Oceanithermus profundus gen. nov., sp. nov., a thermophilic, microaerophilic, facultatively chemolithoheterotrophic bacterium from a deep-sea hydrothermal vent

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A novel moderately thermophilic, organotrophic, microaerophilic, facultatively chemolithotrophic bacterium, designated strain 506^T, was isolated from a deep-sea hydrothermal vent site at 13°N in the East Pacific Rise. Cells were Gram-negative, non-motile rods. The organism grew in the temperature range 40–68 °C, with an optimum at 60 °C, and in the pH range 5.5–8.4, with an optimum around pH 7.5. The NaCl concentration for growth was in the range 10–50 g l⁻¹, with an optimum at 30 g l⁻¹. Strain 506^T grew chemoorganoheterotrophically with carbohydrates, proteinaceous substrates, organic acids and alcohols using oxygen or nitrate as electron acceptor. Alternatively, strain 506^T was able to grow lithoheterotrophically with molecular hydrogen as the energy source. The G + C content of the genomic DNA was 62.9 mol%. Phylogenetic analysis of the 16S rDNA sequence placed strain 506^T in the family *Thermaceae*. On the basis of phenotypic and phylogenetic data, strain 506^T (= DSM 14977^T = VKM B-2274^T) is proposed as the type strain of a novel species in a new genus, *Oceanithermus profundus* gen. nov., sp. nov.

The family *Thermaceae* currently comprises thermophilic, rod-shaped organisms with a strictly respiratory type of metabolism. The first representative, *Thermus aquaticus*, was isolated from a hot spring in Yellowstone National Park (Brock & Freeze, 1969), but representatives of this group have been found in many natural and artificial thermal habitats (Williams & Da Costa, 1992). At the time of writing, the genus *Thermus* is represented by eight species with high optimal growth temperatures (65–75 °C), while four species with lower temperature optima (50–65 °C) are classified in the genus *Meiothermus* (Nobre *et al.*, 1996). A new genus, *Marinithermus*, has recently been described; its unique representative, *Marinithermus hydrothermalis*, isolated from a deep-sea hydrothermal vent, thrives between 50 and

72.5 °C (optimum 67.5 °C) (Sako *et al.*, 2003). Most strains of the family *Thermaceae* are organotrophs, though the ability to grow lithotrophically with sulfur compounds was reported for *Thermus scotoductus* (Skirnisdottir *et al.*, 2001). Some strains are also able to grow anaerobically with nitrate or nitrite as the terminal electron acceptor (Da Costa & Rainey, 2001). In this paper, we report the isolation, from a deep-sea hydrothermal vent, of a novel microaerophilic representative of the family *Thermaceae* that is capable of lithotrophic and organotrophic growth.

Samples were collected during the Amistad cruise (1999) at the 13°N hydrothermal vent field (12°48'N, 103°56'W) on the East Pacific Rise at a depth of 2600 m. Samples of hydrothermal fluids and chimneys were transferred in tightly stoppered 50 ml plastic tubes and stored at 4 °C. The enrichment medium contained the following (g l⁻¹ unless indicated): NH₄Cl, 0.33; KCl, 0.33; KH₂PO₄, 0.33; CaCl₂·2H₂O, 0.33; MgCl₂·6H₂O, 0.33; NaCl, 25.0;

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NaNO₃, 3.0, sodium acetate, 3.0, yeast extract, 0.1; NaHCO₃, 0.5; trace elements (Balch *et al.*, 1979), 10 ml l⁻¹; vitamins (Wolin *et al.*, 1963), 10 ml l⁻¹. The medium was prepared anaerobically (Balch *et al.*, 1979) and dispensed into 15 ml Hungate tubes; the headspace (5 ml) was filled with N₂ (atmospheric pressure). No reducing agents were added to the medium. The pH of the medium was 6.5. Single colonies were isolated using the same medium solidified with 1.5 % agar (Difco) by using a serial 10-fold dilution technique in agar shake tubes. Agar shake tubes were incubated at 60 °C for 5 days. The morphology of the novel isolate was examined using a light microscope (Mikmed-1 model; LOMO). The ultrastructure of whole cells and thin sections was studied as described elsewhere (Bonch-Osmolovskaya *et al.*, 1990). Oxidase activity was assayed with discs impregnated with dimethyl *p*-phenylenediamine (bioMérieux). Catalase activity was assayed by mixing a pellet of a freshly centrifuged culture with a drop of hydrogen peroxide (10 %, v/v). Physiological tests were performed in BM medium, which consisted of enrichment medium from which the sodium acetate and sodium nitrate had been omitted. Potential proteinaceous growth substrates were added to the BM medium at a concentration of 1 g l⁻¹; carbohydrates, sodium salts of organic acids and alcohols were present at a concentration of 3 g l⁻¹. When molecular hydrogen served as the substrate, the headspace (10 ml) was filled with a H₂/CO₂ mixture (4:1, v/v). Possible electron acceptors were added at a concentration of 2 g l⁻¹. All experiments were performed in triplicate.

To determine the ability to grow microaerophilically, air was added to the headspace (10 ml) of tubes filled with BM medium (5 ml). The final oxygen concentration was varied from 0.5 to 9 %. The ability to grow aerobically was determined either on plates with agarized BM medium (2 % agar; Difco) supplemented with sucrose (2 g l⁻¹) and tryptone (1 g l⁻¹) or in 100 ml flasks containing the same medium (10 ml). Growth was monitored by measuring the increase in OD₆₀₀ by using a Spectronic 401 spectrophotometer (Bioblock). All growth experiments were performed in duplicate using Bellco tubes under microaerophilic conditions. The pH range for growth was determined in BM medium supplemented with 2 g sucrose and 2 g sodium nitrate l⁻¹ using various buffers (MES for pH 5.0–6.0, PIPES for pH 6.5 and 7.0, HEPES for pH 7.5, Tris for pH 8.0 and 8.5) at a concentration of 10 mM. Appropriate amounts of 1 M Na₂CO₃ were added to adjust the pH of the medium to 9.0 and 9.5. The pH was determined at room temperature. To determine the optimum NaCl range for growth, NaCl concentrations were varied while the concentrations of the other inorganic components were maintained. The effects of different pH values and concentrations of NaCl were determined at 60 °C. Gaseous and liquid fermentation products were detected by means of GLC (Miroshnichenko *et al.*, 1994). Gaseous and liquid products of nitrate reduction were studied as described elsewhere (Miroshnichenko *et al.*, 2003). Respiratory

quinones and polar lipids were extracted and analysed as described previously (Tindall, 1990a, b). Fatty acids were extracted and analysed as methyl esters as described previously. The presence of unsaturation was confirmed by hydrogenation (Brian & Gardner, 1968) and the position of unsaturation was located by the formation of dimethyl disulfide adducts, using the method of Nichols *et al.* (1986) and the instrumentation and conditions described previously (Strömpl *et al.*, 1999).

DNA was isolated after disruption of cells using a French pressure cell (Thermo Spectronic) and purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed by HPLC as described by Tamaoka & Komagata (1984). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and sequencing of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced directly using a *Taq* Dye-Deoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. An Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of sequence reaction products. The 16S rDNA sequence of strain 506^T was aligned manually with nucleotide sequences obtained from the GenBank and EMBL databases. The method of Jukes & Cantor (1969) was applied to calculate evolutionary distances. Phylogenetic trees were constructed using the methods of DeSoete (1983) and Felsenstein (1993).

Twelve samples from hydrothermal vents containing water and chimney material were used for the inoculation of enrichment medium (5 %, v/v) and incubated at 60 °C. After 5 days incubation, samples of the inner and outer chimney part from the 'Genesis' site produced abundant growth of morphologically homogeneous micro-organisms. After several successive transfers, the enrichment culture was serially diluted and the highest dilutions were transferred to the same medium solidified with agar. After 5 days incubation at 60 °C, single colonies appeared. Colonies were spherical and non-pigmented, with diameters ranging from 0.3 to 1 mm. A single colony from the last dilution was transferred into liquid enrichment medium. The purification procedure was repeated twice, after which the culture was considered to be pure. Purity was confirmed by microscopic examination of the culture growing on medium containing glucose (3 g l⁻¹), pyruvate (3 g l⁻¹) and yeast extract (3 g l⁻¹). The purified micro-organism was designated as strain 506^T.

Cells of strain 506^T were non-motile rods, 0.5–0.7 µm in diameter and of various lengths. On negatively stained electron microscope preparations, small spheres were often seen along the cell wall and between the cells (Fig. 1a). Flagella were never observed. When grown on proteinaceous substrates, old cultures of strain 506^T formed filaments and large spheres (Fig. 1c) resembling the 'rotund bodies' typical

Fig. 1. Negatively stained whole cell (a), thin section of a whole cell (b) and phase-contrast micrograph (c) of strain 506^T. Bars, 0.5 μm.

of aged cells of *Thermus* species (Brock & Edwards, 1970). Thin-section electron micrographic preparations revealed a Gram-negative cell wall structure. The cell wall had a complex multilayered structure with a characteristic corrugated outer layer (Fig. 1b). Spores were never observed. The isolate was microaerophilic, only being able to grow at oxygen concentrations below 6 %. No growth was observed in an atmosphere of air, either in liquid medium or on plates. In an agar tube (containing 5 ml BM medium supplemented with 2 g sucrose and 1 g tryptone l⁻¹) with air in the headspace (10 ml), growth occurred only in a zone located 20 mm below the agar/air interface. Alternatively, strain 506^T grew anaerobically using nitrate as the electron acceptor. Strain 506^T grew within a temperature range of 40–68 °C, optimal growth being observed at 60 °C. At 60 °C, it grew between pH 5.5 and 8.4, with an optimum around 7.5. Strain 506^T required NaCl for growth and grew at NaCl concentrations ranging from 10 to 50 g l⁻¹, with an

optimum at 30 g l⁻¹. Strain 506^T was oxidase- and catalase-positive. It was able to utilize a wide spectrum of carbohydrates in the presence of either nitrate or oxygen. The highest cell yield was observed in the presence of nitrate with fructose, maltose, sucrose, trehalose, galactose, rhamnose or xylose. It also utilized glucose, lactose and starch, but did not grow with ribose, galactose, arabinose, dextrin or cellobiose. Acetate and propionate were produced during growth with sucrose as a growth substrate and nitrate as the electron acceptor. Nitrite was the only product of denitrification. Strain 506^T grew well with complex proteinaceous substrates such as beef extract, tryptone or papaic digest of soybean (1–1.5 g l⁻¹). However, growth was strongly inhibited by higher concentrations of these substrates. The isolate did not grow with Casamino acids or yeast extract as sole sources of carbon and energy, though 100 mg yeast extract l⁻¹ was required for growth. Strain 506^T was able to utilize acetate, pyruvate and propionate as growth substrates. It also grew with methanol, ethanol and mannitol, though the cell yield was lower. Strain 506^T was able to grow lithoheterotrophically using molecular hydrogen as the energy source, yeast extract as the carbon source and nitrate as the electron acceptor. Other electron acceptors (sulfate, elemental sulfur, thiosulfate, nitrite) did not support growth, regardless of growth substrate.

Menaquinones were the sole respiratory lipoquinones detected, with MK-8 predominating (95 %) and MK-9 being present in smaller proportions (5 %). The fatty acids comprised mainly iso- and anteiso-branched fatty acids; iso-unsaturated fatty acids were also present (Table 1). The polar lipid pattern was fairly simple, comprising only phospholipids; glycolipids were not detected (Fig. 2). The major phospholipid had an *R_f* value identical to that of the

Table 1. Fatty acid composition of strain 506^T

Fatty acid	Percentage of total
iso 14:0	1.0
iso 15:1ω7*†	7.7
iso 15:0	33.2
anteiso 15:0	5.1
15:0	1.3
iso 16:1ω8†	2.6
iso 16:0	1.5
16:0	3.3
iso 17:1ω7cis	18.8
iso 17:1ω9cis/ω7trans	1.4
iso 17:1‡	1.0
iso 17:0	12.3
anteiso 17:0	5.4
18:0	1.5
iso 19:1ω7†	1.1

*May comprise two overlapping compounds.

†*Cis/trans* configuration not determined.

‡Location of the double bond not determined.

Fig. 2. Polar lipid composition of strain 506^T. Separation in two dimensions: first dimension, chloroform/methanol/water (65:25:4, by vol.); second dimension, chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Identification of the polar lipids was achieved using spray reagents as described in the text. PL1, PL2 and PL3 indicate unidentified phospholipids: these spots were phosphate-positive only, no other spray reagents gave positive results. The structures of these polar lipids are not currently known, but PL3 has an R_f value identical to that of the major phospholipid of *Thermus* and *Meiothermus* species.

major phospholipid present in members of the genera *Thermus* and *Meiothermus*. The presence of MK-8 as the predominant respiratory quinone is consistent with reports of MK-8 in members of the genera *Thermus*, *Meiothermus* (Hensel *et al.*, 1986; Chung *et al.*, 1997) and *Marinithermus* (Sako *et al.*, 2003). MK-8 is also the dominant respiratory lipoquinone present in members of the genus *Deinococcus* (Embley *et al.*, 1987; Ferreira *et al.*, 1997). However, the presence of MK-9, albeit at only 5 %, appears to be a unique feature of the novel isolate in comparison with the genera *Deinococcus*, *Thermus* and *Meiothermus*. MK-9 was not reported in *Marinithermus hydrothermalis* (Sako *et al.*, 2003). The presence of iso- and anteiso-branched fatty acids is a feature of members of the genera *Deinococcus*, *Thermus*, *Meiothermus* and *Marinithermus* (Embley *et al.*, 1987; Ferreira *et al.*, 1997; Donato *et al.*, 1990, 1991; Sato *et al.*, 2003). However, members of the genera *Thermus*, *Meiothermus* and *Marinithermus* described to date do not produce significant amounts of iso-unsaturated fatty acids, as strain 506^T does. In contrast, members of the genus *Deinococcus* produce a number of isomers of iso-unsaturated fatty acids, although their distribution may differ between species. Members of the genera *Thermus* and *Meiothermus* described to date produce both phospholipids and glycolipids (Donato *et al.*, 1990, 1991). Although

members of the genus *Deinococcus* may also produce glycolipids in addition to a novel series of phosphoglycolipids (Embley *et al.*, 1987; Ferreira *et al.*, 1997), the latter are absent in members of the genera *Thermus* and *Meiothermus*. Strain 506^T does not produce glycolipids in significant proportions, which makes it clearly distinguishable from members of the genera *Thermus* and *Meiothermus*. No information on phosphoglycolipids or glycolipids is available for *Marinithermus hydrothermalis*. The presence of the same major phospholipid (according to TLC studies) in strain 506^T and members of the genera *Thermus* and *Meiothermus* is also consistent with the 16S rDNA sequence data. However, the absence of glycolipids strengthens the case for placing the novel strain in a new genus.

The G + C content of the DNA of isolate 506^T was found to be 62.9 mol%. The similarity between the 16S rRNA gene sequence of strain 506^T and that of *Marinithermus hydrothermalis* JCM 11576^T was 90.3 %; similarity to sequences of members of the *Thermus* and *Meiothermus* branches was 87.0–89.6 %. The branching point of strains 506^T and JCM 11576^T changed with the algorithm and number and phylogenetic status of outgroup reference sequences, and often the two lineages do not cluster together. In some dendrograms, strain 506^T was a deep branch in the *Thermus* clade (using a neighbour-joining algorithm with different *Deinococcus* species as an outgroup), while strain 506^T branched deeply in the *Meiothermus* branch using the DeSoete algorithm with *Aquifex* species and deinococci as an outgroup (Fig. 3). In neither dendrogram did strain 506^T branch within the

Fig. 3. Neighbour-joining dendrogram of 16S rDNA relatedness, showing the position of strain 506^T next to its phylogenetic neighbours, members of the genera *Meiothermus*, *Thermus* and *Deinococcus* in the *Deinococcus*–*Thermus* phylum of the *Bacteria*. Percentages of bootstrap support for branch points (1000 resamplings) above 70 % confidence are indicated. Bar, 10 nucleotide substitutions per 100 sequence positions. The tree was rooted with the 16S rDNA sequences of *Aquifex pyrophilus*.

radiation of either *Thermus* or *Meiothermus*. Only low bootstrap values were found for the branching point of strain 506^T in each of the phylogenetic patterns obtained.

Because of its thermophily and metabolic flexibility, strain 506^T is well adapted to the marine hydrothermal environment. Being microaerophilic, the novel isolate may gain significant advantages under conditions where oxygen is limited by its low solubility at high temperature and the reducing nature of hydrothermal fluids and gases. On the other hand, the ability of strain 506^T to use nitrate as an alternative electron acceptor allows it to sustain growth even under anaerobic conditions, which may occur because of spatial and temporal variations of oxygen concentration in hydrothermal vent habitats. The range of compounds that can support growth of the novel isolate is very broad and includes not only organic nutrients derived from biogenic remains, but also non-fermentable substrates (acetate, propionate) produced by the metabolic activity of other inhabitants of hydrothermal vents, e.g. archaea of the genus *Thermococcus* and bacteria of the order *Thermotogales*. Finally, strain 506^T may gain an advantage even under conditions of strong organic substrate limitation, switching from the chemoorganoheterotrophic type of metabolism to the chemolithoheterotrophic mode, utilizing molecular hydrogen of hydrothermal origin. Thus, its greater metabolic versatility may allow it to compete successfully with obligately anaerobic heterotrophs inhabiting the microbial community of deep-sea hydrothermal vents.

Phylogenetic analysis placed isolate 506^T in the family *Thermaceae*. Indeed, it shares common phenotypic features with members of this family, including the characteristic cell wall structure, with a 'cobblestone' outer layer, the formation of 'rotund bodies', the possession of catalase and oxidase activities and the ability to grow anaerobically in the presence of nitrate, reducing it to nitrite. However, in contrast to all members of this group, strain 506^T, being microaerophilic, is not able to grow with oxygen at atmospheric pressure. It exhibits the ability to utilize a broader range of carbohydrates, organic acids and alcohols. Another significant characteristic of the metabolism of the novel isolate is its ability to grow lithoorganotrophically with molecular hydrogen as the electron donor. On the basis of the phenotypic and genomic differences and the distinct phylogenetic position of isolate 506^T, we propose a new genus, *Oceanithermus* gen. nov., with *Oceanithermus profundus* gen. nov., sp. nov. as the type species.

Description of *Oceanithermus* gen. nov.

Oceanithermus gen. nov. (O.ce.a.ni.ther'mus. L. n. *oceanus* the ocean; Gr. fem. n. *therme* heat; N.L. masc. n. *Oceanithermus* warmth-loving organism living in the ocean).

Cells are non-motile, Gram-negative rods, 0.5–0.7 µm in diameter and of variable length. Moderate thermophile. Neutrophile. Adapted to the salinity of sea water. Microaerophile. Flagella and spores are not observed. Able

to utilize a broad range of carbohydrates, some proteinaceous substrates, organic acids and alcohols. Capable of anaerobic growth with nitrate, which is reduced to nitrite. Capable of chemolithoheterotrophic growth with molecular hydrogen. Sole respiratory lipoquinones present are menaquinones, with MK-8 predominating; MK-9 may account for about 5 % of the total. Fatty acids are iso- and anteiso-branched; unsaturated iso-branched fatty acids are also present. Phospholipids are the only polar lipids present. The major phospholipid present has an *R_f* identical to that of the major phospholipid present in *Thermus* and *Meiothermus* species. The G + C content of the DNA of the type strain of the type species is 62.9 mol%. 16S rDNA analysis places *Oceanithermus* in the family *Thermaceae*. The type species is *Oceanithermus profundus*.

Description of *Oceanithermus profundus* sp. nov.

Oceanithermus profundus (pro.fun'dus. L. gen. n. *profundus* of the abyss, the depths of the ocean).

Displays the following properties in addition to those given in the genus description. Filaments and 'rotund bodies' are formed by old cells grown on proteinaceous substrates. The optimal growth temperature is 60 °C. The optimal pH is 7.5 and the optimum salinity is 30 g l⁻¹. Oxidase- and catalase-positive. Oxidizes carbohydrates, starch, peptides, organic acids and alcohols. The chemical composition of the species is identical to that of the genus. The G + C content of the DNA of the type strain is 62.9 mol%. The type strain, strain 506^T (= DSM 14977^T = VKM B-2274^T), was isolated from a deep-sea hydrothermal vent of the East Pacific Rise.

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