusions are prostheca-like. Such structures are quite common among mesophilic bacteria (22) and their purpose is, apparently, to increase the cell surface area, which is important for the nutrition of oligotrophs. In extremely thermophilic archaea, the role of prostheca-like structures may be similar to that in eubacteria: namely giving the cell a larger surface area. The new isolates from New Zealand shallow-water hot

Fig. 4. SDS-PAGE of W-12 (lane 3) and P-4 (lane 4) cell-envelope proteins. High- M_r standards, lane 1; low- M_r standards, lanes 2, 5. Arrows indicate regularly structured S-layer proteins (RS proteins).

vents are typical of *Thermococcus* species in their nutritional features. In the presence of elemental sulfur they grow preferentially on peptides, but do not oxidize them totally to CO_2 and H_2S , producing volatile fatty acids, which is typical for the fermentation of amino acids. The new strains W-12 and P-4 did not show any growth in the absence of elemental sulfur. However, the production of volatile fatty acids during growth indicates that fermentation does occur and is dependent on the availability of elemental sulfur as an external electron acceptor. It follows that, in their physiology as well as in many other characteristic features, the new isolates are similar to representatives of the genera *Thermococcus*, *Staphylothermus* and *Pyrococcus*. The ability to form a network of fimbriae is similar to that of *Pyrodictium* (23) but, besides a

lower temperature optimum for growth, both isolates differ from representatives of this genus as well as from *Pyrococcus* (4), *Staphylothermus* (5) and *Hyperthermus* (28) by having a significantly higher G + C content.

On the basis of the 16S rDNA partial sequence comparison and the high similarity values to *Thermococcus* species, it is clear that the new isolates are members of the genus *Thermococcus*. The physiological features of the new isolates are also similar to those of members of the genus *Thermococcus*, and they are therefore assigned to this genus. The 16S rDNA sequence data are useful for assigning the new isolates at the genus level but the similarities are too high to allow differentiation at the species level. Those species of *Thermococcus* showing greater than 99% 16S rDNA sequence similarity to the new isolates were compared by DNA–DNA hybridization, the results of which clearly show the lack of relationship at the species level between strains W-12, P-4 and these *Thermococcus* species (Table 1). Strain W-12 differs from all *Thermococcus* species in its ability to form prostheca-like structures, from *T. celer* and *T. fumi-colans* by the obligate requirement for sulfur, from '*Thermococcus litoralis*' and *Thermococcus chitinophagus* by the significant difference in G + C content of the DNA and the requirement for sulfur, and from *T. stetteri* by the cell envelope structure and growth substrates. The differences in growth substrates also differentiate strain W-12 from *T. profundus*, and the strain can be differentiated from *T. chitinophagus* and *T. peptonophilus* on the basis of its cell envelope structure, growth substrates and sulfur requirement. The high pH range for growth and low G + C content of the DNA differentiates '*Thermococcus alcaliphilus*' from the two new isolates. The same characteristics described for strain W-12 above can be used to differentiate strain P-4 from the other *Thermococcus* species, although its cell envelope is similar to those of *T. chitinophagus*, *T. peptonophilus* and *T. stetteri*. Strains W-12 and P-4 differ in their cell envelope structure and in the formation of prosthecae structures by strain W-12. The results of DNA–DNA reassociation experiments along with the characteristics described above clarify the species status of both of the new isolates. Two new species of the genus

Table 1. Percentage DNA–DNA hybridization of the new isolates with ^3H -radiolabelled DNA from type strains of some species of the genus *Thermococcus*

Strain	<i>T. celer</i>	<i>T. stetteri</i>	<i>T. peptonophilus</i>	<i>T. profundus</i>	<i>T. chitinophagus</i>	Strain W-12
<i>T. celer</i>	100	—	4	2	3	—
<i>T. stetteri</i>	5	100	—	—	—	—
<i>T. peptonophilus</i>	5	—	100	12	3	—
<i>T. profundus</i>	4	—	11	100	4	—
<i>T. chitinophagus</i>	3	—	3	1	100	—
Strain W-12	5	10	13	10	2	100
Strain P-4	2	10	8	10	2	54

Thermococcus are proposed: *Thermococcus gorgonarius*, with the type strain W-2, and *Thermococcus pacificus*, with the type strain P-4.

Description of *Thermococcus gorgonarius* sp. nov.

Thermococcus gorgonarius (gor.go.na'ri.us. L. masc. adj. *gorgonarius* pertaining to Gorgon, a Greek mythological creature with snakes for hair).

Cells are irregular cocci, 0.3–1.2 µm in diameter, motile with polar flagella. The cell envelope consists of one layer of subunits. The major protein of the cell wall has an M_r of 75000. Protrusions of different kinds: prostheca-like, chains of bubbles, fimbriae network. Obligate anaerobe. Archaeon. Grows at temperatures 68–95 °C, optimally at 80–88 °C, and at pH 5.8–8.5, optimally at pH 6.5–7.2. Grows on peptides; produces weak growth on pyruvate. Does not utilize glucose, maltose, starch, Casamino acids, volatile fatty acids, lactate, alcohols, or molecular hydrogen. In closed vessels growth is dependent on the presence of elemental sulfur which is reduced to H_2S . Growth products on peptone are CO_2 , H_2S , acetate, propionate, isobutyrate and isovalerate. G+C content of DNA is 50.6 mol %. Source of isolate: sand from the shore of Whale Island, New Zealand. The type strain is strain W-12 (= DSM 10395^T).

Description of *Thermococcus pacificus* sp. nov.

Thermococcus pacificus (pa.ci'fi.cus L. masc. adj. for Pacific Ocean, from the south-west part of which the new organism was isolated).

Cells are irregular cocci, 0.7–1.0 µm in diameter, motile with a bundle of flagella. The cell envelope consists of one layer of subunits. The major protein of the cell wall has an M_r of 56000. Obligate anaerobe. Archaeon. Grows at temperatures between 70 and 95 °C, optimally at 80–88 °C, and at pH 6.0–8.0 with an optimum at pH 6.5. Grows on peptides and starch. Does not utilize glucose, maltose, Casamino acids, volatile fatty acids, pyruvate, lactate or molecular hydrogen. In closed vessels, growth is dependent on the presence of elemental sulfur which is reduced to H_2S . Growth products on peptone are CO_2 , H_2S , acetate, propionate, isobutyrate, and isovalerate. G+C content of DNA is 53.3 mol %. Source of isolate: bottom deposits of the Bay of Plenty, New Zealand. The type strain is strain P-4 (= DSM 10394^T).

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