Vulcanithermus mediatlanticus gen. nov., sp. nov., a novel member of the family *Thermaceae* from a deep-sea hot vent

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A novel thermophilic, microaerophilic, facultatively chemolithoheterotrophic bacterium designated strain TR^T was isolated from a sample of a deep-sea hydrothermal chimney collected at the Rainbow vent field on the Mid-Atlantic Ridge (36°14'N). Gram-negative, non-spore-forming, non-motile rods occurred singly or in pairs. The organism grew in the temperature range 37–80 °C with an optimum at 70 °C and at pH 5.5–8.4 with an optimum around 6.7. The NaCl range for growth was 10–50 g l⁻¹ with an optimum of 30 g l⁻¹. Strain TR^T grew chemoorganoheterotrophically with carbohydrates, proteinaceous substrates, organic acids and alcohols using oxygen or nitrate as electron acceptors. The isolate was able to grow at oxygen concentrations from 0.5 to 21 %. Oxygen concentrations that promoted fastest growth ranged from 4 to 8 % under agitation. The novel isolate was able to grow lithoheterotrophically with molecular hydrogen as the energy source. The G + C content of the genomic DNA was 68.4 mol%. Phylogenetic analysis of the 16S rDNA sequence placed strain TR^T within the phylum *Deinococcus*–*Thermus* of the *Bacteria*. On the basis of phenotypic and phylogenetic data, it is proposed that this isolate should be described as a member of a novel species of a new genus as *Vulcanithermus mediatlanticus* gen. nov., sp. nov. The type strain is TR^T (= DSM 14978^T = VKM B-2292^T = JCM 11956^T).

Members of the family *Thermaceae* are heterotrophic, aerobic, thermophilic, non-spore-forming bacteria that are widely distributed in natural and man-made thermal systems around the world (Williams & da Costa, 1992). Many have been isolated from terrestrial freshwater environments, although some *Thermus* strains originated from shallow-water hot springs (da Costa & Rainey, 2001). However, a strain of *Thermus thermophilus* was isolated from a deep-sea vent at Guaymas Basin (Marteinsson *et al.*, 1999), confirming the presence of thermophilic aerobes in the hydrothermal environment (Marteinsson *et al.*, 1995). All representatives of the family *Thermaceae* isolated to date

from shallow-water hot vents are halotolerant, growing optimally in the absence of NaCl (da Costa & Rainey, 2001). The only obligately marine representatives of this group, *Marinithermus hydrothermalis* (Sako *et al.*, 2003) and *Oceanithermus profundus* (Miroshnichenko *et al.*, 2003b), were isolated very recently from the deep-sea hydrothermal environment. In this paper, we describe a novel member of the family *Thermaceae*, recovered from an *in situ* collector deployed on a hydrothermal vent of the Mid-Atlantic Ridge, that is also obligately dependent on a salinity equivalent to that of sea water.

An *in situ* growth chamber or vent cap (Reysenbach *et al.*, 2000) designed to concentrate the micro-organisms discharged by hydrothermal emissions was deployed in May 2001 on the Mid-Atlantic Ridge (36°16'N, 33°54'W) by the remote-operated vehicle (ROV) *Victor* during the Iris cruise. After *in situ* incubation, the vent cap was closed by the

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hydraulic arm of the ROV before transportation to the surface. Once on board, the content (aquarium filtering wool and liquid) was removed aseptically, immediately transferred to 50-ml glass vials and flooded with a sterile solution of 3% (w/v) sea salts (Sigma). The vials were then closed tightly with butyl rubber stoppers (Bellco), pressurised with N₂ (100 kPa), reduced with sodium sulphide and stored at 4 °C until processed further.

Enrichment medium (EM) contained (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; NaNO₃, 3.0; yeast extract, 0.1; trace elements (Balch *et al.*, 1979), 10 ml; and vitamins (Wolin *et al.*, 1963), 10 ml. The medium was prepared anaerobically (Balch *et al.*, 1979) and dispensed in Bellco tubes; the headspace (25 ml) was filled with H₂/CO₂ (80:20, overpressure of 2 atm). No reducing agents were added to the medium. The pH of the medium was 6.5. Single colonies were isolated by using a serial tenfold dilution technique in agar shake tubes with the same medium solidified with 1.5% agar (Difco) and supplemented with sodium acetate (1 g l⁻¹). The gas phase in this case was replaced by N₂ (100%). Agar shake tubes were incubated at 60 °C for 5 days. The morphology of the novel isolate was examined using an Olympus BX-60 microscope. The ultrastructure of whole cells and thin sections was studied as described elsewhere (Bonch-Osmolovskaya *et al.*, 1990). Physiological studies of the novel isolate were carried out using medium GM, containing (g l⁻¹): NaCl, 30; yeast extract, 0.5; tryptone, 1; sucrose, 2; and KNO₃, 1. MOPS (10 mM) was used as buffer. The pH of the medium was adjusted with 5 M NaOH before autoclaving. The medium was prepared anaerobically but without any reducing agent. Potential proteinaceous growth substrates were added at a concentration of 1 g l⁻¹, carbohydrates, sodium salts of organic acids and alcohols at a concentration of 3 g l⁻¹. When molecular hydrogen served as a substrate, the headspace (10 ml) was filled with a H₂/CO₂ mixture (80:20, v/v). To determine the influence of oxygen on growth, oxygen was added at concentrations from 0.5 to 21% to the headspace (25 ml) of Bellco tubes filled with GM medium (5 ml) from which KNO₃ was omitted. Possible electron acceptors (thiosulphate, sulphate or nitrite) were added in medium GM without KNO₃ at a concentration of 0.2%. Elemental sulphur (10 g l⁻¹) was also tested as a possible electron acceptor. Oxidase activity was assayed using disks impregnated with dimethyl-*p*-phenylene diamine (bioMérieux). Catalase activity was assayed by mixing a pellet of a fresh culture with a drop of hydrogen peroxide (10%, v/v). All experiments were performed in triplicate.

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 at a concentration of 10 mM (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). Appropriate amounts of 1 M Na₂CO₃ were added to adjust the pH of the medium to 9.0 and 9.5. pH values were determined at room temperature.

To determine the optimum NaCl range for growth, NaCl concentrations were varied while maintaining the concentrations of other inorganic components. The effects of different pH values and concentrations of NaCl were determined at 60 °C. Growth was monitored by measuring the increase in OD₆₀₀ using a Spectronic 401 spectrophotometer (Bioblock). Gaseous and liquid fermentation products were detected by GLC (Miroshnichenko *et al.*, 1994). Gaseous and liquid products of nitrate reduction were studied as described elsewhere (Miroshnichenko *et al.*, 2003a). 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 disulphide (DMDS) 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 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). Phylogenetic analysis followed the methods of Miroshnichenko *et al.* (2003a).

After deployment on the Rainbow vent site, the aquarium filtering wool within the vent cap was heavily coated with very fine black sulphide particles. This material was used for inoculating the EM medium. The tubes were incubated at 60 °C. After 3 days, the enrichment produced abundant growth of morphologically different rod-shaped microorganisms. After several successive transfers of the enrichment culture, the gas phase was replaced with N₂ and acetate was added as a substrate. Stable growth of large, non-motile rods was obtained on this medium. This culture was serially diluted and the highest dilutions were transferred to the same medium solidified with agar. After 5 days of incubation at 60 °C, round, slightly creamy colonies appeared. Single colonies (0.3–1 mm in diameter) from the last dilution were transferred into the same medium without agar. The purification procedure was repeated twice before the culture was considered to be pure. Purity was confirmed by microscopic examination of the culture after growth on a test medium containing glucose (3 g l⁻¹), pyruvate (3 g l⁻¹) and yeast extract (3 g l⁻¹). The isolated strain was designated TR^T.

Cells of strain TR^T were non-motile rods, 0.5–0.7 µm in diameter and variable in length (Fig. 1a). Flagella, spores and 'rotund bodies', which are typical of the majority of members of the family *Thermaceae* (Brock & Edwards, 1970), were never observed. Thin-section electron preparations

Fig. 1. Negatively stained whole cells of strain TR^T (a) and thin section of the whole cell (b). Bars, 0.5 μm.

revealed a Gram-negative structure of cell wall with a 'cobblestone' outer layer, consistent with that of representatives of the family *Thermaceae* (da Costa & Rainey, 2001) (Fig. 1b). The isolate was able to grow at oxygen concentrations from 0.5 to 21 %. Oxygen concentrations that promoted fastest growth ranged from 4 to 8 % under agitation. However, the growth yield was two to three times higher when the strain was cultivated with 12–16 % oxygen. A long lag phase (20 h) was observed when air was applied as headspace. The novel isolate was able to grow anaerobically in the presence of nitrate serving as electron acceptor. Strain TR^T grew within a temperature range of 37–80 °C, with an optimum at 70 °C. At 70 °C, it grew between pH 5.5 and 8.4, with an optimum around 6.7. Strain TR^T required NaCl for growth and grew at NaCl concentrations ranging from 1 to 5 % with an optimum at 3 %. Strain TR^T was able to utilize a wide spectrum of carbohydrates in the presence of both nitrate and oxygen. It grew well on media containing complex proteinaceous substrates (tryptone, yeast extract, bio-trypticase and Bactopeptone) and sugars (fructose, maltose, sucrose, trehalose, galactose, arabinose and xylose). It also utilized glucose, lactose, starch and maltodextran, but growth was lower with these substrates. No fermentation products were detected during growth of the novel isolate with sucrose and nitrate. It was able to utilize acetate, pyruvate, succinate, fumarate and butyrate as growth substrates. It also grew with methanol, ethanol, propanol and mannitol. The novel isolate did not grow with casein, malate, lactate, propionate or cellobiose. Strain TR^T was able to grow lithoheterotrophically (100 mg yeast extract l⁻¹ was necessary) using molecular hydrogen as an energy source and nitrate or oxygen as electron acceptors. Nitrate, when present, was reduced to nitrite, which was the only product of denitrification. Other possible products of nitrate reduction (NO₂⁻, N₂, NO, N₂O) were not detected. Other

electron acceptors (sulphate, elemental sulphur, thiosulphate, nitrite) did not support growth, regardless of the growth substrate.

Menaquinones were the sole respiratory lipoquinones detected, with MK-8 predominating; a small amount of a second quinone with a retention time identical to that of MK-8 (VIII-H₂) from members of the family *Halobacteriaceae* was also present. However, this compound could not be identified unambiguously. The fatty acids comprised mainly iso- and anteiso-branched fatty acids, with iso-unsaturated and cyclopropane-ring-containing fatty acids also being present (Table 1). The combination of GLC-MS on the underivatized fatty acids and the DMDS adducts does not allow unambiguous identification of either the position of the cyclopropane rings or the position of branching. However, cyclopropane rings are generally derived from unsaturated fatty acids in which the aliphatic backbone has the same number of carbon atoms. One may, therefore, speculate that the cyclopropane-containing fatty acids are iso-branched, although further work is needed to confirm this. The polar lipid pattern was fairly simple, comprising both phospholipids and glycolipids (Fig. 2). The major phospholipid had an *R_f* value identical to that of the major phospholipid present in members of the genera *Oceanithermus*, *Thermus* and *Meiothermus*, whereas the major glycolipid had an *R_f* value close, but not identical, to that of the major glycolipid of *Thermus aquaticus* DSM 625^T (data not shown).

The presence of MK-8 as the predominant respiratory

Table 1. Fatty acid composition of strain TR^T

Fatty acid	Content (%)
iso 15:1ω7*†	5.5
iso 15:0	22.7
anteiso 15:0	2.4
15:0	0.9
iso 16:1ω8†	2.3
iso 16:0	2.5
16:0	1.1
iso 17:1ω7c‡	15.0
iso 17:1ω9c/ω7t	2.6
iso 17:1§	1.6
iso 17:0	20.5
anteiso 17:0	5.2
Branched cyclo 17:0all	15.2
Branched cyclo 17:0bll	1.4
18:0	1.0

*May comprise two overlapping compounds.

†*Cis/trans* configuration not determined.

‡A second, minor compound co-elutes; structure not determined.

§Location of the double bond not determined.

||The methods employed do not allow unambiguous location of the cyclopropane ring, nor the position of branching.

Fig. 2. Polar lipid composition of strain TR^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 polar lipids was by use of spray reagents (see Methods). PL1, PL2 and PL3, Phospholipids, phosphate-positive only, no other spray reagents gave positive results. GL 1, GL2 and GL3, Spots giving positive reactions characteristic of glycolipids (anisaldehyde/sulphuric acid-positive, α -naphthol-positive and slowly periodate-Schiff-positive). The structures of these polar lipids are currently not known, but PL3 has an R_f identical to that of the major phospholipid of *Oceanithermus*, *Thermus* and *Meiothermus* species (see text for discussion).

quinone is consistent with reports of MK-8 in members of the genera *Thermus*, *Meiothermus*, *Marinithermus* and *Oceanithermus* (Hensel *et al.*, 1986; Chung *et al.*, 1997; Sako *et al.*, 2003; Miroshnichenko *et al.*, 2003b). The presence of iso- and anteiso-branched fatty acids is a feature of members of these genera and also *Deinococcus* (Embley *et al.*, 1987; Ferreira *et al.*, 1997; Donato *et al.*, 1990, 1991; Sako *et al.*, 2003; Miroshnichenko *et al.*, 2003b). However, in contrast to members of the genera *Marinithermus*, *Thermus* and *Meiothermus* described to date, strain TR^T produces significant amounts of iso-unsaturated fatty acids. The fatty acid composition of *Oceanithermus profundus* shows similar trends to that of strain TR^T; however, branched-chain and cyclopropane-ring-containing compounds were detected only in strain TR^T. 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 from members of the genera *Thermus* and *Meiothermus*. Strain TR^T produced a glycolipid with an R_f value similar to that of the compound detected in *T. aquaticus*, its presence distinguishing strain TR^T from

members of the genera *Meiothermus* and *Oceanithermus*. The presence of the same major phospholipid (based on TLC studies) in strain TR^T and members of the genera *Oceanithermus*, *Thermus* and *Meiothermus* is also consistent with results from 16S rDNA sequence data. Taken together, the presence of MK-8, the occurrence of phospholipids and glycolipids similar to those found in *T. aquaticus* and the presence of both saturated and unsaturated iso- and anteiso-branched fatty acids are indicative of the unique chemical composition of strain TR^T and may serve to distinguish it for all other genera in the family *Thermaceae*. The DNA base composition of strain TR^T was 68.4 mol% G + C.

Phylogenetically, strain TR^T was a member of the phylum *Deinococcus-Thermus* of the *Bacteria*. Similarity values of the 16S rRNA gene sequence of this strain to those of the representatives of other genera in this phylum were below 90 %, illustrating the isolated position of the novel isolate. In phylogenetic trees, strain TR^T forms a separate branch at the root of the family *Thermaceae* (Fig. 3). While membership of the phylum is supported by high bootstrap values, there was no indication of a close relationship to any of the existing genera of the family *Thermaceae*.

The hottest areas of the hydrothermal habitat (emitted fluids and active chimneys) have been long considered to represent extremely reduced biotopes, where the presence of aerobic micro-organisms seemed unlikely. However, reports have now shown that ecological niches for thermophilic aerobes could exist in this habitat (Marteinsson *et al.*, 1995, 1996, 1999; Sako *et al.*, 2003; Miroshnichenko *et al.*, 2003b). Such niches might occur in narrow zones with moderate temperatures, where hot, reduced fluid interacts with cold, oxygenated oceanic waters (Marteinsson *et al.*, 1995). Such an environment, where sharp oxygen gradients with tremendous temporal and spatial variability occur, may facilitate colonization by organisms able to thrive in more than

Fig. 3. Phylogenetic position of strain TR^T within the phylum *Deinococcus-Thermus*. Only type species of existing genera belonging to this group and related phyla are shown. The dendrogram is derived from a distance-matrix analysis of almost complete 16S rRNA gene sequences. Values at branch-points are bootstrap percentages (1000 resamplings). The sequence of *Archaeoglobus profundus* (AF322392) was used as out-group (not shown). Bar, 10 % estimated sequence divergence.

one type of chemical environment. Phylogenetically distinct thermophilic bacteria able to grow in both microaerobic and anaerobic conditions have recently been isolated from deep-sea hydrothermal systems. These are two lithoautotrophic thermophilic species of a new genus *Persephonella* (Götz *et al.*, 2002) and *Oceanithermus profundus*, a species of a new genus of the family *Thermaceae* also capable of chemolithotrophy (Miroshnichenko *et al.*, 2003b). Strain TR^T is an additional deep-sea hydrothermal vent representative, capable of nutritional versatility. Phylogenetically, strain TR^T belongs to the family *Thermaceae*, sharing many common features with its members. Within this family, *Oceanithermus profundus* exhibits some features similar to those of the novel isolate: ability to grow microaerobically, ability to grow lithoheterotrophically with molecular hydrogen, obligate requirement for NaCl and capacity for anaerobic respiration in the presence of nitrate. However, strain TR^T differs from it by its higher temperature of growth, the ability to grow under air and chemical composition. Based on phenotypic and genomic differences and the distinct phylogenetic position of isolate TR^T, we propose a new genus, *Vulcanithermus* gen. nov., with the type species *Vulcanithermus mediatlanticus* sp. nov.

Description of *Vulcanithermus* gen. nov.

Vulcanithermus (Vul.ca.ni.ther'mus. L. n. *Vulcanus* the Roman god of fire; Gr. adj. *thermos* hot; N.L. masc. n. *Vulcanithermus* heat-loving organism, living in the vicinity of volcanic areas).

Cells are non-motile, Gram-negative rods, 0.5–0.7 µm in diameter and variable in length. Moderately thermophilic. Neutrophilic. Adapted to the salinity of sea water. Microaerophilic. Capable of aerobic growth. 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. Fatty acids are iso- and anteiso-branched, with unsaturated iso-branched fatty acids also present, as are branched-chain cyclopropane fatty acids. Phospholipids and glycolipids 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, while the major glycolipid has an *R_f* value close, but not identical, to that of the major glycolipid present in *T. aquaticus*. The G+C content of the DNA of the type species is 68.4 mol%. 16S rDNA sequence analysis places *Vulcanithermus* in the family *Thermaceae*. The type species is *Vulcanithermus mediatlanticus*.

Description of *Vulcanithermus mediatlanticus* sp. nov.

Vulcanithermus mediatlanticus (me.di.at.lan'ti.cus. L. adj. *medius* middle; L. masc. adj. *atlanticus* Atlantic; N.L. adj. *mediatlanticus* from the middle of the Atlantic).

Displays the following properties in addition to those given in the genus description. Optimal growth temperature is 70 °C. The optimal pH is around 6.7. The optimum salinity is 3 % NaCl. 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 68.4 mol%. The type strain, TR^T (=DSM 14978^T=VKM B-2292^T=JCM 11956^T), was isolated from the Rainbow deep-sea hydrothermal vent field on the Mid-Atlantic Ridge.

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