Thermovibrio ruber gen. nov., sp. nov., an extremely thermophilic, chemolithoautotrophic, nitrate-reducing bacterium that forms a deep branch within the phylum Aquificae

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INTRODUCTION

Within the Bacteria, the deepest lineages in 16S-rDNA-based phylogenetic trees are represented by the orders Aquificales and Thermotogales (Burggraf et al., 1992). As implied by the name Aquificales, most members of this order are capable of growing under microaerobic conditions using molecular hydrogen, sulfur or thiosulfate as electron donors, and oxygen or nitrate as electron acceptors (Eder & Huber, 2002; Huber & Eder, 2002). These rod-shaped organisms have an optimum growth temperature of ≥ 70 °C and are therefore extreme thermophiles or hyperthermophiles. Representatives of the order Thermotogales are obligate anaerobes that grow heterotrophically by fermentation of a broad range of organic nutrients. They are rod-shaped cells that are surrounded by an outer sheath which balloons over the ends of the cells (Huber & Stetter, 1999). Similar to genera of the order Aquificales, the known genera of the order Thermotogales harbour mesophilic, thermophilic and hyperthermophilic organisms. Based on its 16S rDNA sequence, the genus Desulfurobacterium (represented by Desulfurobacterium thermolithotrophicum) was shown to be related to the orders Aquificales and Thermotogales. However, its morphology and physiology were shown to be different from existing genera belonging to these orders (L’Haridon et al., 1998). Therefore, Desulfurobacterium was designated as genus incertae sedis in the new edition of Bergey’s Manual of Systematic Bacteriology (L’Haridon & Jeanthon, 2001). Desul-
**furobacterium thermolithotrophum** is a thermophilic, strictly anaerobic autotroph that grows by reduction of elemental sulfur, thiosulfate and sulfate, forming H$_2$S. 16S rDNA sequences related to the 16S rDNA sequence of this organism were detected in environmental samples obtained from an in situ growth chamber placed on a deep-sea hydrothermal vent on the Mid-Atlantic Ridge (23° N) (Reysenbach et al., 2000).

Here, we describe the isolation and properties of a novel, extremely thermophilic, chemolithoautotrophic, rod-shaped organism which reduces nitrate to ammonium and is, based on its 16S rDNA sequence, related to *D. thermolithotrophum*.

**METHODS**

**Collection of samples.** During cruise SO-133 of the research vessel *Sonne*, 13 anaerobic samples (reduced with Na$_2$S) from submarine, hot, heavily gassing hydrothermal vents were taken in the Papua New Guinea region. These samples included sandy sediments and venting water (original temperatures of 70–110 °C) from the Capit beach off Lihir Island (depth up to 6 m) and from the beach of Rabaul on the island New Britain (original temperature around 80 °C). The samples were brought to our laboratory anaerobically without temperature control.

**Culture conditions.** The novel isolate (strain ED11/3LLK$^T$) was enriched and cultivated in strictly anaerobic SME medium (based on Stetter et al., 1983), prepared according to Balch & Wolfe (1976). The medium is composed of the following salts (1$^\text{m}$): NaCl, 27.7 g; MgSO$_4$, 7H$_2$O, 70 g; MgCl$_2$, 6H$_2$O, 5.5 g; KH$_2$PO$_4$, 0.5 g; CaCl$_2$, 2H$_2$O, 0.75 g; KCl, 0.65 g; (NH$_4$)$_2$SO$_4$, 0.25 g; NaBr, 0.1 g; H$_2$BO$_3$, 0.03 g; SrCl$_2$, 6H$_2$O, 15 mg; KI, 50 μl (1 mg ml$^{-1}$). For cultivation of the novel isolate, strain ED11/3LLK$^T$, 1 g of NaN$_2$O$_4$ was added to the medium to act as an electron acceptor. Reduction of the medium was carried out by the addition of 20 ml Na$_2$S (2.5%, w/v); the pH of the medium was adjusted at room temperature to pH 6 with H$_2$SO$_4$. Routinely, the new strain was cultivated in 120 ml serum bottles containing 20 ml medium pressurized with H$_2$/CO$_2$ (80:20, v/v; 250 kPa). Heterotrophic growth of the strain was tested using Na$_2$CO$_3$ (80:20, v/v; 200 kPa) as the gas phase. Organic substrates and alternative electron acceptors (thiosulfate, sulfate, elemental sulfur or nitrate) were added to the medium to final concentrations of 0.1%. Usually, incubations were carried out at 75 °C with shaking (100 r.p.m.). To determine the optimal ion strength for growth of strain ED11/3LLK$^T$, different NaCl concentrations were used in the medium. In a parallel experiment, the concentrations of all salts added to the medium were decreased and increased. Batch cultures were grown in a 300 l enamel-protected fermenter (HTE, Bioengineering, Wald, Switzerland) at 75 °C with stirring (150 r.p.m.) and gassing with H$_2$/CO$_2$ (80:20, v/v; 2 1 min$^{-1}$).

**Light and electron microscopy.** Cells of strain ED11/3LLK$^T$ were routinely observed under 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 described previously (Huber, H. et al., 2000).

**Analyses of metabolic products.** The formation of ammonium by strain ED11/3LLK$^T$ was determined quantitatively by the addition of 0.1 ml of a freshly prepared mixture of 0.5 ml NaOH (27%, w/v) and 0.5 ml potassium tetraiodomercurate (II) solution (Nessler’s reagent) to 0.5 ml of culture medium. The presence of ammonium in the medium was indicated by an orange precipitate. Quantitative ammonium determination was carried out spectrophotometrically after the addition of NADH, 2-oxoglutarate and glutamate dehydrogenase to the sample, yielding glutamate and NAD (assay kit from Fa. Biopharm, Darmstadt, Germany). The nitrate content of the medium was determined quantitatively by enzymic bioanalysis (reduction of nitrate with NADPH and nitrate reductase) (Fa. Biopharm), following precipitation of chloride ions by the addition of silver fluoride to the medium. The production of molecular nitrogen and N$_2$O in the medium was determined by GC (HP 5890 chromatograph) using a Molecularsieb 5A column and a Poropak Q column, respectively. Nitrite was determined qualitatively with Lunge’s reagent. The formation of H$_2$S in the medium was monitored qualitatively by using lead acetate paper (Macherey & Nagel).

**DNA isolation and DNA base content.** DNA was prepared as described by Wildgruber et al. (1982). The G+C content (mol%) of the genomic DNA of strain ED11/3LLK$^T$ was determined by melting-point analysis (Marmur & Doty, 1962) and by direct analysis of the nucleotides, as described previously (Huber, R. et al., 2000).

**16S rRNA gene sequence analysis.** The nearly complete 16S rRNA gene of the novel isolate was amplified by PCR (Saiki et al., 1985, 1988). The primers used in the amplification corresponded to positions 9–27 (GRGTTTGTACCTGCGTCAG; Eder et al., 1999) and positions 1512–1492 (ACGGHTACCTTGATCCTGGTACGATT; Lane, 1991) in the 16S rRNA gene sequence of *Escherichia coli* (Brosius et al., 1981). Both strands of the PCR products were sequenced directly with the following primers: 9bF (Eder et al., 1999); 519uF (Eder et al., 1999); 531uR (ACCGCGGGCKGCTGGC); 704bR (TCTACGYATTTACGTYT); and 1513uR (Eder et al., 1999). The resulting sequence (length, 1512 bases) was aligned with a set of about 11000 homologous full and partial primary sequences using the program *arbor* (Ludwig & Strunk, 2001). Dendrograms for the 16S rDNA sequences were computed by using the neighbour-joining, maximum-parsimony and maximum-likelihood methods included within the *arbor* package. Phylogenetic distances were determined using distant-matrix analysis without applying a correction factor. The 16S rRNA gene sequence of strain ED11/3LLK$^T$ has been deposited in the EMBL database under accession number AJ316619.

**RESULTS**

**Enrichment and isolation of ED11/3LLK$^T$.** To enrich for chemolithoautotrophic, extremely thermophilic organisms, serum bottles with 20 ml SME medium supplemented with 0.1% nitrate or 1% (w/v) sulfur were inoculated with about 1 g of sandy sediments taken from the beach off Lihir Island, Papua New Guinea. The enrichment cultures were incubated with shaking at 65, 80 and 90 °C. After 3 days incubation, slightly curved rods grew in the medium supplemented with nitrate and incubated at 80 °C (sample ED11). High amounts of ammonium could be
detected in the culture medium. The enrichment culture was successfully transferred into fresh medium and the organism was purified by the ‘optical-tweezer’ technique (Huber et al., 1995). The resulting isolate was designated strain ED11/3LLKT.

**Morphology**

Cells of strain ED11/3LLKT were slightly curved rods of about 1.5–3 μm in length and 0.5–0.8 μm in width (Fig. 1a–d). They stained Gram-negative and usually occurred singly or in pairs. The cells were motile and possessed up to six flagella (Fig. 1b). The flagella were located at one or both poles of the cells; hence, monopolar and bipolar flagellated cells were observed (Fig. 1a, b). Formation of endospores by the cells was not observed. The cell architecture of strain ED11/3LLKT was studied by the freeze-etch/freeze-fracture technique (Fig. 1c, d). The densely packed cytoplasm of the cells was surrounded by a cell envelope consisting of a cytoplasmic membrane, a periplasmic space (width, 25–30 nm) and an outer membrane, an arrangement typical for Gram-negative cells. There was no evidence that cells of strain ED11/3LLKT possessed an S layer. Cell pellets of strain ED11/3LLKT had an intense red colour.

**Metabolism**

Strain ED11/3LLKT was an obligate anaerobe that grew chemolithoautotrophically by reduction of nitrate using molecular hydrogen as an electron donor, and cell densities of 2–5 × 10^6 cells ml^-1 were usually obtained, occasionally reaching 8 × 10^6 cells ml^-1. Under these conditions, ammonium was identified as the sole metabolic end product of strain ED11/3LLKT, and was formed in stoichiometric amounts (Fig. 2). Nitrite, N\(_2\)O and molecular nitrogen could not be detected quantitatively or qualitatively after growth in the culture medium. In addition, strain ED11/3LLKT was able to grow by reduction of sulfur using molecular hydrogen as an electron donor, producing H\(_2\)S as the final product. However, cell densities of less than 1 × 10^6 cells ml^-1 were obtained for strain ED11/3LLKT when it was grown in the presence of sulfur and molecular hydrogen. When strain ED11/3LLKT was grown in the presence of molecular hydrogen, no growth was observed when nitrite, thiosulfate, sulfite (0–1% each) or oxygen (0.001–5%, v/v) were the electron acceptors. Growth of strain ED11/3LLKT was stimulated by the addition of complex organic nutrients, such as meat extract, yeast extract, peptone and Casamino acids (each 0.1%), to the medium (final cell densities of around 2 × 10^7 cells ml^-1). No effect on the growth or final cell concentrations of strain ED11/3LLKT were observed when it was grown in the presence of sugars (glucose or sucrose) and organic acids (lactate, acetate or pyruvate; 0.1% each). The addition of formate...
(0.05%) to the medium inhibited growth of the strain. No growth was observed on organic substrates, such as meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, agarose, formate, acetate, sucrose or glucose, when cultures of strain ED11/3LLK were pressurized with hydrogen-free gas (N₂/CO₂ = 80:20; 200 kPa).

**Optimal growth conditions**

Strain ED11/3LLK grew between 50 and 80 °C, with an optimum growth temperature of 75 °C and a doubling time of 1 h. No growth was obtained when the strain was incubated at ≤ 45 °C or at ≥ 82 °C. Growth of the strain occurred in the presence of between 20 and 4-7% (w/v) NaCl in the medium, corresponding to 0-6–1.6 × SME medium, with the optimum around 3% (w/v) NaCl or 1 × SME medium (data not shown). Strain ED11/3LLK grew between pH 5 and 6.5. Nitrate concentrations of between 0.001 and 1% in the medium resulted in propagation of the isolate; optimal growth was obtained when 0.01–0.1% nitrate was present in the medium. Growth of the strain could be detected when hydrogen concentrations of between 0.1 and 80% (v/v; Fig. 3) were introduced into the medium, with a broad optimum concentration observed (between 5 and 80%) under autotrophic conditions. In the presence of hydrogen concentrations of 0.01% or less, no growth of strain ED11/3LLK was observed. Cultures of strain ED11/3LLK grown at hydrogen concentrations of between 0.1 and 5% (v/v) produced cells that were about half the length of those grown in the presence of 80% (v/v) hydrogen. When peptone was added to the culture medium (0.05%), final cell densities for strain ED11/3LLK increased up to 2 × 10⁶ cells ml⁻¹ [at 80% (v/v) hydrogen; Fig. 3].

**Sensitivity to antibiotics**

Cells of strain ED11/3LLK were sensitive to ampicillin and chloramphenicol (each at 100 µg ml⁻¹), whereas they were resistant to the translation inhibitor kanamycin (at 100 µg ml⁻¹).

**Storage**

Stock cultures of strain ED11/3LLK containing 5% (v/v) DMSO and stored at −140 °C over liquid nitrogen served as viable inocula for at least 1 year.

**DNA base content**

The G+C content of the genomic DNA of strain ED11/3LLK was 45 mol% upon its determination by melting-point analysis; it was 47 mol% upon determination by direct analysis of the mononucleotides.

**Phylogenetic analysis**

Analyses of 16S rDNA sequence alignments using all three major approaches for tree reconstruction (i.e. neighbour-joining, maximum-parsimony and maximum-likelihood) clearly indicated that strain ED11/3LLK was a member of the *Bacteria*, branching deeply within the phylum *Aquificae* (Fig. 4). In all calculations, the closest phylogenetic relative of strain ED11/3LLK was *Desulfurobacterium thermolithotrophum*, which exhibited a phylogenetic distance of about 6% to the isolate. These two organisms form a cluster that also includes environmental sequences obtained from a deep-sea vent at the Mid-Atlantic Ridge (Reysenbach et al., 2000) (phylogenetic distances to ED11/3LLK around 5%). This phylogenetic group represents a deep independent branch besides the order *Aquificae* (and the order *Thermotogales*), regardless of the tree reconstruction method used (Fig. 4). This deep branch was also obvious from the obtained phylogenetic distances, which were between 21 and 28% to members of the order *Aquificae* and between 23 and 28% to members of the order *Thermotogales*. Comparable distances were observed between the orders *Thermotogales* and *Aquificae* (25–35%).

Remarkably, all four sequences within the *Desulfurobacterium thermolithotrophum*–ED11/3LLK lineage share a structural feature in their 16S rDNA which has been assigned as a defining signature for members of the order *Aquificae*, namely the helix at positions 198–219 of the 16S rDNA sequence (*E. coli* numbering) (Burggraf et al., 1992; Reysenbach et al., 1994). However, recently described *Aquificae* sequences, including the 16S rDNA sequence of *Hydrogenothermus marinus* (Stöhr et al., 2001), lack this signature and a major part of the corresponding helix.
**DISCUSSION**

Strain ED11/3LLK\(^T\) is an extremely thermophilic, obligately anaerobic, nitrate-reducing bacterium that forms ammonium as its sole metabolic end product. On the basis of these characteristics, it resembles the bacterial genus *Ammonifex*, which so far only harbours one species, *Ammonifex degensii* (Huber et al., 1996). However, strain ED11/3LLK\(^T\) is phylogenetically distinct from *Ammonifex degensii*, based on 16S rDNA sequence comparisons. The closest phylogenetic relative of strain ED11/3LLK\(^T\) is *Desulfurobacterium thermolithotrophum*, an organism whose genus is currently designated *incertae sedis* (L’Haridon & Jeanthon, 2001). *Desulfurobacterium thermolithotrophum* forms a branch between the orders *Aquificales* and *Thermotogales* in 16S-rDNA-based phylogenetic trees. Strain ED11/3LLK\(^T\) and *Desulfurobacterium thermolithotrophum* share a Gram-negative cell wall, have a similar optimal growth temperature, are obligate anaerobes and strict chemolithoautotrophs, and reduce elemental sulfur with molecular hydrogen; they both also share several sequence identities within their 16S rDNA sequences (L’Haridon et al., 1998). However, strain ED11/3LLK\(^T\) can be differentiated from *Desulfurobacterium thermolithotrophum* on the basis of its cell shape, the absence of an \(S\) layer, the electron acceptors it uses for energy production and its G+C content. Therefore, strain ED11/3LLK\(^T\) represents a separate, novel genus within the phylum *Aquificae*, for which we propose the name *Thermovibrio*. The type species of the genus is *Thermovibrio ruber* ED11/3LLK\(^T\) (= DSM 14644\(^T\) = JCM 11468\(^T\)).

As indicated by 16S rDNA sequence comparisons, *Thermovibrio ruber* and *Desulfurobacterium thermolithotrophum* are not members of the order *Thermotogales* (Huber & Stetter, 1992), which is in line with the morphological and physiological differences these two genera exhibit compared to the characteristics of members of the order *Thermotogales*. In contrast to the members of the order *Aquificales* (Reysenbach, 2001), *Thermovibrio ruber* and *Desulfurobacterium thermolithotrophum* are unable to grow microaerobically or aerobically and cannot use oxygen as an electron acceptor. However, they do use molecular hydrogen as an electron donor and have some 16S rDNA signatures characteristic of members of the order *Aquificales*. Therefore, as supported by detailed 16S-rDNA-based phylogenetic analyses, *Thermovibrio ruber* and *Desulfurobacterium thermolithotrophum* are members of the phylum *Aquificae*, but may represent a new order within this phylum (phylogenetic distance to *Aquificales*, 21–28%). However, the description of a new order within this phylum should not be made until further isolates have been detected that cluster with *Thermovibrio ruber* and *Desulfurobacterium thermolithotrophum*. The characterization of these novel isolates will also give insights into the physiological and biochemical characteristics of this novel taxon.

Several deeply branching, extremely thermophilic and hyperthermophilic archaeal and bacterial genera/species have been described that are able to use nitrate as an electron acceptor under anaerobic conditions. These include members of the orders *Aquificales* (*Aquifex pyrophilus* and *Hydrogenobacter thermophilus*), *Desulfurococcales* (*Pyrobaculum fumarii*) and *Thermoproteales* (*Pyrococcus furiosus* and *Pyrobaculum aerophilum*) (Blöchl et al., 1997; Huber et al., 1992; Suzuki et al., 2001; Völkl et al., 1993). All of these organisms can also grow aerobically by hydrogen oxidation. In contrast, *Thermovibrio ruber* is an obligate anaerobe that is unable to use oxygen as an electron acceptor. In the 16S-rDNA-based phylogenetic tree shown in Fig. 4, *Thermovibrio ruber* exhibits a very short lineage, a characteristic typical of organisms considered to be rather primitive (Stetter, 1994). Since nitrate is considered to have existed in the primitive atmosphere of the Earth (Mancinelli & McCoy, 1988), the discovery of *Thermovibrio ruber* is further evidence that the ability of
organisms to reduce nitrate could have developed very early in the history of life as an energy-yielding pathway.

The biotope of *Thermovibrio ruber* is located close to several currently inactive seamounts, such as Edison, Conical or TUBAF, and is part of the so-called New Ireland Fore-Arc, with the area around Lihir Island being the most active volcanic zone in this fault system. We were able to isolate a number of truly thermophilic and hyperthermophilic organisms from the same sample as strain ED11/3LLK.T. These included members of the archaeal genera *Pyrococcus* and *Archaeoglobus*, new species of the bacterial genus *Thermodesulfobacterium* and the novel genus ‘Thermospirillum’ (H. Huber, S. Diller and R. Rachel, unpublished data). Hence, this study documents, for the first time, the presence of extremely thermophilic and hyperthermophilic micro-organisms in this geographical area and the existence of a phylogenetically and physiologically highly diverse community in this biotope. In hydrothermal fluids, carbon dioxide and hydrogen are commonly found and nitrate and elemental sulfur are present in marine biotopes (Corliss et al., 1979; Jannasch & Mottl, 1985). Because of this, *Thermovibrio ruber* represents an important primary producer within its biotope, since it can provide both biomass and ammonium to other inhabitants of this environment, even at very low nitrate concentrations.

Description of *Thermovibrio* gen. nov.

*Thermovibrio* (Therm.o.vib´ri.o. Gr. fem. n. therme heat; L. v. vibra re to vibrate; M.L. masc. n. vibrio that which vibrates; N.L. masc. n. thermovibrio a thermophilic curved rod).

Cells are curved rods, occurring singly or in pairs. They stain Gram-negative and do not form spores. Cells are extremely thermophilic, obligately anaerobic and chemolithoautotrophic. Optimum growth pH around 6. Growth by reduction of nitrate and formation of ammonium. The G+C content of the type species is 46 mol%. Based on 16S-rDNA-based phylogenetic analyses, the type species of the genus, together with *Desulfurobacterium thermolithotrophum*, forms a separate lineage within the phylum *Aquificae*, which probably represents a new order within this phylum. Type species of the genus is *Thermovibrio ruber*.

Description of *Thermovibrio ruber* sp. nov.

*Thermovibrio ruber* (ru´ber. L. masc. adj. ruber red, describing the colour of the cells).

Cells are curved rods of about 0.5–0.8 μm in width and 1–3 μm in length. Highly motile by monopolar or bipolar polymeric flagellation. Gram-negative. Occurs singly and in pairs. No evidence for a regularly arrayed surface protein. Cell masses have an intense red colouration. Grows between 50 and 80 °C and pH 5 and 6.5, and in the presence of between 2 and 4.7% NaCl. Optimal growth conditions are around 75 °C, pH 6 and 3% NaCl. Strictly anaerobic. Chemolithoautotrophic growth in the presence of hydrogen and carbon dioxide with nitrate or elemental sulfur as the electron acceptor, with formation of ammonium or H₂S, respectively. Sulfate, sulfite, thiosulfate, nitrite and oxygen are not used as electron acceptors. No chemo-organotrophic growth on meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate or glucose. Sensitive to ampicillin and chloramphenicol (100 μg ml⁻¹ each). Resistant to 100 μg kanamycin ml⁻¹. DNA base content is 46 mol% G+C (determined by the thermal denaturation method and by HPLC). Type strain is ED11/3LLK.T (= DSM 14644T = JCM 11468T), which was isolated from sandy sediments taken from the beach off Lihir Island, Papua New Guinea.

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