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Pyrobaculum gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100°C

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Abstract. Seven members of a new group of rod-shaped hyperthermophilic neutrophilic archaebacteria were isolated from boiling neutral to alkaline solfataric waters from the Azores, Iceland, and Italy. The organisms are strict anaerobes, growing optimally at 100° C. The cells are motile due to peritrichous or bipolar polytrichous flagellation. The isolates grow facultatively chemolithoautotrophically or obligately heterotrophically. Molecular hydrogen or complex organic substances are used as electron donors. During heterotrophic growth, elemental sulfur, thiosulfate, sulfite, L(−)cystine and oxidized glutathione may serve as electron acceptors depending on the individual strain. Elemental sulfur is strictly required as an electron acceptor for autotrophic growth. The G+C content of the DNA is around 46 mol%. The isolates represent a new genus which we have named *Pyrobaculum* (the "fire stick"). Two species are described: the facultatively autotrophic *Pyrobaculum islandicum* (DSM 4184), which is the type species, and the obligately heterotrophic *Pyrobaculum organotrophum* (DSM 4185).

Key words: Archaebacteria – Hyperthermophilic – Solfatara fields – Geothermal – Sulfur

Up to now, hyperthermophilic bacteria growing optimally at 100°C and above have been isolated exclusively from submarine hydrothermal systems. They can be separated into two different groups of archaebacteria: (a) *Pyrodictium*, consisting of disc-shaped, network-forming, hydrogen-sulfur autotrophs with an optimal growth temperature of 105°C and an upper temperature border of growth at around 110°C (Stetter 1982; Stetter et al. 1983), and (b) *Pyrococcus*, consisting of coccoid, motile, fermentative organisms with an optimal growth temperature at 100°C and an upper temperature limit of growth at 103°C (Fiala and Stetter 1986). Here, we describe the isolation and properties of rod-shaped continental hyperthermophilic archaebacteria growing optimally at 100°C. They were obtained from samples taken at a geothermal power plant and within solfatara fields.

Materials and methods

Strains. Thermoproteus tenax DSM 2078, *Thermoproteus neutrophilus* DSM 2338, *Staphylothermus marinus* DSM 3639 and *Escherichia coli* ATCC 11775 were from the strain collection of our institute.

Culture conditions. The new isolates were cultivated using the anaerobic technique of Balch and Wolfe (1976). They were grown in a basal mineral medium (Allen 1959), containing Na_2S (0.05%) and resazurin (0.0001%). If not otherwise mentioned, the mineral medium was supplemented with yeast extract (Bacto Difco, 0.02%), peptone (Bacto Difco, 0.05%) and sulfur (2.0%). The pH was adjusted to 6 with H_2SO_4.

Ten milliliter cultures were grown in stoppered 28 ml serum tubes (Balch et al. 1979) pressurized with N_2 (300 kPa) and incubated without shaking. Batch cultures were grown in a 300 l enamel-protected fermentor (HTE, Bioengineering, Wald, Switzerland) under stirring (80 rev/min) and gassing with N_2 (3 l/min).

Light microscopy. Cultures were routinely observed using a Zeiss standard phase contrast microscope with an oil immersion objective 100/1.3. Phase contrast photographs were taken with a Zeiss MC 63 camera after preparation of the slides according to the method of Pfennig and Wagener (1986).

Electron microscopy. Cells and thin sections were prepared and photographed as described previously (Huber et al. 1986).

Determination of growth. Bacterial growth was followed by cell counting in a "Neubauer" counting chamber (depth: 0.02 mm).

Preparation of cell extracts. Cell extracts were prepared according to Fiala et al. (1986).

Analyses of metabolic products. The metabolic products were analysed by gas chromatography or by titration as described elsewhere (Huber et al. 1986).

Analysis of muramic acid. Muramic acid was analyzed as described by König and Stetter (1982).

Determination of oxygen sensitivity. Stoppered serum bottles (120 ml) containing 20 ml of anaerobic exponentially growing cultures (1 to 5×10^7 cells/ml) were opened in a heated waterbath (95°C). The cultures were then flushed vigorously with air (2 min). The bottles were closed again

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Table 1. Origin of the new isolates

Source		Original temperature (°C)	Original pH	Strain designation
Country	Locality			
Azores	Ribeira Quente; pond with superheated water	100	6	KB18
Iceland	Krafla geothermal power plant; water from overpressure valve	100	8.5	GEO2, GEO3, GEO4
	Grandalur (Hveragerthi); strongly gassed spring hole	93	6	H10
	Banks of Varma (Hveragerthi); large brownish waterhole	96	9	H16
Italy	Pisciarelli Solfatara (Naples); greyish groundwater, about 50 cm deep within the solfataric soil	95	6	PC1

and pressurized with air (300 kPa). The cultures were incubated at 100° C in a shaker (300 rev/min) and the titre of viable cells was determined several times by serial dilutions.

Isolation of DNA. DNA was isolated as described (Stetter et al. 1981).

DNA base composition. The GC-content was determined by melting point analysis (Marmur and Doty 1962) and by direct analysis after digestion of the DNA with nuclease P1 and separation by high performance liquid chromatography (Zillig et al. 1980b).

DNA homology. DNA-DNA hybridization was carried out after radioactive labelling of the DNA (Kelly et al. 1970) with a nick translation reagent kit (Bethesda Research Laboratories) using the filter technique (Gillespie and Gillespie 1971; Birnstiel et al. 1972) as described (König 1984).

Results

Collection of samples and isolation of the new bacteria

One hundred and two mud- and water samples were taken anaerobically (Stetter 1982) from solfatara fields at the Azores, Iceland, Italy and the USA and from a geothermal power plant at Krafla, Iceland (orig. pH: 1.5–9.0; orig. temp.: 72° C–100° C). The samples were transported to the laboratory without temperature control. In order to enrich hyperthermophiles, serum tubes containing 10 ml of anaerobic culture medium with yeast extract, peptone and elemental sulfur as carbon- and energy sources (pH 6; gas phase N_2, 300 kPa) were inoculated with 1 ml of the samples and incubated at 100° C. After 3–11 days, rod-shaped bacteria were visible in 7 of 102 culture attempts. The enrichment cultures were purified by repeated serial dilutions (Stetter 1982). The isolate GEO3 was cloned by plating on culture medium solidified by starch (20% w/v; final concentration), containing yeast extract, peptone and sodium thiosulfate (0.1% w/v; final concentration). Tiny greenish-black colonies were formed after anaerobic incubation (7 days, 85° C; Balch et al. 1979). Isolates GEO3 and H10 from Iceland were obtained first in pure culture and as they differed from each other they were studied in detail.

Distribution

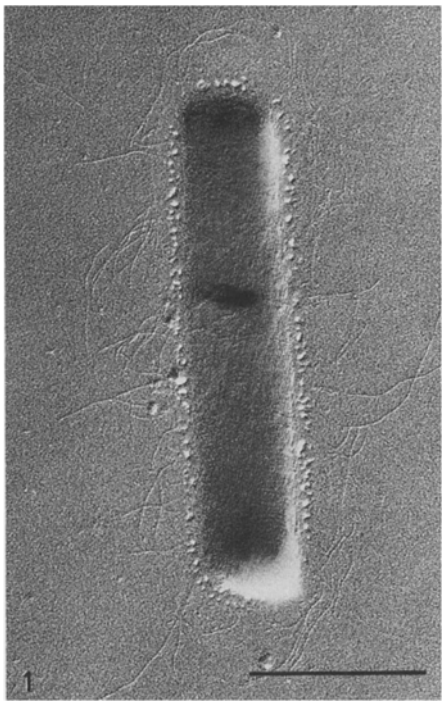
The new organisms were isolated (Table 1) from an outflow of superheated water of an overpressure valve at the Krafla geothermal power plant and from the Hveragerthi solfatara field (both: Iceland), from the Ribeira Quente solfataras (Azores) and Pisciarelli Solfatara (Italy). Attempts to isolate similar organisms from samples collected in the Yellowstone National Park, USA (Obsidian Creek; Nymph Lake; Frying Pan Spring; Beryl Spring; Firehole Pool; Midway Geyser; West Thumb; Lake Yellowstone) were not successful.

Culture and storage

In order to obtain storage cultures, 10 ml culture media were inoculated (2%) and incubated at 100° C for 2 (isolate GEO3) or 3 (isolate H10) days. The cultures were then kept at 4° C and served as an inoculum for at least 3 months. Batch cultures (300 l) of isolate GEO3 yielded about 60 g cell mass (wet weight; electron acceptor: thiosulfate 0.1% w/v), while those of isolate H10 yielded only about 35 g (electron acceptor: S^0 2% w/v). Cell masses of H10 were grey in colour while those of GEO3 exhibited an intensive dark green colour, probably due to the presence of a green pigment.

Morphology

The new isolates were rod-shaped almost rectangular cells, about 1.5 to 8 µm long and 0.5 µm wide. More than about 80% of the cells of H10 were 3 to 5.5 µm, whereas those of GEO3 were 2.5 µm long. Strain H10 exhibited peritrichous flagellation (flagella about 13 nm in diameter; up to 5 µm long: Figs. 1, 2), while strain GEO3 showed bipolar polytrichous flagellation with up to three flagella (about 13 nm in diameter; up to 15 µm long; Fig. 3) at each end. Motility was observed under the light microscope. It was strongly enhanced by heating the microscopic slide to 90° C. Cells of isolate GEO3 exhibited motility during the whole growth phase while cells of H10 were only motile during the early exponential growth phase. During growth, cells formed aggregates arranged in V-shape with various angles (Fig. 4), and, especially in cultures of H10, in X- and raft-shape (Fig. 5). In exponentially growing cultures about 1% of the rods developed terminal spherical bodies. They were similar to the "golf clubs" reported for *Thermoproteus tenax* (Zillig et al. 1981) and may represent buds. No septa formation was observed. Probably cells multiplied by constriction and budding. Cells are Gram-negative and are surrounded by an S-layer (Sleytr and Messner 1983) of protein subunits (Fig. 6).



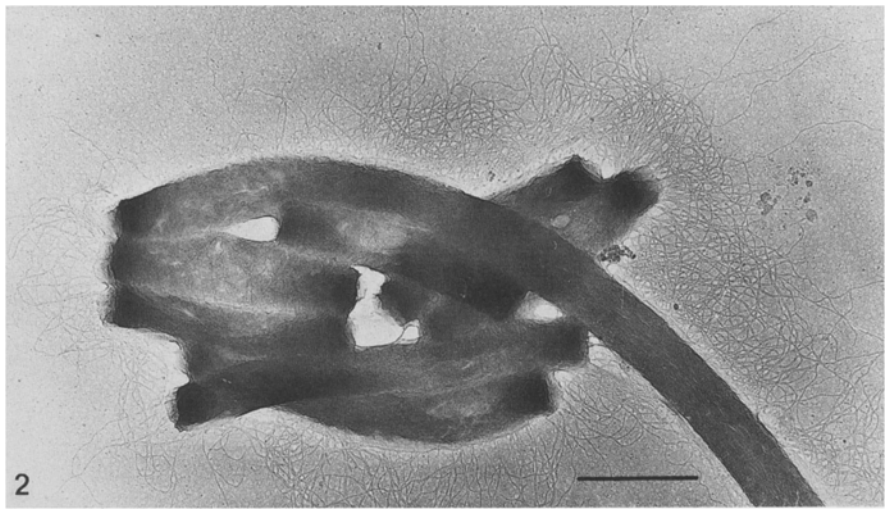


Fig. 1
Electron micrograph of *Pyrobaculum organotrophum* (isolate H10), platinum-shadowed, showing peritrichous flagellation. Bar: 1 µm

Fig. 2
Electron micrograph of *Pyrobaculum organotrophum* (isolate H10), platinum-shadowed, Bar: 1 µm

Growth temperatures

In closed culture bottles isolate GEO3 grew at temperatures between 74° C and 102° C with an optimum at 100° C (about 260 min doubling time). The isolate H10 grew between 78° C and 102° C with an optimum at 102° C (690 min doubling time; Fig. 7). At 74° C, the doubling time of isolate GEO3 was extremely long (around 3 weeks; data not shown). In a 1 l fermentor, isolate GEO3 grew with a doubling time of only 150 min (not shown) when gassed continously with H_2/CO_2 (80:20).

pH of growth

Growth of all new isolates was observed between pH 5 and 7 with an optimum at around 6 (not shown).

Growth in the presence of salt

Isolates GEO3 and H10 grew in culture medium with up to 0.8 and 0.5% (w/v) NaCl, respectively.

Metabolism

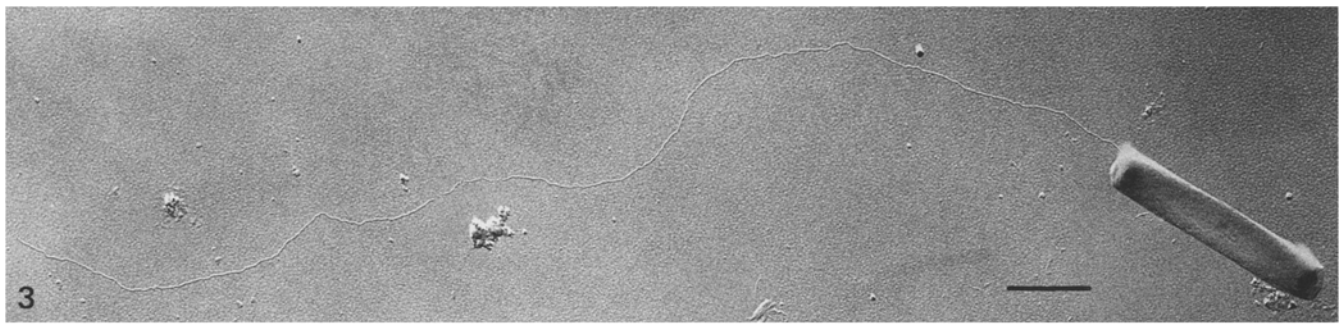
The new isolates were anaerobes. They were able to grow by the reduction of elemental sulfur (Fig. 8). Thus they were sulfur respirers (Pfennig and Biebl 1976). All of them were able to grow organotrophically, using complex organic material as hydrogen donors. Growth was obtained on yeast extract, peptone, extract of meat and cell homogenates (0.25 g cells wet weight/100 ml culture medium) of eubacteria (*Lactobacillus bavaricus, Thermotoga maritima*) and archaebacteria (*Methanothermus fervidus, Methanococcus thermolithotrophicus, Staphylothermus marinus*). No growth was obtained on galactose, glucose, maltose, starch glycogen (each 0.5% w/v), ethanol, methanol, formamide, formate, malate, propionate, L(+)lactate, acetate (each 0.2% w/v) and casamino acids (0.1% w/v). The metabolic end products were identified as H_2S (about 2 µmol/ml) and CO_2. Organic acids, alcohols or H_2 were not detected and therefore not formed in significant amounts. Isolates GEO2, GEO3, GEO4 and H16 were able to grow alternatively chemolithoautotrophically in mineral medium in the presence of elemental sulfur, molecular hydrogen and CO_2, using the formation of H_2S as an energy source (Fischer et al. 1983). Up to 63 µmol H_2S were formed per ml culture medium (isolate GEO3). In contrast, isolates H10, PC1 and KB18 were obligate organotrophs.

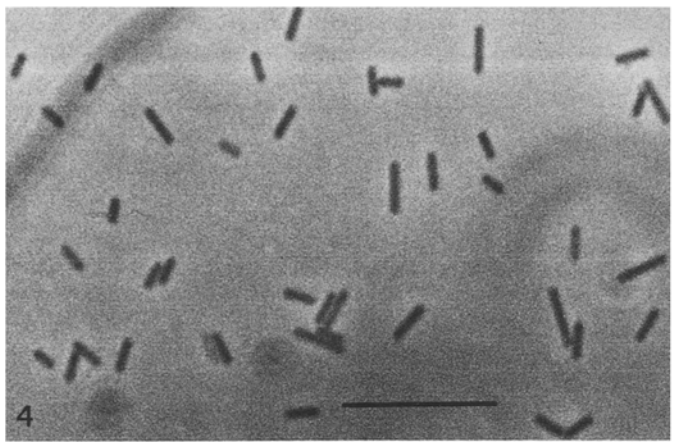
Terminal hydrogen acceptors

For chemolithotrophic growth on H_2, elemental sulfur was essential as it is the case also for *Thermoproteus tenax* (Fischer et al. 1983; Huber et al. 1987). Sulfur could not be replaced by sulfite, thiosulfate, sulfate, tetrathionate, dimethyl sulfone, L(−)cystine or oxidized glutathione (isolate GEO3). During organotrophic mode of nutrition, sulfur was replaceable by different sulfur compounds, depending on the isolates. In GEO2, GEO3, and GEO4, S^0 as H^+-acceptor could be replaced by sulfite, thiosulfate, L(−)-cystine and oxidized glutathione. H10 grew only in the presence of L(−)cystine or glutathione, while H16, KB18 and PC1 were able to grow only in the presence of thiosulfate, when S^0 was absent. None of the isolates were able to use DL-lanthionine, fumarate, tetrathionate, dimethyl sulfone or sulfate as a hydrogen acceptor.

Oxygen sensitivity

The new isolates did not grow when traces of oxygen were present. This was indicated by the red colour of resazurin (redox potential above −40 mV). The cells remained viable for about 20 min, when S^0-containing exponentially growing cultures of isolates GEO3 and H10 were exposed to oxygen by aerating at 100° C (Fig. 9). After 40 min, only 10% of the





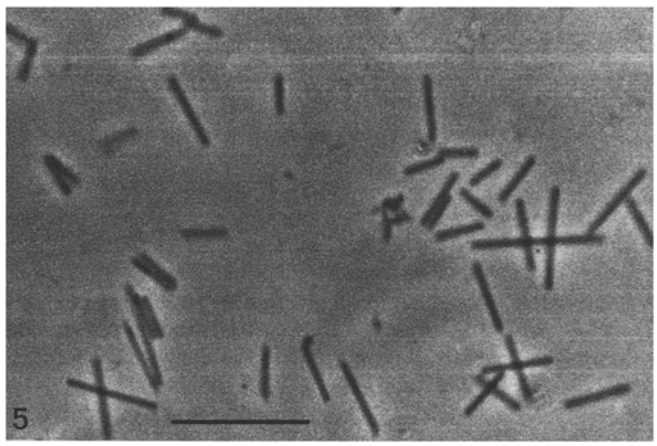


Fig. 3. Electron micrograph of *Pyrobaculum islandicum* (isolate GEO3), platinum-shadowed, showing one polar flagellum. Bar: 1 μm

Fig. 4. Phase contrast micrograph of cells of *Pyrobaculum islandicum* (isolate GEO3). Bar: 10 μm

Fig. 5. Phase contrast micrograph of cells of *Pyrobaculum organotrophum* (isolate H10) Bar: 10 μm

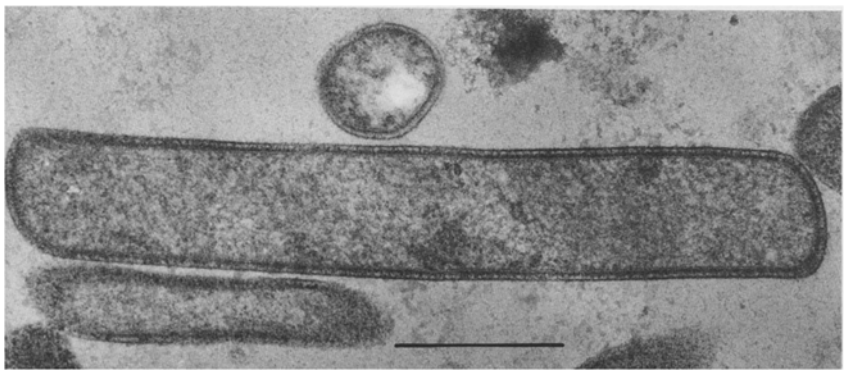


Fig. 6
Thin section of *Pyrobaculum islandicum* (isolate GEO3), contrasted with lead citrate and uranyl acetate. Bar: 0.5 μm

cells in both cultures were still alive and there were no survivers after 90 min of aeration. In a parallel experiment, sulfur was removed by centrifugation and the cultures were then exposed to oxygen at 100° C (Fig. 9). Under these conditions, after 20 min the concentration of viable cells of isolates GEO3 and H10 had decreased to 1% and 0.1%, respectively. After 40 min, all cells of H10 were killed and after 60 min, also all cells of GEO3 were inactivated. The protective action of S^0 against cell inactivation by oxygen may be due to the reduction of oxygen by the H_2S formed by the cells, which for unknown reasons was limited to the first 20 min.

Sensitivity to antibiotics

Isolates GEO3 and H10 were resistant against penicillin G, streptomycin, phosphomycin, chloramphenicol, and vancomycin, which were added (each 100 μg/ml) to exponentially growing cells at 80° C. In the presence of rifampicin (100 μg/ml), cells of both strains stopped growing. After 2 days, the cells began to multiply again due to thermal destruction of rifampicin. Inactivation of rifampicin was demonstrated in the culture medium of *Escherichia coli*: after preincubating the antibiotic-containing culture medium (100 μg/ml) at

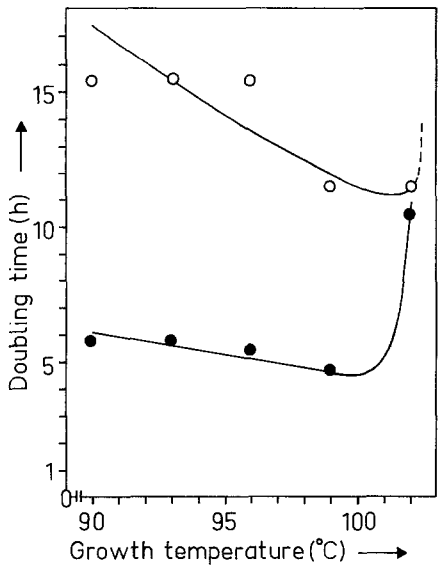


Fig. 7. Optimal growth temperatures of *Pyrobaculum islandicum*, isolate GEO3 (●) and Pyrobaculum organotrophum, isolate H10 (○). The doubling times were calculated from the slopes of the growth curves (not shown)

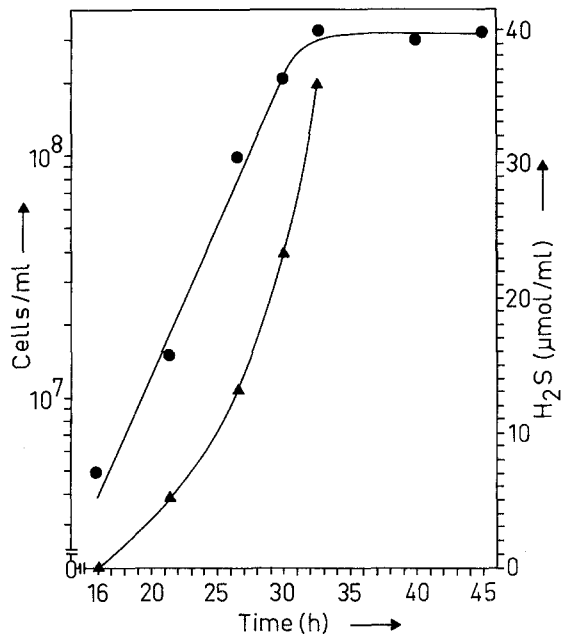


Fig. 8. Formation of H_2S during organotrophic growth of a batch culture (1 l) of *Pyrobaculum islandicum* (isolate GEO3), on yeast extract (0.02%), peptone (0.05%) and sulfur. Gas phase: H_2/CO_2 80:20. Growth (●) was determined in a counting chamber. H_2S (▲) was determined titrimetrically (Williams 1979)

75° C for 24 h, no inhibition was observed after inoculation with *Escherichia coli* (ATCC 11775) at 37° C.

DNA base composition

The DNA of isolates GEO2, GEO3, GEO4, H10, KB18, and PC1 had G+C-contents between 44 and 48 mol% as determined by the T_M method and by analysis of the mononucleotides after digestion of the DNA with nuclease P1 (Table 2; Marmur and Doty 1962; Zillig et al. 1980b).

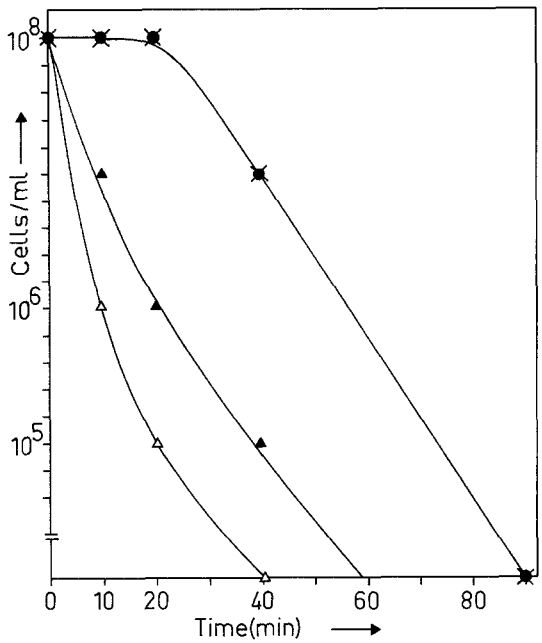


Fig. 9. Oxygen sensitivity of cells of *Pyrobaculum islandicum*, isolate GEO3 (●, ▲) and *Pyrobaculum organotrophum*, isolate H10 (×, △). (●, ×) Viable cells during exposure to oxygen in the presence of S^0. (▲, △) Viable cells in the presence of oxygen in the absence of S^0

Table 2. GC-content (mol%) of different *Pyrobaculum* isolates

Isolate	T_M method	Direct analysis
GEO2	46	48
GEO3	45	47
GEO4	44	47
H10	46	48
H16	n.d.	n.d.
KB18	44	48
PC1	48	n.d.

Sensitivity to diphtheria toxin

After addition of diphteria toxin to cell homogenates of GEO3 and H10, a protein which was most likely elongation factor G was ADP-ribosylated (F. Klink, unpublished work).

DNA homology

By DNA-DNA hybridization (Table 3), the new isolates H10 and GEO3 showed about 65% homology, indicating that they belong to different species. No significant homology could be detected with members of the genera *Thermoproteus* and *Staphylothermus* (Table 3).

Cell wall

Cells of isolates GEO3 and H10 do not contain muramic acid, indicating that they lack murein.

Lipids

Isolates GEO3 and H10 contain isopranyl ether lipids typical of archaebacteria (T. A. Langworthy, unpublished work; Langworthy 1982).

Table 3
Levels of DNA homology for the new isolates GEO3 (*Pyrobaculum islandicum*) and H10 (*Pyrobaculum organotrophum*) and the type strains of *Thermoproteus tenax*, *Thermoproteus neutrophilus* and *Staphylothermus marinus*

Source of filter-bound DNA	*% Homology with the following sources of* ^{32}P-labelled DNA:			
	Isolate H10	Isolate GEO3	*Thermoproteus tenax* DSM 2078	*Thermoproteus neutrophilus* DSM 2338
Isolate H10	100	64	9	15
Isolate GEO3	67	100	8	14
Thermoproteus tenax DSM 2078	10	8	100	9
Thermoproteus neutrophilus DSM 2338	16	17	8	100
Staphylothermus marinus DSM 3639	5	5	5	4
Calf thymus	6	5	5	5

Discussion

The new isolates are Gram-negative rod-shaped hyperthermophilic bacteria. They grow optimally at 100° C with an upper temperature limit of about 102° C. No growth occurs below 78° C or 74° C, depending on the type of strain. The new organisms were isolated from superheated or almost boiling neutral to slightly alkaline anaerobic solfataric waters. Since only very few samples gave rise of positive enrichment cultures, the new isolates may be rather rare at the surface and may possibly grow mainly within the depth of the solfataric soils, where water temperatures above 100° C may be possible due to the hydrostatic pressure and where the pH is only slightly acidic (Stetter et al. 1986). Due to their low salt tolerance, the new organisms appear to be unable to grow within submarine hydrothermal systems. This is in contrast to the biotope of the other hyperthermophiles (Stetter 1982; Stetter et al. 1983; Fiala and Stetter 1986). They are therefore adapted to the (low salt) continental solfataric springs (Brock 1978). The isolates have striking morphological similarities to rod-shaped bacteria that had developed on microscope slides immersed in superheated neutral springs (Brock 1978). They belong to the archaebacterial kingdom (Woese et al. 1978) as demonstrated by the lack of a murein cell wall in favour of a protein cell envelope (Kandler 1982), the occurrence of isopranyl ether lipids (Langworthy 1982), the existence of an ADP-ribosylable elongation factor G (Kessel and Klink 1982) and the resistance to the antibiotics penicillin, streptomycin, phosphomycin, vancomycin, and chloramphenicol. The cells are sensitive to rifampicin. This sensitivity is unusual for archaebacteria and may be possibly explained by a target different from the RNA polymerase similar to *Halobacterium halobium* (Zillig et al. 1980a). Within the archaebacteria, the new isolates resemble the genus *Thermoproteus* judged by their S^0-respirative metabolism, their straight rectangular rod-shape and their ability to form terminal buds. In contrast to *Thermoproteus*, the new organisms do not form filaments. They differ in their motility and have a 10% lower G+C content in their DNA (*Thermoproteus*: 56 mol% G+C). Moreover, their optimal growth temperature is 12° C higher than *Thermoproteus*. The phylogenetic distance to members of the genus *Thermoproteus* is evident by the lack of significant DNA homology. On the basis of the results presented in this paper we describe a new genus which we have named *Pyrobaculum*, the "fire stick". The syllable "Pyro" serves to denote the ability to grow at temperatures above 100° C (Stetter et al. 1983). Two distinct new species can be distinguished by morphology, flagellation, metabolic and physiological properties and by DNA homology studies: the facultatively heterotrophic *Pyrobaculum islandicum* and the strictly heterotrophic *Pyrobaculum organotrophum*. Within its superheated biotope, *Pyrobaculum islandicum* may act as a primary producer of organic matter during chemolithoautotrophic growth on S^0, CO_2 and H_2. In the presence of organic material, however, it is able to use also other S-compounds like thiosulfate and sulfite present in geothermal waters (Zinder and Brock 1977).

Description of a new genus and two new species

Pyrobaculum, Stetter, Huber and Kristjansson (gen. nov.), Py.ro.ba'cu.lum Gr. neutr. n. *pyr* fire; L. neutr. n. *baculum* stick; M. L. neutr. n. *Pyrobaculum* the fire stick. Cells are Gram-negative rods with almost rectangular ends, occurring singly and in V-, X- and raft-shaped aggregates. Terminal spheres ("golf-clubs") appear during the exponential growth phase. No septa formation observed. Cells are about 1.5–8 μm in lenght and about 0.5 μm in width. Motile due to flagellation. Colonies are grey or greenish-black. Cells surrounded by an S-layer of protein subunits. Growth between 74° C and 102° C (optimum: 100° C), pH 5–7 and 0–0.5% NaCl. Strictly anaerobic. Facultative and obligate heterotrophs. Heterotrophic growth on yeast extract, peptone, extract of meat and archae- and eubacterial cell homogenates by respiration of elemental sulfur, L(−)cystine or oxidized glutathione or, depending on the strains, on sulfite and thiosulfate. Lithoautotrophic growth on H_2, CO_2 and elemental sulfur by H_2/S-autotrophy. Cells are insensitive against penicillin G, streptomycin, phosphomycin, vancomycin, and chloramphenicol and sensitive against rifampicin (most likely unspecific inhibition). Elongation factor G is ADP-ribosylated. No murein present. Isopranyl ether lipids in the cell membrane. 44–48 mol% G+C. Members of the genus *Pyrobaculum* occur in neutral to slightly alkaline boiling solfataric waters. The type species is *Pyrobaculum islandicum*.

Pyrobaculum islandicum, Stetter, Huber and Kristjansson (sp. nov.), is.la'n.di.cum M. L. neutr. adj. *islandicum* Icelandic, describing the place of its first isolation. Cells are usually about 2.5 μm long and exhibit bipolar polytrichous flagellation, each flagellum up to 15 μm long and about 13 nm in width. V-shaped aggregates of 2 cells with different angles are formed. Packed cells exhibit a green colour. Growth at 74° C and in the presence of 0.8% (w/v) NaCl. Optimal doubling time (in closed culture vessels) about

260 min at 100°C. Facultatively organotrophic. During organotrophic growth, S^0, thiosulfate, sulfite, L(−)-cystine and oxidized glutathione serve as electron acceptors. Isolates were obtained from boiling solfataric and geothermal waters in Iceland. The type strain is *Pyrobaculum islandicum*, GEO3, DSM 4184, Göttingen, FRG.

Pyrobaculum organotrophum, Stetter, Huber, and Kristjansson (sp. nov.), or.ga.no.tro'ph.um, Gr. neutr. n. *organon* tool (pertaining to organic chemical compounds in M. L.), Gr. masc. n. *trophos* one who feeds; M. L. neutr. adj. *organotrophum* feeding on organic material. Cells are usually between 3 and 5.5 µm long and are peritrichous flagellated (flagella up to 5 µm long, about 13 nm in width). Often raft-shaped aggregates with up to 20 cells. Packed cells show a grey colour. No growth at 74°C or in the presence of 0.8% (w/v) NaCl. Optimal doubling time (in closed culture vessels) about 690 min at 100°C. Strictly organotrophic. During growth, S^0, L(−)cystine and oxidized glutathione serve as electron acceptors. 65% DNA homology with *Pyrobaculum islandicum*. Isolates were obtained from boiling solfataric waters in Iceland, Italy and the Azores. The type strain is *Pyrobaculum organotrophum*, H10, DSM 3185, Göttingen, FRG.

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