Hippea maritima gen. nov., sp. nov., a new genus of thermophilic, sulfur-reducing bacterium from submarine hot vents

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Three strains of moderately thermophilic, sulfur-reducing bacteria were isolated from shallow-water hot vents of the Bay of Plenty (New Zealand) and Matupi Harbour (Papua New Guinea). Cells of all isolates were short, Gram-negative, motile rods with one polar flagellum. All strains were obligate anaerobes and grew optimally at pH 5.8–6.2, 52–54 °C and 2.5–3 % (w/v) NaCl. Growth substrates were molecular hydrogen, acetate and saturated fatty acids; one of the strains, isolated from Matupi Harbour, was able to utilize ethanol. Elemental sulfur was required for growth. H₂S and CO₂ were the only growth products. No growth occurred in the absence of 100 mg yeast extract l⁻¹. The G+C content of the DNA determined for the type strain MH₂^T was 40.4 mol%. Results of 16S rDNA sequencing indicated that these strains represent a distinct lineage most closely related to the genus *Desulfurella*. On the basis of the results of morphological, physiological and phylogenetic studies, a new genus, *Hippea* gen. nov., is proposed with the type species *Hippea maritima* gen. nov., sp. nov., of which the type strain is MH₂^T (= DSM 10411^T).

Keywords: *Hippea maritima* gen. nov., sp. nov., thermophilic bacteria, sulfur reduction, submarine hot vents

INTRODUCTION

Anaerobic, thermophilic, sulfur-reducing bacteria were found to inhabit diverse volcanic environments where organic matter and elemental sulfur are available. These include cyanobacterial mats, communities of sulfur-accumulating thermophilic bacteria, bottom deposits of volcanic lakes and submarine hot vents (Bonch-Osmolovskaya, 1994; Miroshnichenko *et al.*, 1994, 1998b). All organisms described to date belong to the genus *Desulfurella*, which contains four species, *Desulfurella acetivorans* (Bonch-Osmolovskaya *et al.*, 1990), *Desulfurella multipotens* (Miroshnichenko *et al.*, 1994), *Desulfurella kamchatkensis* (Miroshnichenko *et al.*, 1998a) and *Desulfurella propionica* (Miroshnichenko *et al.*, 1998a). All these organisms are Gram-negative short rods, able to oxidize a wide spectrum of

non-fermentable substrates, including molecular hydrogen, volatile and long-chain fatty acids and some organic acids. They are moderate thermophiles and neutrophiles, growing at temperatures of 40–75 °C and at pH 6.0–7.5. All representatives of *Desulfurella* inhabit terrestrial hot springs and do not require NaCl for growth. In this publication we present a description of a new group of thermophilic, sulfur-reducing bacteria inhabiting marine thermal environments.

METHODS

Sampling and enrichment. All samples used in this work were obtained during the eighteenth cruise of the Russian scientific vessel 'A. Nesmeyanov' in the regions of shallow-water hot vents of the south-western Pacific Ocean. Samples from two different sites were used for the enrichment and isolation of marine, thermophilic, sulfur-reducing bacteria. These samples were from submarine hot vents of the Bay of Plenty, New Zealand, located at 40 m depth and obtained by scuba-divers, and the tidal zone of Matupi Harbour, Papua New Guinea. Temperatures in the sampling points were 56 °C (Bay of Plenty) and 55 °C (Matupi Harbour) and the pH was 6.0 and 6.5, respectively. Samples from the Bay of Plenty

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The EMBL accession number for the 16S rDNA sequence of strain MH₂^T is Y18292.

were taken in glass tubes 20 × 250 mm and stopped with rubber stoppers. In this work, the upper part of the sand column, enriched with elemental sulfur, was used. Samples from Matupi Harbour were collected in glass vials during low tide and the vials were closed with butyl stoppers and screw caps. Samples were used for inoculation immediately on board the vessel. For enrichment of thermophilic, sulfur-reducing bacteria the following medium was used (in 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; sodium acetate, 5.0 (or ethanol, 0.5%); yeast extract, 0.1; Na₂S · 9H₂O, 0.5; NaHCO₃, 0.5; resazurin, 0.002; trace elements (Balch *et al.*, 1979), 1 ml l⁻¹; vitamins (Wolin *et al.*, 1963), 1 ml l⁻¹. Elemental sulfur was added as an aqueous suspension of sulfur flowers to a final concentration of 10 g l⁻¹. The pH was adjusted to 6.5 with 2.5 M H₂SO₄. After dispensing into 15 ml Hungate tubes with screw caps, the headspace (5 ml) was filled with an N₂/CO₂ gas mixture (4:1, v/v). Inoculated tubes were incubated at 55 °C. Growth was followed by light microscopy of the culture broth and by measurement of hydrogen sulfide.

Isolation of pure cultures. Pure cultures of sulfur-reducing bacteria were obtained from liquid enrichments by serial dilution in medium of the same composition, solidified with 1.5% (w/v) agar (Difco), and consequent isolation of single colonies. In this case, elemental sulfur was replaced by polysulfide (Widdel & Pfennig, 1992).

Analytical methods. The cell density was determined by direct cell counting in a light microscope. Gaseous and liquid fermentation products were detected by means of GLC (Miroshnichenko *et al.*, 1994). Hydrogen sulfide was measured by a colorimetric method (Trüper & Schlegel, 1964).

Morphology and ultrastructure studies. Morphology of new isolates was studied with a light microscope. The ultrastructure of whole cells was studied with a JEM-100 electron microscope (JEOL), preparations being stained with phosphotungstate as described previously (Bonch-Osmolovskaya *et al.*, 1990). For ultrathin sectioning, cells were fixed in 3% glutaraldehyde/5% formaldehyde in PBS for 1 h on ice. After washing with the same buffer, samples were dehydrated with a graded series of acetone on ice. In-block staining was performed overnight with 2% uranyl acetate in 70% acetone. Samples were embedded in Spurr's resin. After polymerization of the resin at 70 °C for 8 h, samples were trimmed and cut with glass knives (Ultracut S; Leica). Sections were collected onto 300-mesh copper grids and post-stained with uranyl acetate and lead citrate. Samples were examined in a Zeiss EM 910 transmission electron microscope at an acceleration voltage of 80 kV and at calibrated magnifications.

Physiology of growth. Organic growth substrates were added at 0.5% concentration, organic acids as their sodium salts. When molecular hydrogen served as substrate, the headspace (10 ml) was filled with an H₂/CO₂ gas mixture (4:1, v/v). Possible electron acceptors were added at 0.2% concentration.

G+C content of the DNA. Determination of the G+C content was carried out as described previously (Miroshnichenko *et al.*, 1994).

16S rDNA sequence determination. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced by using the *Taq* DyeDeoxy terminator cycle

sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequencing reaction products were electrophoresed by using the Applied Biosystems 373A DNA Sequencer.

Phylogenetic analysis. The ae2 editor (Maidak *et al.*, 1994) was used to align the 16S rDNA sequence of strain MH₂^T against the 16S rDNA sequences of representatives of the various subclasses of the *Proteobacteria* available from public databases. The final alignment of the sequence was against the previously published 16S rDNA sequences of *Desulfurella* species (Rainey *et al.*, 1993; Miroshnichenko *et al.*, 1998a). Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The least-squares distance method of De Soete (1983) was used for construction of the phylogenetic dendrogram from distance matrices.

Nucleotide sequence accession numbers. The accession numbers of the reference strains used in the phylogenetic analyses are as follows: *Agrobacterium tumefaciens* (D14506); *Alcaligenes faecalis* (M22467); *Campylobacter jejuni* (L14630); *Desulfobacterium autotrophicum* (M94409); *Desulfobulbus propionicus* (M34410); *Desulfomonile tiedjei* (M26635); *Desulfovibrio desulfuricans* (M34113); *Desulfovibrio halophilus* (U48243); *Desulfovibrio vulgaris* (M34399); *Desulfurella acetivorans* (72768); *Desulfurella kamchatkensis* (Y16941); *Desulfurella multipotens* (Y16943); *Desulfurella propionica* (Y16942); *Escherichia coli* (X80725); *Rhodocyclus purpureus* (M34132); *Syntrophobacter wolnini* (X70905); and *Wolinella succinogenes* (M88159).

RESULTS

Enrichment and isolation of pure cultures of marine, thermophilic, sulfur-reducing bacteria

After inoculation with samples from submarine hot vents, growth of motile short rods was observed with acetate or ethanol as the energy substrate after 3–5 d incubation at 55 °C, accompanied by the formation of significant quantities of hydrogen sulfide (up to 30–40 mM). When serial dilutions were transferred to agar medium in roll-tubes, round, flat, white-greyish colonies appeared, up to 0.5 mm in diameter. After the isolation of colonies in liquid medium, three pure cultures were obtained. The isolate from Matupi Harbour growing on ethanol was designated MH₃, the strain from the same sample growing on acetate was MH₂^T and the only isolate from the Bay of Plenty that grew on acetate was designated BP₁.

Morphology and ultrastructure

Cells of all isolates were short rods, 1–3 × 0.4–0.8 µm, single or in pairs, motile with one long polar flagellum (Fig. 1a). Gaps (empty spaces) could often be observed in the cytoplasm (Fig. 1b). Thin sections revealed the Gram-negative structure of the cell wall (Fig. 1c). The cytoplasm was of an uneven density. Formation of spores was never observed.

Physiology of growth

All isolates grew in the temperature range 40–65 °C and grew optimally at 52–54 °C. Growth was observed in the pH range 5.7–6.5 with an optimum at 5.8–6.2.

Fig. 1. Negatively stained whole cells (a) and thin-sectioned cells (b, c) of strain MH_2^T . Bars, 0.5 μm .

NaCl was required in the concentration range 2.0–3.0% (w/v). No growth was observed without pre-reduction of the medium, which completely excluded oxygen. Addition of 0.02% (w/v) yeast extract was found to be required.

Growth substrates and products

In the presence of elemental sulfur, isolate MH_3 was able to grow on, in addition to ethanol, molecular hydrogen, acetate, pyruvate and saturated fatty acids

Fig. 2. Phylogenetic dendrogram based on 16S rDNA sequence comparisons indicating the position of strain MH_2^T within the radiation of the *Proteobacteria*. The scale bar indicates 10 inferred nucleotide substitutions per 100 nucleotides.

(stearate and palmitate). Isolates MH_2^T and BP_1 grew on the same substrates with the exception of ethanol. Weak growth of all the isolates was observed with fumarate as the substrate and elemental sulfur as the electron acceptor. None of isolates grew on formate, propionate, butyrate, pyruvate, lactate, succinate, glucose, starch, peptone, methanol or hexadecane. On all the substrates, CO_2 and H_2S were the only products detected.

Electron acceptors

Elemental sulfur was required for growth by all three isolates studied. No growth occurred in the absence of sulfur on any of the substrates tested, neither could it be substituted for by any of following electron acceptors: thiosulfate, sulfite, sulfate, fumarate, cystine, nitrate or ferric iron.

G + C content of DNA

The G + C content of the DNA of strain MH_2^T was found to be 40.4 mol %.

16S rDNA sequence analysis

Almost complete 16S rDNA sequences were determined for the three strains. The three sequences were identical. The 16S rDNA sequence of strain MH₂^T comprised 1614 nucleotides between *E. coli* positions 32 and 1530. Comparison of this sequence with representatives of the *Proteobacteria* showed it to have highest similarity to the previously determined sequences of the four species of the genus *Desulfurella*. The identities were in the range 87.5–88.1%. The identities between the sequence of strain MH₂^T and the sequences of other representatives of the *Proteobacteria* included in Fig. 2 were in the range 77.3–82.7%. The phylogenetic dendrogram shown in Fig. 2 shows strain MH₂^T to represent a distinct lineage within the *Proteobacteria* that clusters with the members of the genus *Desulfurella* as its closest relative. The bootstrap value for the clustering of strain MH₂^T with the *Desulfurella* species cluster was 100% on the basis of 1000 data samplings.

DISCUSSION

Elemental sulfur is the most common form of sulfur in volcanic environments, both terrestrial and submarine. Thermophilic, sulfur-reducing bacteria belonging to the genus *Desulfurella* inhabit different terrestrial thermal environments, rich in organic substrates and elemental sulfur, making sulfur reduction an important part of the anaerobic carbon cycle (Bonch-

Osmolovskaya, 1994; Miroshnichenko *et al.*, 1998b). The habitats studied here were also very rich in organic matter; elemental sulfur was constantly supplemented by the oxidation of volcanic hydrogen sulfide by the oxygen in cold ocean water. We found that moderately thermophilic, sulfur-reducing bacteria were also present in these habitats. Their catabolic capacities are almost as broad as those of *Desulfurella* and thus made marine sulfur reducers an important component of the anaerobic microbial food web in submarine hot vents.

In many features, the new isolates resembled members of the genus *Desulfurella*. Their cells were short, Gram-negative rods, motile with one polar flagellum, as are the majority of *Desulfurella* strains. They were shown to be obligate anaerobes and moderate thermophiles showing optimal growth at 55 °C. They were capable of lithotrophic growth with molecular hydrogen as the energy substrate and elemental sulfur as the electron acceptor. They could also utilize such non-fermentable substrates as acetate and long-chain saturated fatty acids, oxidizing them completely to CO₂ with H₂S being the only other product of growth. However, some significant phenotypic and genotypic differences between the new isolates and the previously described *Desulfurella* species could be found (Table 1). The new marine isolates were dependent on the presence of 2–3% NaCl in the medium. One new isolate was able to grow on ethanol, a feature never found in any strain belonging to the genus *Desulfurella*. The pH range and optimum for growth were lower for marine thermo-

Table 1. Comparative characteristics of terrestrial and marine thermophilic sulfur-reducing bacteria

The terrestrial, thermophilic, sulfur-reducing bacteria of the genus *Desulfurella* (Terrestrial) are compared with the new marine isolates (Marine). (+), Weak growth.

Feature	Terrestrial	Marine
Morphology	Short rods, motile or non-motile	Short, motile rods
Optimal growth temperature (°C)	55–60	52–54
Optimal growth pH	6.8–7.0	5.8–6.0
Requirement for NaCl	—	+
Requirement for yeast extract	—	+
Growth substrates:		
H ₂	+	+
Acetate	+	+
Propionate	+*	—
Butyrate	+*	—
Saturated fatty acids	+	+
Ethanol	—	+*
Pyruvate	+*	—
Lactate	+*	—
Succinate	+*	—
Fumarate	+*	(+)
Requirement for elemental sulfur	—*	+

* Not true for all species/strains.

philic sulfur reducers than for terrestrial ones. They also differed in their ability to grow autotrophically on mineral medium: marine strains, in contrast to the terrestrial strains, required the presence of yeast extract.

There was also a large difference between the G+C content of the DNA of the new isolates and the previously described *Desulfurella* species. The G+C content of the marine isolates differed by 8–9 mol% from that of the *Desulfurella* species (40 vs 31–32 mol%, respectively).

The genus *Desulfurella* has been shown to represent a distinct branch within the class *Proteobacteria* (Rainey *et al.*, 1993). Comparison of 16S rDNA sequences of the newly isolated strains with those of the *Desulfurella* species and representatives of the *Proteobacteria* shows that all three isolates with identical sequences belong to the same species and represent an individual lineage branching with the *Desulfurella* lineage. Together with *Desulfurella*, the new isolates represent a distinct lineage within the *Proteobacteria* closest to the δ -subclass. Additional gene sequences will be required to determine whether this lineage represents a new subclass of the *Proteobacteria*.

The new isolates are not related closely enough to the genus *Desulfurella* (87% sequence identity) to be considered members of that genus. On the basis of phenotypic and genomic differences, including very different G+C contents of the DNA and a distinct phylogenetic position, we propose a new genus *Hippea* gen. nov., with type species *Hippea maritima* gen. nov., sp. nov.

Description of *Hippea* gen. nov.

Hippea (Hip'pea. L. fem. n. after Hans Hippe, a German microbiologist, in recognition of his significant contribution to the characterization of new, obligately anaerobic prokaryotes and the understanding of their physiology).

Cells are Gram-negative rods. Moderate thermophiles. Neutrophiles to moderate acidophiles. Obligate anaerobes. Metabolize by reduction of elemental sulfur. Capable of lithotrophic growth with H_2 and sulfur. Oxidize completely volatile fatty acids, fatty acids and alcohols. Growth products are CO_2 and H_2S . The G+C content of the DNA of the type strain of the type species is 40 mol%. Inhabit submarine hot vents. The type species is *Hippea maritima*.

Description of *Hippea maritima* sp. nov.

Hippea maritima (ma.ri'ti.ma. L. fem. adj. *maritima* inhabiting marine environments).

Cells are short, Gram-negative rods, single or in pairs, motile with one polar flagellum. Moderate thermophiles with growth temperature range of 40–65 °C and optimal growth at 52–54 °C. Neutrophiles to moderate

acidophiles with pH growth range of 5.4–6.5 and optimal growth at pH 6.0. Obligate anaerobes. Require 2.5–3% (w/v) NaCl for growth. Metabolize by reduction of elemental sulfur. Growth substrates are H_2/CO_2 , acetate, stearate and palmitate. Weak growth on fumarate. Some strains are able to utilize ethanol. No growth with formate, propionate, butyrate, pyruvate, lactate, succinate, glucose, starch, peptone, methanol or hexadecane. Elemental sulfur is required for growth and cannot be substituted for by thiosulfate, sulfate, sulfite, fumarate, cystine, nitrate or ferric iron. Growth products are CO_2 and H_2S . Yeast extract (0.02% w/v) is required for growth. The G+C content of the DNA of the type strain is 40 mol%. Inhabits submarine hot vents rich in organic compounds and elemental sulfur. Isolated from hot vents in the tidal zone of Matupi Harbour, Papua New Guinea and shallow-water hot vents of the Bay of Plenty, New Zealand. The type strain of *Hippea maritima* is MH₂^T (= DSM 10411^T).

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