

***Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic Archaea, represented by two new species, *Ignicoccus islandicus* sp. nov. and *Ignicoccus pacificus* sp. nov.**

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Two species of novel, chemolithoautotrophic, sulfidogenic micro-organisms were isolated from submarine hydrothermal systems in the Atlantic (at the Kolbeinsey Ridge north of Iceland) and in the Pacific (at 9 °N, 104 °W). The coccoid cells grew within a temperature range of 70–98 °C (optimum around 90 °C). They gained energy by reduction of elemental sulfur using molecular hydrogen as the electron donor. 16S rDNA-based sequence comparisons revealed that the organisms are members of the crenarchaeal branch of the Archaea. They represent a new, deeply branching lineage within the family of the Desulfurococcaceae. In DNA–DNA hybridization experiments both strains exhibited low levels of hybridization to each other and to further representatives of this family. Therefore, they represent a new genus, for which the name *Ignicoccus* gen. nov. is proposed. At present it consists of two new species, *Ignicoccus islandicus* sp. nov. (type strain is Kol8^T = DSM 13165^T = ATCC 700957^T) and *Ignicoccus pacificus* sp. nov. (type strain is LPC33^T = DSM 13166^T = ATCC 700958^T).

Keywords: *Archaea*, *Crenarchaeota*, hyperthermophilic, marine, chemolithoautotrophic

INTRODUCTION

Within the *Archaea*, hyperthermophilic micro-organisms growing optimally at temperatures between 80 and 110 °C are found in both kingdoms, the *Euryarchaeota* and the *Crenarchaeota* (Stetter, 1988, 1996; Woese *et al.*, 1990). Within the *Euryarchaeota* they are represented by deep-branching organisms like *Methanopyrus kandleri* and members of the genera *Thermococcus*, *Pyrococcus*, *Methanothermus*, *Methanococcus* and *Archaeoglobus*. So far, the cultivated *Crenarchaeota* are exclusively extremely thermophilic or hyperthermophilic (Huber & Stetter, 1999). 16S rRNA-based sequence comparisons have revealed that three orders are evident within the *Crenarchaeota* (Burggraf *et al.*, 1997): *Sulfolobales*,

consisting of lobed or coccoid-shaped thermoacidophiles, *Thermoproteales*, which harbours all rod-shaped organisms of the kingdom, and the recently described order *Desulfurococcales* (Huber & Stetter, 2000). *Desulfurococcales* is composed of two families: the *Pyrodictiaceae*, members of which grow optimally above 100 °C, and the representatives of the *Desulfurococcaceae*, which exhibit optimal growth between 85 and 100 °C (Burggraf *et al.*, 1997). The representatives of the *Desulfurococcaceae* are usually regular to irregular cocci, which occur singly or in pairs. Some species form chains or aggregates in addition. With the exception of the aerobic *Aeropyrum pernix*, all members of the *Desulfurococcaceae* are obligate anaerobes, growing heterotrophically by sulfur respiration of various organic compounds (producing H₂S) or by fermentation. In both cases, yeast extract, peptides or sugars serve as substrates. Organic acids or alcohols are found as metabolic products (Huber & Stetter, 1999).

The EMBL accession numbers for the 16S rDNA sequences of *Ignicoccus islandicus* and *Ignicoccus pacificus* are X99562 and AJ271794, respectively.

In this study we describe the isolation and properties of novel, chemolithoautotrophic members of the *Desulfurococcaceae* which grow by sulfur reduction with molecular hydrogen as electron donor.

METHODS

Sources of samples. At the Kolbeinsey Ridge, north of Iceland, eight samples of submarine sandy sediments and venting water (original temperatures around 90 °C) were taken by the research submersible 'Geo' at depths between 103 and 106 m (Fricke *et al.*, 1989; Burggraf *et al.*, 1990). Furthermore, black smoker samples were obtained during dive 3072 of the submersible 'Alvin' at the East Pacific Rise at 9° N, 104° W at a depth of 2500 m. The samples were brought to our laboratory anaerobically without temperature control.

Strains and culture conditions. The new isolates were enriched and cultivated in strictly anaerobic half-strength SME medium (Stetter *et al.*, 1983; Pley *et al.*, 1991), prepared according to Balch & Wolfe (1976). The medium contains the following components (l⁻¹): NaCl, 13.85 g; MgSO₄·7H₂O, 3.5 g; MgCl₂·6H₂O, 2.75 g; KH₂PO₄, 0.5 g; CaCl₂·2H₂O, 0.38 g; KCl, 0.33 g; (NH₄)₂SO₄, 0.25 g; NaBr, 0.05 g; H₃BO₃, 0.015 g; SrCl₂·6H₂O, 7.5 mg; KI (1 mg ml⁻¹), 25 µl; elemental sulfur, 5.0 g. Reduction of the medium was carried out by addition of 20 ml Na₂S (2.5%, w/v); afterwards, the pH was adjusted at room temperature to 5.5 with sulfuric acid. The organisms were grown routinely in 120 ml serum bottles containing 20 ml medium pressurized with H₂/CO₂ (80:20, v/v; 250 kPa). Incubation was carried out at 90 °C with shaking (100 r.p.m.). Heterotrophic growth was tested under a gas phase of N₂/CO₂ (80:20, v/v; 200 kPa).

Unless otherwise stated, organic substrates and alternative electron acceptors (thiosulfate, sulfite, sulfate, nitrate and nitrite) were added at final concentrations of 0.1%. Batch cultures were grown in a 300 l enamel-protected fermenter (HTE Bioengineering) at 90 °C with stirring (100 r.p.m.) and gassing with H₂/CO₂ (80:20; 2 l min⁻¹).

Light and electron microscopy. Cells were routinely observed with an Olympus BX 60 phase-contrast microscope with an oil immersion objective, UPlanFl 100/1.3. Bacterial growth was followed by direct cell counting using a Thoma chamber (depth 0.02 mm). Electron microscopy was performed as follows. For direct visualization, cells were chemically fixed by adding glutaraldehyde (2%, v/v, final concn) to the culture medium, concentrated by centrifugation, applied onto a carbon-coated copper grid and shadowed with 1 nm Pt/C (angle 15°). For ultrathin sections, cells were cultivated in cellulose capillary tubes, high-pressure frozen, freeze-substituted in acetone containing 1% OsO₄ and embedded in Epon/Araldite (Rieger *et al.*, 1997). Sections were stained with uranyl acetate and lead citrate. For freeze-etching, a concentrated cell suspension was frozen in liquid nitrogen, freeze-etched for 4 min at -97 °C and shadowed with 1 nm Pt/C (angle 45°) and with 10 nm C (angle 90°). Replicas were cleaned overnight on sulfuric acid (70%, w/v). Electron micrographs were taken on a model CM12 transmission electron microscope (Philips) with an acceleration voltage of 120 kV.

H₂S determination. The formation of H₂S was qualitatively monitored by addition of 20 µl saturated lead acetate solution to 0.5 ml samples, yielding a dark brown precipitate.

For quantitative determinations, H₂S was analysed by titration (Williams *et al.*, 1979).

Lipid analysis. Core lipids were analysed according to Trincone *et al.* (1992).

DNA isolation and DNA base composition. DNAs were prepared as described previously (Wildgruber *et al.*, 1982). The G+C content of genomic DNAs was determined by melting point analysis (Marmur & Doty, 1962) and by direct analysis of the nucleotides after digestion of the DNA with nuclease P1 and separation by HPLC chromatography (Vökl *et al.*, 1993). Calf thymus DNA was used as reference.

DNA-DNA hybridization. DNA-DNA hybridization was carried out using the filter technique (Gillespie & Gillespie, 1971; Birnstiel *et al.*, 1972) as described by Pley *et al.* (1991). Nucleic acids were hybridized under optimal conditions (25 °C below *T_m* in 3 × SSC buffer at 65 °C) (Marmur & Doty, 1961; Brenner, 1973; Meyer & Schleifer, 1978) using the DNAs of Kol8^T and LPC33^T as probes.

16S rRNA analysis. The nearly complete 16S rRNA genes of the new isolates were PCR-amplified (Saiki *et al.*, 1985, 1988). The primers used in the amplification corresponded to positions 8–23 (TCYGGTTGATCCTGCC), and 1512–1492 (ACGGHTACCTTGTTACGACTT) for Kol8^T or 1406–1390 (ACGGGCGGTGTGTRCAA) for LPC strains (*Escherichia coli* 16S rRNA numbering; Brosius *et al.*, 1981). Both strands of the PCR products were directly sequenced as described by Burggraf *et al.* (1997). The sequences (Kol8^T, 1465 bases; LPC33^T, 1311 bases; LPC37, 1297 bases) were aligned with a set of representative archaeal sequences using the ARB program (W. Ludwig & O. Strunk, 1998, <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/>). Dendrograms were computed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods included in the ARB package.

RESULTS

Enrichment and isolation

To enrich chemolithoautotrophic, sulfur-reducing hyperthermophiles, serum bottles with 20 ml half-strength SME medium supplemented with 1% (w/v) sulfur (gas phase H₂/CO₂) were inoculated with about 1 g of the sandy sediments from the Kolbeinsey Ridge or rocky black smoker material obtained from the deep sea of the East Pacific Rise. The enrichment attempts were incubated with shaking at 90 °C. After 2 d, irregular cocci had grown in 3 out of 10 samples (Kol8^T, LPC33^T and LPC37) and large amounts of H₂S could be detected qualitatively in the culture medium. The enrichment cultures were successfully transferred into fresh medium and cells were first purified by serial dilution carried out three times using the same medium. In addition, the 'optical tweezer' technique was used for final purification (Huber *et al.*, 1995). The isolates were designated as the samples.

Morphology

Cells of both new isolates were irregular cocci usually occurring singly or in pairs. They exhibited cell diameters between 1.2 and 3 µm (Kol8^T), and 1 and

2 µm (LPC33^T). The organisms stained Gram-negative. They were both motile, possessing one bundle of up to nine flagella (for isolate Kol8^T; Fig. 1a). The cell architecture of the isolates was studied by sectioning cells after high-pressure freezing and freeze-substitution (Fig. 1b) and by the freeze-etch/freeze-fracture technique (Fig. 1c). The cytoplasm was densely packed and surrounded by a membrane (approx. 6–8 nm wide). The periplasm had a variable width, ranging between 20 nm and 350 nm. It contained numerous, round or elongated vesicles, 50–60 nm in diameter and up to 300 nm in length, each surrounded by a membrane (Fig. 1b). Rarely, these periplasmic vesicles were in close contact with the cytoplasmic membrane. In sections, the outermost part of the cell envelope occasionally had a weak double-layer appearance. This 'sheath' was frequently found to be fractured into two leaflets in freeze-etch/freeze-fracture experiments (Fig. 1c) and, therefore, most likely represented a (lipid) membrane. Close inspection of the fracture planes revealed that this membrane was tightly packed with particles, most likely proteins. A two-dimensional crystalline arrangement, as observed in S-layer sheets of most *Archaea* and many *Bacteria*, was never observed. The core lipids of isolate Kol8^T are acyclic 2,3-di-*O*-phytanil-*sn*-glycerol and glycerol-dialkyl glycerol tetraether in a relative ratio of about 1:1 (A. Gambacorta, personal communication).

Metabolism

The new isolates were obligate anaerobes, growing chemolithoautotrophically by sulfur reduction using molecular hydrogen as electron donor (final cell concentrations up to 4×10^7 cells ml⁻¹). Up to 25 µmol H₂S (ml culture medium)⁻¹ was formed by isolate Kol8^T at the end of the exponential growth phase. In the presence of H₂ and S⁰, growth of all isolates was stimulated by the addition of meat extract, tryptone and glycogen (each 0.1%; final cell densities around 6×10^7 – 8×10^7 cells ml⁻¹). The addition of yeast extract (0.1%) resulted in shorter doubling times for both strains, although higher final cell densities were only observed for LPC33^T. No effect on growth or final cell concentrations was obtained in the presence of Casamino acids, casein, starch, gelatin, maltose or glucose (each 0.1%) for all strains. The addition of formate or acetate (0.05%) decreased final cell densities to about 1×10^7 cells ml⁻¹. No growth was observed on organic substrates like meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate or glucose when cultures were pressurized with hydrogen-free gas (N₂/CO₂, 80:20; 200 kPa). In a similar way, sulfur could not be replaced by oxygen (0.5–5% v/v), thiosulfate, tetrathionate, sulfite, sulfate or nitrate (each 0.1%) as electron acceptors. Ampicillin, rifampicin and vancomycin (final concn 50 µg ml⁻¹) were not inhibitory to growth of the strains.

Optimal growth conditions

Isolate Kol8^T grew between 70 and 98 °C. No growth was observed at 65 °C or below, nor at 100 °C or above (Fig. 2). Isolates LPC33^T and LPC37 were able to grow between 75 and 98 °C with an optimum at 90 °C (Fig. 2). When the medium was supplemented with 0.1% meat extract, the shortest doubling times of 1.4 and 0.8 h were observed for isolates Kol8^T and LPC33^T at 90 °C, respectively (Fig. 2). Under chemolithoautotrophic conditions, the shortest doubling times were 1.7 and 1.6 h at 90 °C, respectively (data not shown). Kol8^T grew at NaCl concentrations 0.3 and 5.5% (w/v) (Fig. 3), while strain LPC33^T exhibited a minimum NaCl requirement of 1.0% and a maximum of 5.0% (Fig. 3). For all strains the optimal NaCl concentration was around 2% and cells lysed at NaCl concentrations above 6.0% in the medium. Kol8^T grew between pH 3.8 and 6.5 with an optimum around pH 5.8 (Fig. 4). The optimal pH for growth of LPC33^T was 6.0 with a minimum at pH 4.5 and a maximum at pH 7.0 (Fig. 4).

Storage

Stock cultures, containing 5% (v/v) dimethylsulfoxide and stored at –140 °C over liquid nitrogen served as viable inocula for at least 3 years.

DNA base composition

The G + C content of genomic DNA of isolate Kol8^T was 41 mol%, calculated by melting point analysis and by direct analysis of the mononucleotides. Isolates LPC33^T and LPC37 exhibited 45 mol% G + C in their genomic DNA.

DNA–DNA similarity

DNA–DNA hybridization experiments revealed no significant hybridization signals between isolate Kol8^T and isolates LPC33^T and LPC37 (similarity 11 and 8%, respectively). In contrast, a DNA–DNA similarity of 76% was obtained between LPC33^T and LPC37. The DNA of each of the new isolates yielded no significant hybridization signal (similarity between 5 and 9%) with DNA of the following *Crenarchaeota* and *Euryarchaeota*: *Staphylothermus marinus*, *Desulfurococcus mobilis*, *Thermococcus celer* and *Pyrococcus furiosus*.

Phylogenetic analysis

Comparison of 16S rDNA sequences between the new isolates and representatives of the *Crenarchaeota* and *Euryarchaeota* using all three major approaches for tree reconstruction clearly indicated that the new

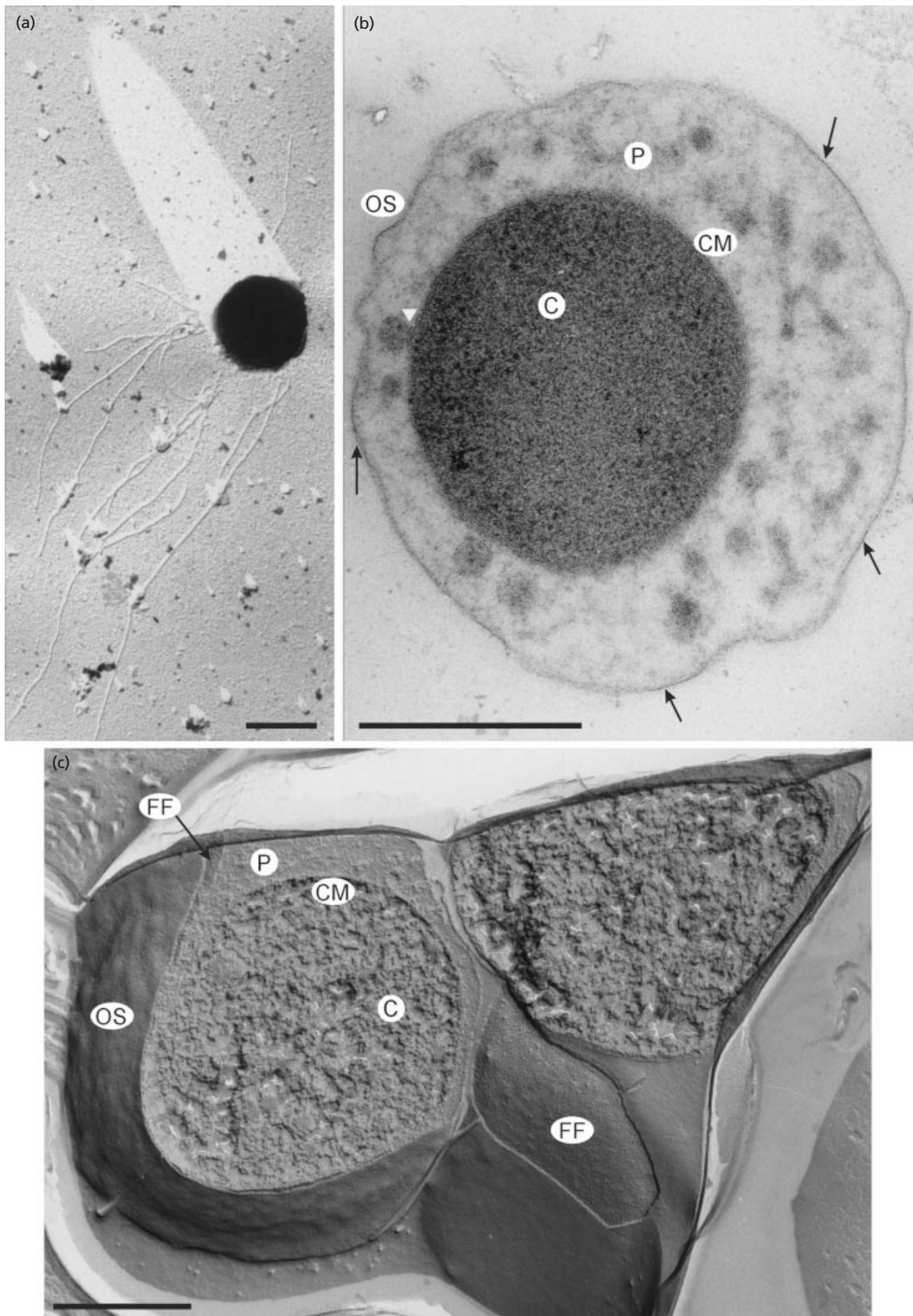


Fig. 1. For legend see facing page.

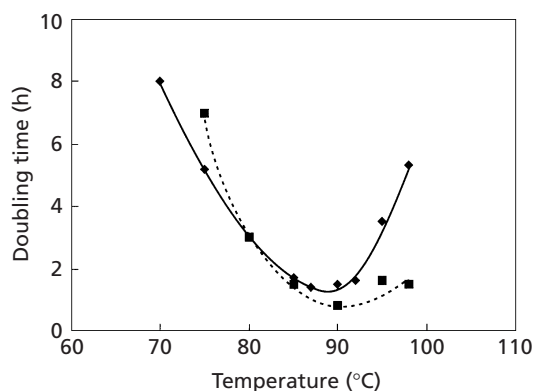


Fig. 2. Optimal growth temperature of *Ignicoccus islandicus* strain Kol8^T (◆) and *Ignicoccus pacificus* strain LPC33^T (■) during growth on H₂ and sulfur at pH 5.5 and 2% NaCl. Doubling times were calculated from the slopes of the growth curves (not shown).

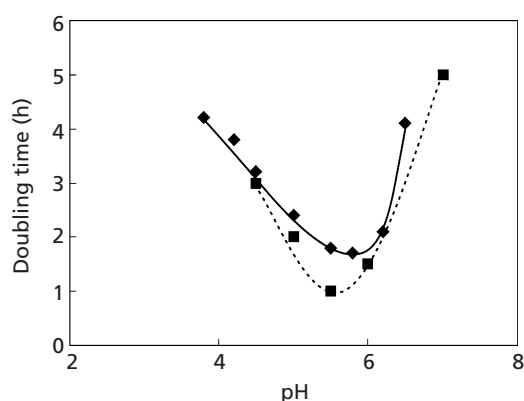


Fig. 4. Effect of pH on growth of *Ignicoccus islandicus* strain Kol8^T (◆) and *Ignicoccus pacificus* strain LPC33^T (■) at 90 °C and 2% NaCl. Doubling times were calculated as described in the legend to Fig. 2.

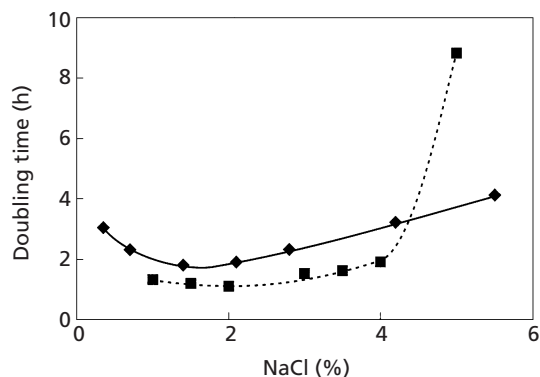


Fig. 3. Effect of NaCl concentration on growth of *Ignicoccus islandicus* strain Kol8^T (◆) and *Ignicoccus pacificus* strain LPC33^T (■) at 90 °C and pH 5.5. Doubling times were calculated as described in the legend to Fig. 2.

strains belong to the crenarchaeal branch of the archaeal domain (Fig. 5). In all calculations they are members of the new order *Desulfurococcales*, representing a deep branch within the *Desulfurococcaceae* (Fig. 5). They exhibit sequence differences between 6 and 10% to any of the other members of this family (data not shown). In contrast, the differences among the new isolates were between 0.2 (LPC33^T and LPC37) and 2.0% (Kol8^T and LPC strains).

DISCUSSION

The novel hyperthermophilic isolates Kol8^T, LPC33^T and LPC37 represent the first obligate chemolithoautotrophic sulfur reducers within the crenarchaeal family of the *Desulfurococcaceae*. They are representatives of this family due to their coccoid cell shape, the lack of a cell sacculus, their negative Gram reaction, their optimal growth temperature of around 90 °C and their 16S rDNA sequences (Huber & Stetter, 2000). They can be differentiated from the other members of the *Desulfurococcales* by their unique cell wall architecture (Baumeister & Lembcke, 1992) and the extremely limited spectrum of utilized electron donors and acceptors: molecular hydrogen is the only electron donor and no acceptors other than elemental sulfur can be used. Therefore, the organisms are obligate hydrogen-sulfur autotrophs, producing H₂S. Some organic components stimulate growth by decreasing the shortest doubling times and yielding higher final cell densities. However, they cannot be used as sole energy sources. In contrast, all other anaerobic members of the *Desulfurococcaceae* known so far grow organotrophically. In addition to organic acids or alcohols, several representatives produce H₂S from elemental sulfur (e.g. *Desulfurococcus*, *Staphylothermus*, 'Thermodiscus' or *Stetteria*), while the members of the genera *Sulfophobococcus* and *Thermosphaera* are inhibited in the presence of elemental sulfur (Huber & Stetter, 1999). This physiological separateness of the new isolates is confirmed by 16S rDNA sequence analyses, where the new isolates

Fig. 1. (a) Electron micrograph of a cell of isolate Kol8^T, exhibiting numerous flagella. The sample was fixed with 2% glutaraldehyde and shadowed with Pt/C. Bar, 1 μm. (b) Electron micrograph of a thin section of a freeze-substituted cell of isolate Kol8^T. C, cytoplasm; CM, cytoplasmic membrane; P, periplasm; OS, outer sheath; white arrowhead, contact site of a periplasmic vesicle with the cytoplasmic membrane; black arrows, areas of the outer sheath with double-layer appearance. Bar, 0.5 μm. (c) Electron micrograph of freeze-etched cells of isolate Kol8^T. C, cytoplasm; CM, cytoplasmic membrane; P, periplasm; FF, fracture face of the outer sheath; OS, outer sheath. Bar, 0.5 μm.

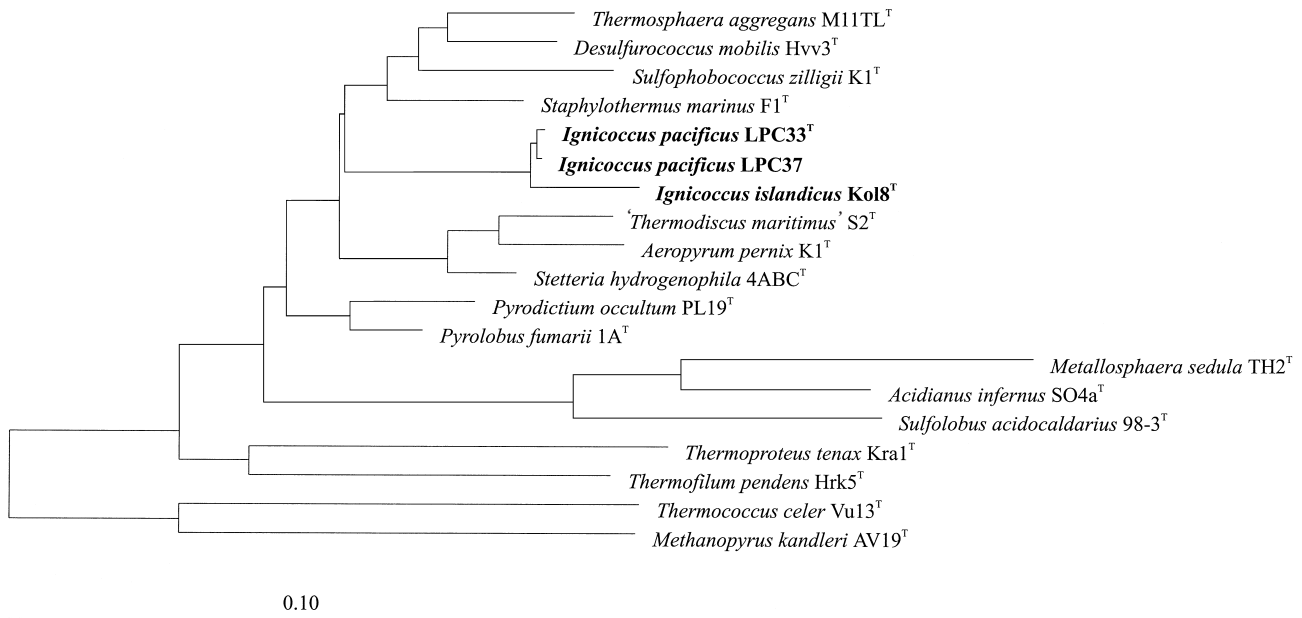


Fig. 5. Phylogenetic position of *Ignicoccus* species (isolates Kol8^T, LPC33^T and LPC37), calculated by the neighbour-joining method using Jukes & Cantor correction. Bar, 10 estimated substitutions per 100 nt.

exhibit sequence differences of at least 6% to any other representative of the *Desulfurococcaceae* but only about 2% to each other. This indicates that the new isolates belong to the same new genus, for which we propose the name *Ignicoccus* gen. nov., the 'fireball', expressing the extremely high temperature optimum of all strains. The first isolate, Kol8^T, can be distinguished from the two LPC strains by a generally bigger cell diameter, a lower minimum growth temperature of 70 °C, growth at lower NaCl concentrations (0.3% in contrast to 1.0%), a low DNA–DNA similarity to these two organisms and a 4 mol% lower genomic DNA G+C content. It represents the type species of the new genus, which we have named *Ignicoccus islandicus* (type strain is Kol8^T = DSM 13165^T = ATCC 700957^T), referring to the location of its isolation.

Isolates LPC33^T and LPC37 show similar physiological and molecular characteristics. Furthermore, in DNA–DNA hybridization experiments, a similarity of 76% was obtained for these two organisms. This indicates that they belong to the same second species, which we have named *Ignicoccus pacificus*, referring to the location of its isolation. The type strain is LPC33^T (= DSM 13166^T = ATCC 700958^T).

Ignicoccus islandicus and *Ignicoccus pacificus* were isolated from hot sediments at the Kolbeinsey Ridge, north of Iceland, and from active black smoker walls from the East Pacific, respectively. These biotopes are located far from each other and represent different geological environments. Therefore, since their substrates (hydrogen, CO₂ and sulfur) are commonly

found in hydrothermal fluids (Jannasch & Mottl, 1985), representatives of *Ignicoccus* may be widely distributed in submarine hydrothermal systems. Due to their chemolithoautotrophic mode of life, they are probably important primary producers of organic matter in these biotopes and are essential for the growth of organotrophic hyperthermophiles, like the other marine representatives of the *Desulfurococcaceae*.

Description of *Ignicoccus* gen. nov. (Huber, Burggraf and Stetter)

Ignicoccus (Ig'ni.coc'cus. L. masc. n. *ignis* fire; Gr. masc. n. *coccus* berry; M.L. masc. n. *Ignicoccus* the berry of the fire).

Irregular cocci, about 1–3 µm in diameter, monopolar polytrichous flagella. Gram-negative. Occurring singly and in pairs. Cell envelope consists of a cytoplasmic membrane, a periplasm (20–350 nm wide) and a sheath resembling an outer membrane. Cells contain phytanyl di- and tetraether lipids. Growth between 70 and 98 °C, pH optimum around 6. Strictly anaerobic. Chemolithoautotrophic growth in the presence of H₂ and CO₂ with sulfur as electron acceptor. Sulfate, sulfite, thiosulfate, tetrathionate, nitrate and oxygen are not used as electron acceptors. H₂S formed during growth. Ampicillin-, rifampicin- and vancomycin-resistant. DNA base composition between 41 and 45 mol% G+C. Based on 16S rDNA sequence comparison, the genus is a member of the family *Desulfurococcaceae*, within the kingdom *Crenarchaeota*. Type

species is *Ignicoccus islandicus* (Kol8^T = DSM 13165^T = ATCC 700957^T).

Description of *Ignicoccus islandicus* sp. nov. (Huber, Burggraf, Mayer and Stetter)

Ignicoccus islandicus (is.lan'di.cus. M.L. masc. adj. *islandicus* Icelandic, pertaining to the location of its first isolation).

Slightly irregular cocci, about 1.2–3 µm in diameter, monopolar polytrichous flagella. Gram-negative. Occurring singly and in pairs. No evidence for a regular arrayed surface protein. Growth between 70 and 98 °C, pH 3.8–6.5 and 0.3–5.5% NaCl; optima are 90 °C, pH 5.8 and 2% NaCl. Strictly anaerobic. Chemolithoautotrophic growth in the presence of H₂ and CO₂ with sulfur as electron acceptor. No chemo-organotrophic growth on meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate and glucose. Sulfate, sulfite, thiosulfate, nitrate and oxygen are not used as electron acceptors. H₂S (up to 25 µmol ml⁻¹) is formed during growth. Growth stimulated by addition of 0.1% meat extract, tryptone or gelatin. DNA base composition 41 mol% G+C. Low DNA–DNA similarity to *Ignicoccus pacificus*. Type strain is isolate Kol8^T (= DSM 13165^T = ATCC 700957^T), isolated from the Kolbeinsey Ridge, north of Iceland.

Description of *Ignicoccus pacificus* sp. nov. (Huber and Stetter)

Ignicoccus pacificus (pa.ci'fi.cus. M.L. masc. adj. *pacificus* from the Pacific, pertaining to the site of its first isolation).

Slightly irregular cocci, about 1–2 µm in diameter, monopolar polytrichous flagella. Gram-negative. Occurring singly and in pairs. No evidence for a regular arrayed surface protein. Growth between 75 and 98 °C, pH 4.5–7.0 and 1.0–5.0% NaCl; optima are 90 °C, pH 6 and 2% NaCl. Strictly anaerobic. Chemolithoautotrophic growth in the presence of H₂ and CO₂ with sulfur as electron acceptor. No chemo-organotrophic growth on meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate or glucose. Sulfate, sulfite, thiosulfate, tetrathionate and nitrate are not used as electron acceptors. H₂S formed during growth. Growth stimulated by addition of 0.1% meat extract, tryptone, gelatin or yeast extract. DNA base composition 45 mol% G+C. No significant DNA–DNA similarity to *Ignicoccus islandicus*. Type strain is isolate LPC33^T (= DSM 13166^T = ATCC 700958^T), isolated from black smoker samples at 9° N, 104° W in the Pacific Ocean.

ACKNOWLEDGEMENTS

The excellent technical assistance of Kerstin Roth, Jutta Schipka and Peter Hummel is highly appreciated. We wish to thank Wolfgang Ludwig for providing the ARB program, Sabine Weber for primary work in electron microscopy,

Manfred Biebl for practical work on strain LPC37 and Agata Gambacorta for lipid analysis. This work was supported by grants of the Deutsche Forschungsgemeinschaft (STE 297/10–3) and the Fonds der Chemischen Industrie.

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