Geoglobus ahangari gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor

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A novel, regular to irregular, coccoid-shaped, anaerobic, Fe(III)-reducing micro-organism was isolated from the Guaymas Basin hydrothermal system at a depth of 2000 m. Isolation was carried out with a new technique using Fe(III) oxide as the electron acceptor for the recovery of colonies on solid medium. The isolate, designated strain 234^T, was strictly anaerobic and exhibited a tumbling motility. The cells had a single flagellum. Strain 234^T grew at temperatures between 65 and 90 °C, with an optimum at about 88 °C. The optimal salt concentration for growth was around 19 g l^{-1}. The isolate was capable of growth with H_2 as the sole electron donor coupled to the reduction of Fe(III) without the need for an organic carbon source. This is the first example of a dissimilatory Fe(III)-reducing micro-organism capable of growing autotrophically on hydrogen. In addition to molecular hydrogen, strain 234^T oxidizes pyruvate, acetate, malate, succinate, peptone, formate, fumarate, yeast extract, glycerol, isoleucine, arginine, serine, glutamine, asparagine, stearate, palmitate, valerate, butyrate and propionate with the reduction of Fe(III). This isolate is the first example of a hyperthermophile capable of oxidizing long-chain fatty acids anaerobically. Isolate 234^T grew exclusively with Fe(III) as the sole electron acceptor. The G+C content was 58·7 mol%. Based on detailed analysis of its 16S rDNA sequence, G+C content, distinguishing physiological features and metabolism, strain 234^T is proposed to represent a novel genus within the *Archaeoglobales*. The name proposed for strain 234^T is *Geoglobus ahangari* gen. nov., sp. nov.

Keywords: *Archaea*, hydrothermal vents, Fe(III) reduction, long-chain fatty acids, autotrophic

INTRODUCTION

Micro-organisms with the ability to grow anaerobically with Fe(III) serving as the sole electron acceptor are found in both the *Bacteria* and the *Archaea* (Lovley, 2000a). Short-chain fatty acids and hydrogen are the primary electron donors that support the growth of mesophilic and thermophilic Fe(III)-reducing bacteria now available in pure culture (Lovley, 2000a). There has been little investigation of hyperthermophilic micro-organisms capable of using Fe(III) as an electron acceptor, even though Fe(III) is considered to be an important electron acceptor in modern hot environments such as the deep subsurface and around hydrothermal systems and may have also been an important electron acceptor on hot, early Earth and other planets (Lovley, 2000b).

Abbreviation: AQDS, anthraquinone-2,6-disulfonate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strain 234^T, *Ferroglobus placidus* DSM 10642^T and *Archaeoglobus profundus* DSM 5631^T are respectively AF220165, AF220166 and AF322392.

Most of the previous evaluations of Fe(III) reduction by hyperthermophiles have consisted of studies of the ability of hyperthermophiles, isolated with electron acceptors other than Fe(III), to reduce Fe(III) with hydrogen (Kashefi & Lovley, 2000; Vargas *et al.*, 1998). All hyperthermophiles that have been investigated to date have the ability to oxidize hydrogen with the reduction of Fe(III) in cell suspension (Vargas *et al.*, 1998). *Pyrobaculum islandicum* in the *Archaea* and *Thermotoga maritima* in the *Bacteria* were found to conserve energy to support growth from hydrogen oxidation coupled to Fe(III) reduction (Kashefi & Lovley, 2000; Vargas *et al.*, 1998). Both of these organisms required the presence of low concentrations of yeast extract for growth on hydrogen and Fe(III). *P. islandicum* was also found to grow in medium in which peptone and yeast extract served as electron donors for Fe(III) reduction, but the specific compounds utilized as electron donors were not identified.

Here, we report the isolation and characterization of a novel, Fe(III)-reducing hyperthermophile, designated strain 234^T, that was enriched and isolated from a vent sample from the Guaymas Basin using a newly developed isolation procedure in which Fe(III) oxide was incorporated into the solidified medium. The use of Fe(III) oxide as the electron acceptor for enrichment and isolation yielded a micro-organism with metabolic capabilities not previously reported for hyperthermophiles and/or Fe(III)-reducing micro-organisms. It is suggested that strain 234^T be assigned to a new genus, designated *Geoglobus*, as *Geoglobus ahangari* gen. nov., sp. nov.

METHODS

Source of organism. Strain 234^T was isolated from samples from a hydrothermal chimney collected by the research submersible *Jason* at Guaymas Basin (27° N, 111° W, depth 2000 m). *Ferroglobus placidus* DSM 10642^T, *Archaeoglobus fulgidus* DSM 4304^T, *Archaeoglobus profundus* DSM 5631^T and *Archaeoglobus veneficus* DSM 11195^T were obtained from the DSMZ, Braunschweig, Germany.

Enrichment of strain 234^T. Strict anaerobic culturing techniques were used throughout (Balch *et al.*, 1979; Miller & Wolin, 1974). Gases were passed through a column of hot copper filings to remove all traces of oxygen. All transfers and sampling of cultures were performed with syringes and needles that had been flushed with oxygen-free gas. The marine enrichment medium contained the following (l^{-1}): NaCl, 19·0 g; $MgCl_2.6H_2O$, 9·0 g; $MgSO_4.7H_2O$, 0·15 g; $CaCl_2.2H_2O$, 0·3 g; KCl, 0·5 g; KH_2PO_4, 0·42 g; $(NH_4)_2SO_4$, 0·10 g; NaBr, 0·05 g; $SrCl_2.6H_2O$, 0·02 g; Difco yeast extract, 0·10 g; $NaHCO_3$, 2·5 g; Na_2SeO_4, 0·02 g. The medium was also supplemented with a vitamin mixture (Lovley & Phillips, 1988), a trace mineral solution (Pledger & Baross, 1989) and poorly crystalline Fe(III) oxide (100 mmol l^{-1}), which was prepared as described previously (Lovley & Phillips, 1986). Pyruvate (10 mM) was the electron donor for the enrichment and isolation. The medium (10 ml) was dispensed into 26 ml anaerobic pressure tubes (Bellco Glass, Inc.) and sparged with N_2/CO_2 (80:20%, v/v) for 12–15 min to remove dissolved oxygen. The tubes were then sealed with thick butyl-rubber stoppers. After autoclaving, the medium in each tube was supplemented with 0·25 mM L-cysteine hydrochloride and 1·3 mM $FeCl_2.2H_2O$ from concentrated anaerobic stock solutions. The final pH of the medium was approx. 6·8–7·0 (at room temperature). With 2·5 g sodium bicarbonate l^{-1} (30 mM) in the growth medium at 85 °C under a N_2/CO_2 (80:20%, v/v) atmosphere, this pH should be around 6·9–7·0.

Isolation of strain 234^T on solid medium. The isolate was purified with a modification of the roll-tube method (Hungate, 1969). The solid medium was made in a two-step process. To prepare the solidifying agent, 1 g GELRITE gellan gum (Sigma) was added to 50 ml anaerobic water in 128 ml serum bottles under a N_2 atmosphere. The bottles were then sealed with thick butyl-rubber stoppers. The other part of the medium, with the constituents at twice the concentration listed above and 20 mM pyruvate, was amended with additional $MgCl_2$ (20 mM) and $CaCl_2$ (6 mM). This medium was then dispensed into pressure tubes in 3·5 ml aliquots under N_2/CO_2 (80:20%, v/v). The solidifying agent and double-strength medium were autoclaved and then placed in a water bath (85–90 °C). After autoclaving, the solidifying-agent solution was amended with L-cysteine hydrochloride (0·5 mM) and $FeCl_2$ (2·6 mM). An inoculum (0·7 ml) from the enrichment cultures was added anaerobically to tubes of double-concentration medium, followed immediately by an aliquot (3·5 ml) of the solidifying agent. The contents were mixed gently and thoroughly at 85–90 °C. Next, 0·7 ml was quickly removed and transferred into a second pressure tube containing 3·5 ml of the double-concentration marine medium and mixed well. The first pressure tube was then rolled at room temperature with a tube spinner (Bellco Glass Inc.), taking care to ensure an even coating of the inner pressure tube wall. The second pressure tube was amended with the solidifying agent (3·5 ml) and the contents were mixed as before. This and subsequent tubes were treated in the same manner as the first tube to generate serial dilutions up to a dilution of 10^{-9}. When complete, the roll tubes were incubated vertically at 85 °C.

Evaluation of electron donors and electron acceptors utilized. In order to determine the ability of the strain to use substrates other than pyruvate, electron donors were added individually from concentrated anoxic and sterile stock solutions. Substrate utilization was monitored by measuring growth and Fe(II) production over the period of incubation at 85 °C and under a N_2/CO_2 (80:20%, v/v) atmosphere. When hydrogen was used as the electron donor, it was provided as H_2/CO_2 (80:20%, v/v) at an overpressure of 101 kPa and yeast extract was omitted from the medium. To evaluate the utilization of electron acceptors, poorly crystalline Fe(III) was omitted from the medium. Alternative electron acceptors were added individually from concentrated anaerobic and sterile stock solutions, using pyruvate as the electron donor at 85 °C under a N_2/CO_2 (80:20%, v/v) atmosphere.

Temperature, pH and salt optima. The influence of temperature on growth was determined over the range 60–95 °C in cultures provided with pyruvate as the electron donor and Fe(III) oxide as the electron acceptor. The cultures were incubated in either temperature-controlled hot-air incubators or water baths using a calibrated thermometer. The pH range for growth of strain 234^T was investigated for the range pH 5·0–8·6 using bicarbonate buffer under a N_2/CO_2 (80:20%, v/v) atmosphere at 85 °C. The effect of NaCl on growth was determined by varying its concentration from 0 to 72 g l^{-1}. The effect of salinity was determined at 85 °C.

Antibiotic susceptibility. To test the effect of antibiotics, a 1 ml aliquot of an exponentially growing culture was transferred into fresh marine medium containing poorly crystalline Fe(III) oxide, pyruvate (10 mM) and filter-sterilized antibiotics. The cultures were incubated at 80 °C and routinely examined for cell growth and Fe(II) accumulation as a result of Fe(III) reduction.

Analytical techniques. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time. The amount of Fe(II) solubilized after 2 h (incubation in dark and at room temperature) in anaerobic oxalate solution was determined with ferrozine as described previously (Phillips & Lovley, 1987). Cells were counted by epifluorescence microscopy as described previously (Lovley & Phillips, 1988). Methane was analysed with a GC (Hewlett Packard model 5890, series II) equipped with a 30-m fused silica capillary column (HP-624; Hewlett Packard) with N_2 (zero grade) as the carrier gas. The oven temperature was 100 °C and methane was determined with a flame-ionization detector.

Light and electron microscopy. Cells were routinely examined with a Zeiss Axioskop 20 phase-contrast microscope (with an oil-immersion objective $\times 100/1{\cdot}25$) equipped with a UV lamp, an LP 420 excitation filter and a BG 38 red-attenuation filter. Electron microscopy was carried out as described previously (Lovley *et al.*, 1993).

G+C content of the DNA. DNA isolation and determination of G+C content were performed on Fe(III) citrate-grown cells by the Identification Service of the DSMZ. In brief, cells were first disrupted using a French pressure cell and purified on hydroxyapatite according to the procedure of Cashion *et al.* (1997). The DNA was digested enzymically and then dephosphorylated, the resulting deoxyribonucleosides were separated and analysed by HPLC according to Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma), with the G+C content of 49·9 mol%, was used as the standard (Mesbah *et al.*, 1989). The mean G+C content ($n = 3$) was calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) according to the method of Mesbah *et al.* (1989).

16S rDNA sequence analysis. Cultures (10 ml) grown with poorly crystalline Fe(III) oxide as the electron acceptor were first treated with 30 ml filter-sterilized oxalate solution (Phillips & Lovley, 1987) in order to remove Fe(III), which inhibits *Taq* polymerase. Cells were collected by centrifugation and genomic DNA was extracted as described previously (Rooney-Varga *et al.*, 1999).

Nearly the entire 16S rDNA of strain 234^T, *F. placidus*, *A. fulgidus*, *A. profundus* and *A. veneficus* was amplified with primers 25e Forward and 1525 Reverse (Achenbach & Woese, 1995). PCR mixtures were adapted from those described previously (Murray *et al.*, 1996). Each 100 µl reaction contained 1·5 mM $MgCl_2$, 2·5 U AmpliTaq (Perkin Elmer Cetus) and glycerol (15%). There was an initial denaturation step at 92 °C for 2 min, followed by 30 cycles of 92 °C for 1 min, 53·5 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 6 min. PCR products were prepared for sequencing using a QIAquick PCR purification kit (Qiagen). Sequencing was performed at the University of Massachusetts Amherst Sequencing Facility.

Complete bidirectional sequences were obtained from each PCR product. Sequences were compared to sequences from the GenBank and RDP databases using BLAST (Altschul *et al.*, 1990) and SIMILARITY_RANK (Maidak *et al.*, 2000) algorithms. Secondary structures were verified prior to manual alignment with related 16S rDNA sequences from GenBank and the RDP using the Genetic Computer Group (GCG) sequence editor (Wisconsin Package version 10). Aligned sequences were imported into PAUP 4.0b 4a (Swofford, 1998), where phylogenetic trees were inferred. The branching order was determined and compared using character-based (maximum-parsimony and maximum-likelihood) and distance-based (using the HKY85 4-parameter, Jukes–Cantor and Kimura two-parameter models) algorithms. The region considered for phylogenetic analysis, according to the conventional numbering system for 16S rDNA sequence analysis, included positions 52–1480; 18% of the alignment, representing hypervariable regions, was masked. However, inclusion of these regions did not affect the branching order. Bootstrap analysis was performed using the distance-based HKY85 4-parameter model, with 100 replicates. In addition to the archaeal species shown in Fig. 5, representative species from the following taxonomic groups were used for construction of this phylogenetic tree: *Methanococcales*, *Methanosarcinales*, *Halobacteriaceae*, *Methanobacteriaceae*, *Thermococcales*, *Methanopyrales* and *Thermoproteales*.

RESULTS

Enrichment and isolation

Several of the Guaymas Basin vent samples provided active Fe(III)-reducing enrichment cultures, but only the results from the enrichment known as 234^T will be summarized here. Within 2 days of inoculating the 234^T sample into pyruvate/Fe(III) oxide medium at 80 °C, the reddish-brown, non-magnetic Fe(III) oxide changed to a black, magnetic mineral, which was probably magnetite (Lovley *et al.*, 1987). Inocula (10%) from the enrichments were transferred into the same medium and incubated at 75, 80, 85, 90 or 100 °C. The enrichment at 85 °C was successfully transferred five times and the fifth transfer was further transferred into serial 10-fold dilutions. The 10^{-8} dilution, which was the highest dilution that exhibited Fe(III) reduction, was serially diluted and the serial dilution procedure was then repeated a third time.

The highest positive dilution from the third serial transfer was used to inoculate roll tubes of pyruvate/Fe(III) oxide medium solidified with GELRITE. After 10 days of incubation at 85 °C, well-isolated colonies (0·5–1·0 mm diameter) appeared in the more-dilute tubes. Individual colonies were picked from the highest positive dilutions and transferred into 2 ml liquid medium that was amended with poorly crystalline Fe(III) oxide and pyruvate. The liquid culture with the highest rate of Fe(III) reduction was passed through the serial dilution procedure three more times to ensure that this was a pure culture. This isolate was designated strain 234^T.

Morphology

Cells of strain 234^T grown with pyruvate/Fe(III) oxide were morphologically uniform (Fig. 1a). Under phase-contrast microscopy, cells of isolate 234^T appeared as

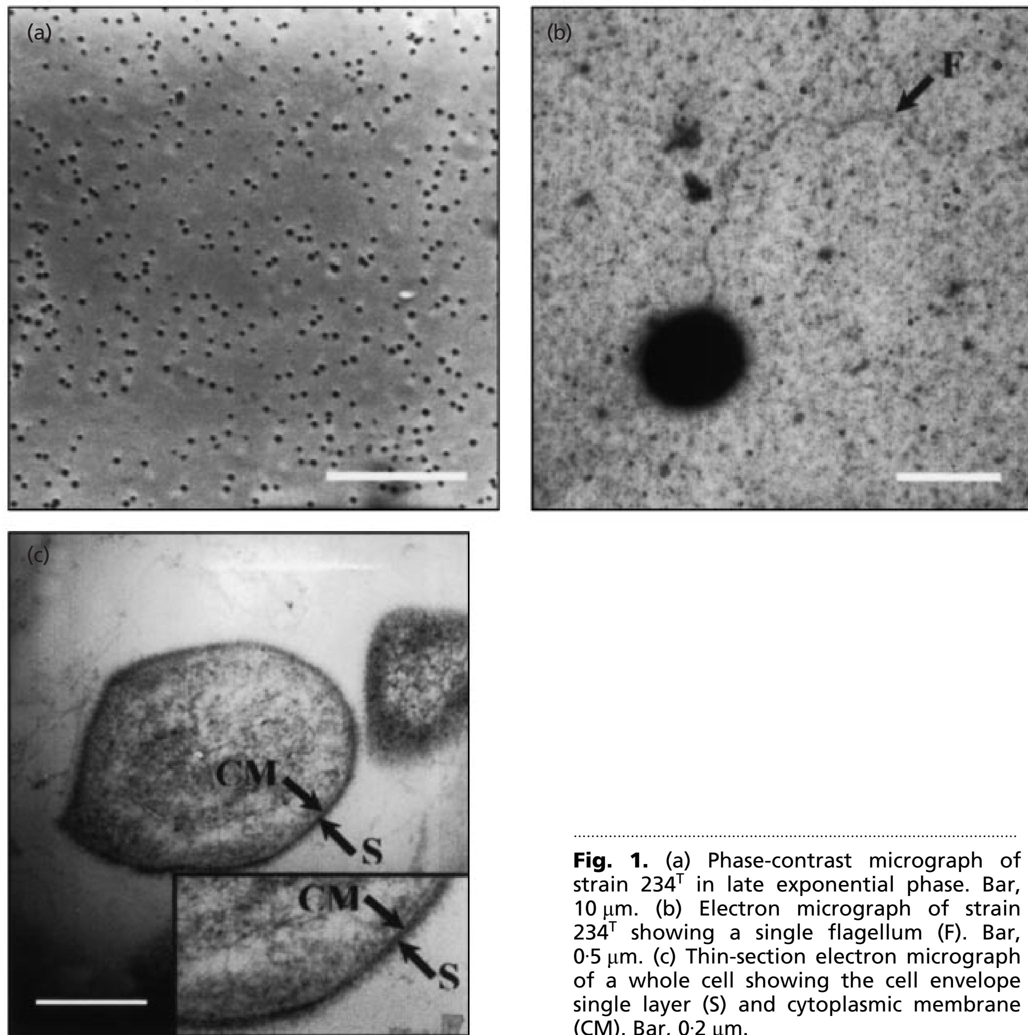

Fig. 1. (a) Phase-contrast micrograph of strain 234^T in late exponential phase. Bar, 10 μm. (b) Electron micrograph of strain 234^T showing a single flagellum (F). Bar, 0·5 μm. (c) Thin-section electron micrograph of a whole cell showing the cell envelope single layer (S) and cytoplasmic membrane (CM). Bar, 0·2 μm.

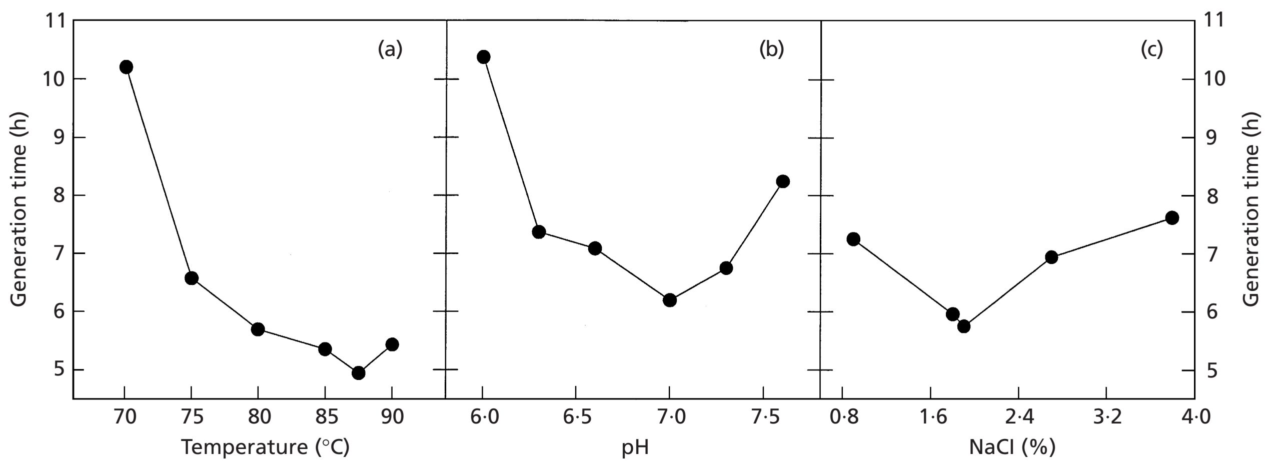

Fig. 2. Effects of temperature (a), pH (b) and NaCl concentration at 85 °C (c) on growth of isolate 234^T. Doubling times were calculated from the slopes of the growth curves (not shown) at pH 7·0 (a, c) or at 85 °C (b). All growth experiment were done with pyruvate as the electron donor and poorly crystalline Fe(III) as the electron acceptor.

regular to irregular cocci, about 0·3–0·5 μm in diameter, usually arranged as single cells or in pairs. The cells had a single flagellum (Fig. 1b). Strain 234^T was highly motile even at room temperature. When examined by phase-contrast microscopy, a tumbling motility was observed.

Table 1. Electron donor and acceptor utilization by strain 234[T]

Compound	Concentration*
Electron donors utilized	
Hydrogen (H_2/CO_2; 80:20)	101 kPa
Pyruvate	10
Acetate	4
Malate	10
Succinate	10
Peptone	0·1 %
Formate	10
Fumarate	10
Yeast extract	0·1 %
Glycerol	20
Isoleucine	7·6
Arginine	5·7
Serine	9·5
Glutamine	6·8
Asparagine	7·6
Stearate	1
Palmitate	1
Valerate	1–10
Butyrate	5
Propionate	5
Electron donors tested but not utilized	
Lactate	10
Citrate	10
Alanine	11·2
Histidine	6·4
Proline	8·7
Glycine	13·3
L-Cysteine	8·3
Aspartic acid	7·5
Glutamic acid	6·8
DMSO	2
Catechol	1
Phenol	0·25–0·50
Toluene	1
Benzene	0·5–1·0
Benzoic acid	0·5–1·0
Fe^{2+} (amorphous FeS)	20
Electron acceptors utilized	
Poorly crystalline Fe(III) oxide	100 mmol l^{-1}
Fe(III) citrate	50
Electron acceptors not utilized	
Oxygen	0·6–1 and 20 %
$S_2O_3^{2-}$	10
SO_4^{2-}	10–20
SO_3^{2-}	2–4
S^0	20 % (w/v)
NO_3^-	10
NO_2^-	1·0
MnO_2 [poorly crystalline Mn(IV)]	20
Anthraquinone-2,6-disulfonate	5
Goethite	50
Haematite	50
Fumarate	10–50
DMSO	1–2
Fe(III) pyrophosphate	10

* Concentration in mM unless otherwise indicated.

Transmission electron microscopy of thin sections revealed that the cell envelope is composed of a cytoplasmic membrane, a periplasmic space and a single outer surface layer, which is typical of the *Archaea* (Fig. 1c).

Temperature, pH and salt optima

With pyruvate as the electron donor and poorly crystalline Fe(III) oxide as the electron acceptor, strain 234[T] grew at between 65 and 90 °C with an optimum temperature of around 88 °C (Fig. 2a). No growth occurred after 2 weeks of incubation at 64 °C or below or at 91 °C or above. The pH range for growth was pH 5·0–7·6, with an optimum at pH 7·0 (generation time around 6·3 h; Fig. 2b). The generation time at pH 5·0 was about 12·45 h (not shown). No growth was detected at pH 7·7 or above. Strain 234[T] grew at NaCl concentrations ranging from 9·0 to 38 g l^{-1}, with an optimum at 19 g l^{-1}, no growth was observed at 4·5 or 72 g NaCl l^{-1} (Fig. 2c).

Antibiotic sensitivity

Growth of strain 234[T] was inhibited by ampicillin (200 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), penicillin G (200 μg ml^{-1}), phosphomycin (200 μg ml^{-1}), rifampicin (100 μg ml^{-1}) and trimethoprim (300 μg ml^{-1}), but cycloheximide (100 μg ml^{-1}), kanamycin (200 μg ml^{-1}), neomycin sulfate (100 μg ml^{-1}), novobiocin (100 μg ml^{-1}), puromycin (10 μg ml^{-1}) and streptomycin (200 μg ml^{-1}) did not inhibit growth. Growth of strain 234[T] was inhibited by vancomycin hydrochloride at 100 and 200 μg ml^{-1} for 48 h and up to 72 h, respectively, after which cells started to grow, possibly due to thermal inactivation of this compound over time.

Electron donors and acceptors utilized

Strain 234[T] readily grew in liquid medium at 85 °C in which pyruvate was the sole electron donor and Fe(III) oxide was the sole electron acceptor (data not shown). There was no significant reduction of Fe(III) with pyruvate in the absence of the organism. Furthermore, there was no reduction of Fe(III) in control tubes that contained cells (previously grown with hydrogen as

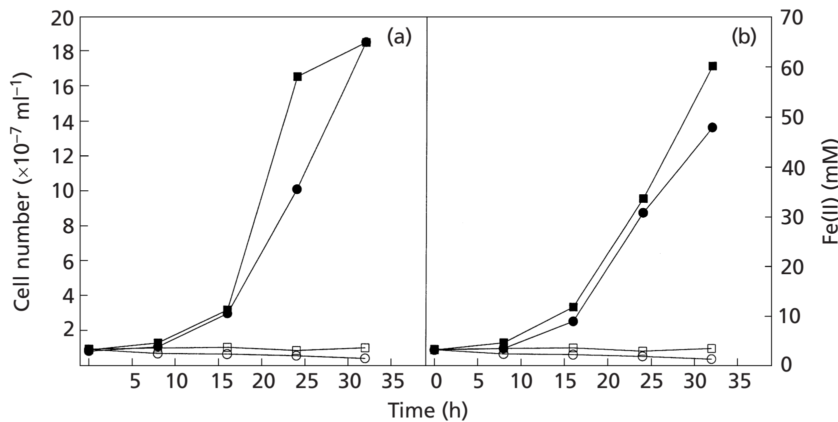

Fig. 3. Growth of strain 234^T at 85 °C in medium with palmitate (a) or stearate (b) as the electron donor and poorly crystalline Fe(III) as the electron acceptor. Results are means of duplicate cultures. Cell numbers (●, ○) and levels of Fe(II) (■, □) are shown; open symbols denote control samples without fatty acid.

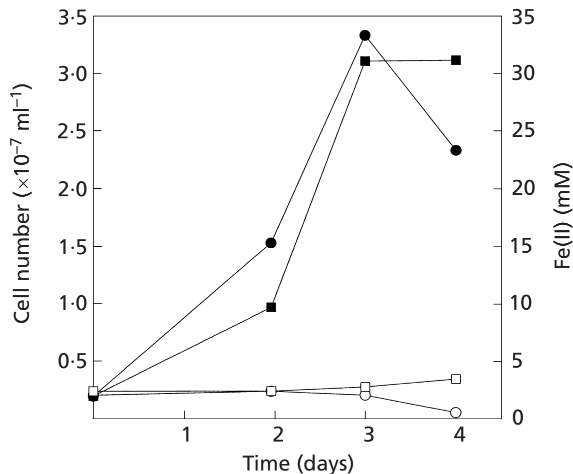

Fig. 4. Growth of strain 234^T at 85 °C in medium with H_2 as the electron donor and poorly crystalline Fe(III) as the electron acceptor. Cell numbers (●, ○) and levels of Fe(II) (■, □) are shown; open symbols denote control samples with H_2 replaced by N_2. Results are means of triplicate cultures.

electron donor) but no pyruvate and under a N_2/CO_2 (80:20%, v/v) atmosphere. In addition to pyruvate, the novel isolate also grew by oxidizing acetate to carbon dioxide with the reduction of Fe(III) (Tor *et al.*, 2001). Strain 234^T could also reduce Fe(III) with a number of other organic electron donors (Table 1). Growth on sugars could not be investigated because the sugars reacted abiotically with the Fe(III) oxide at 85 °C. Several amino acids and short-chain fatty acids such as propionate, butyrate, valerate could serve as the sole electron donor for Fe(III) reduction. The long-chain fatty acids palmitate (generation time 6·5 h; Fig. 3a) and stearate (generation time 7·2 h, Fig. 3b) also served as electron donors for Fe(III) reduction. Growth on long-chain fatty acids was more rapid than the previously reported growth on acetate (Tor *et al.*, 2001). The addition of 0·5 mmol palmitate resulted in the reduction of 37 mmol Fe(III) and the addition of 0·5 mmol stearate resulted in the reduction of 44 mmol Fe(III) in duplicate cultures. Giving the expected stoichiometry of Fe(III) reduced per mole of palmitate oxidized (92 mmol) and based on the expected stoichiometry of Fe(III) reduced per mole of stearate oxidized (104 mmol), and also considering that a portion of the fatty acids was probably assimilated into cell carbon, the obtained stoichiometry is consistent with the oxidation of palmitate and stearate coupled to the reduction of Fe(III).

Strain 234^T also grew with hydrogen as the sole electron donor for Fe(III) reduction. No organic carbon source was required for growth on hydrogen. In the presence of hydrogen, Fe(III) was reduced and Fe(III) reduction was accompanied by cell growth (Fig. 4). There was no Fe(III) reduction or cell growth in the absence of added hydrogen.

Strain 234^T did not grow readily with Fe(III) citrate as the electron acceptor when first tested for this ability. After an extended period of time, strain 234^T was adapted for growth with Fe(III) citrate, but growth and Fe(III) reduction remained much slower than with poorly crystalline Fe(III) oxide.

Several attempts to grow strain 234^T on a wide variety of commonly considered electron acceptors [including sulfate (10–20 mM), thiosulfate (10 mM), sulfite (2–4 mM), S^0 (20%, w/v), nitrate (10 mM), nitrite (1·0 mM) and oxygen (0·6–1·0 and 20%, v/v)] other than poorly crystalline Fe (III) oxide (100 mM) and Fe(III) citrate (50 mM) using H_2 (as H_2/CO_2, 80:20%, v/v; 101 kPa), lactate (10 mM), pyruvate (10 mM), acetate (10 and 20 mM), yeast extract (0·05%) and peptone (0·1%) or a combination of H_2/lactate (2 and 10 mM), H_2/pyruvate (2 and 10 mM), H_2/acetate (2 and 10 mM) and H_2/yeast extract (0·02%) and peptone (0·05%) were unsuccessful. Furthermore, no growth was observed when Fe^{2+} (20 mM amorphous FeS) alone or Fe^{2+} and H_2 (as H_2/CO_2, 80:20%, v/v; 101 kPa) were used as electron donors with nitrate (10 mM) as the electron acceptor.

Similarly, attempts to grow *A. fulgidus*, *A. profundus* and *A. veneficus* on poorly crystalline Fe(III) oxide (100 mM) or Fe(III) citrate (20–50 mM) as an electron acceptor with H_2 (as H_2/CO_2, 80:20%, v/v; 101 kPa),

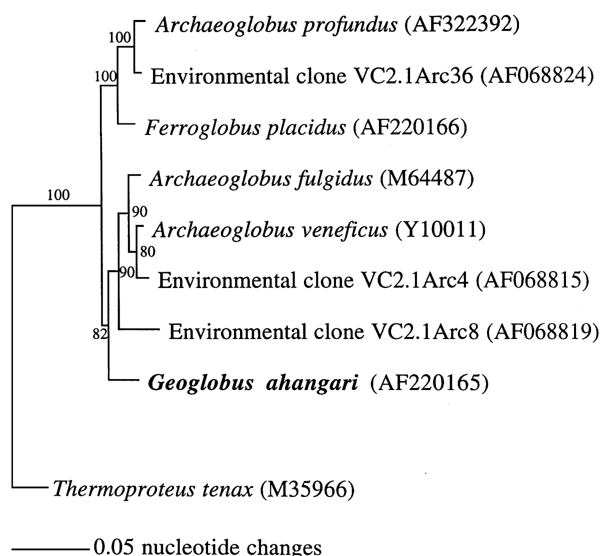

Fig. 5. Phylogenetic tree generated by maximum-likelihood analysis of 16S rDNA sequences showing the relationship of strain 234^T to previously described *Archaea*. Bootstrap values at nodes were calculated with 100 replicates. The branching order was identical when maximum-parsimony and distance-based algorithms were applied to aligned sequences in PAUP 4.0b 4a (Swofford, 1998) with hypervariable regions masked (1230 bases considered).

lactate (10 mM), pyruvate (10 mM), acetate (10 and 20 mM) or yeast extract (0·05 %) and peptone (0·1 %) or a combination of H_2/lactate (2 and 10 mM), H_2/pyruvate (2 and 10 mM), H_2/acetate (2 and 10 mM) or H_2/yeast extract (0·02 %) and peptone (0·05 %) were also unsuccessful.

DNA base composition and phylogenetic analysis

The G + C content of strain 234^T was 58·7 mol %. The aligned 16S rDNA sequences were submitted to the similarity matrix program available on the RDP website and a similarity matrix was generated with 1230 alignment positions considered. Analysis of the 16S rDNA sequence of strain 234^T indicated that its closest known relatives are *A. fulgidus* (96·2 % similar), *A. profundus* (94·1 %), *A. veneficus* (95·1 %) and *F. placidus* (95·1 %), which are members of the domain *Archaea* (Fig. 5).

Fluorescent co-factors

Cells of strain 234^T showed no fluorescence when examined under UV microscopy. In contrast, when *A. fulgidus* cells were used as controls, they showed a bluish-green fluorescence under the same conditions.

Methane formation

No methane was detected (< 0·005 %) in the headspace of cultures of strain 234^T ($3·5 \times 10^8$ cells ml^{-1} over 30 h incubation at 85 °C).

DISCUSSION

The results demonstrate that strain 234^T has several unique physiological characteristics not previously demonstrated in Fe(III)-reducing micro-organisms and/or hyperthermophiles. These include the ability to grow with hydrogen as the sole electron donor and Fe(III) oxide as the electron acceptor in the absence of an organic carbon source and the ability to use long-chain fatty acids as the electron donor. As detailed below, these types of metabolism are likely to have environmental significance. Furthermore, as discussed below, the significant physiological differences between *Archaeoglobus* species and strain 234^T indicate that these organisms should be assigned to different genera, and the differences in phylogeny and some significant differences in physiology suggest that strain 234^T should not be placed in the same genus as *F. placidus*. Thus, strain 234^T should be designated as the type species of a new genus.

Insoluble Fe(III) oxide as an electron acceptor

A key to the isolation of strain 234^T may have been the use of insoluble Fe(III) oxide as the electron acceptor in the solidified isolation medium. Most Fe(III)-reducing organisms, including previously described hyperthermophilic Fe(III) reducers, have been recovered in pure culture with an electron acceptor other than Fe(III) (Lovley, 2000a). In the few cases where Fe(III) has been used as the electron acceptor, soluble, chelated Fe(III) forms have been employed. The technique described here permits direct isolation of Fe(III)-reducing micro-organisms with Fe(III) oxide as the electron acceptor. Fe(III) oxide represents the form of Fe(III) that predominates in most sedimentary environments (Lovley, 1991, 2001) and the use of Fe(III) oxide in the isolation procedure therefore makes it possible to isolate micro-organisms that can reduce Fe(III) in its environmentally relevant form. It is not uncommon that micro-organisms isolated with soluble forms of Fe(III) are not effective in reducing insoluble Fe(III) oxide. Soluble forms of Fe(III) commonly used in the isolation of Fe(III)-reducing micro-organisms such as Fe(III) chelated to nitrilotriacetic acid, citrate or EDTA are toxic to some Fe(III)-reducing micro-organisms (Lovley, 2000a). Thus, the use of soluble Fe(III) forms will prevent isolation of some Fe(III) reducers that may be environmentally significant. For example, strain 234^T grows only poorly in media in which Fe(III) citrate serves as the Fe(III) source.

Of a wide variety of electron acceptors evaluated, strain 234^T grew only with Fe(III). Most of the Fe(III)-reducing micro-organisms presently available in culture have the ability to use electron acceptors other than Fe(III), but this may be a function of the method by which these organisms were isolated. It is notable that strain 234^T, the first organism isolated directly with Fe(III) oxide as the electron acceptor, does not use other commonly considered electron acceptors.

Continued isolations directly with Fe(III) oxide as the electron acceptor may indicate that there are other Fe(III)-reducing micro-organisms that are similarly restricted in their ability to use alternative electron acceptors. The inability of strain 234^T to use the humic-substance analogue anthraquinone-2,6-disulfonate (AQDS) as an electron acceptor was unexpected, as Fe(III)-reducing bacteria (Lovley *et al.*, 1996, 1998) and archaea (Lovley *et al.*, 2000) typically have the ability to use humic substances as an electron acceptor (Lovley, 2000a).

Fatty acids as electron donors

The ability of strain 234^T to oxidize a variety of fatty acids anaerobically has environmental significance. As discussed previously (Tor *et al.*, 2001), hyperthermophiles like strain 234^T that can oxidize acetate anaerobically are likely to be important in the degradation of organic matter in hot, microbial ecosystems, since acetate is a key fermentation product in these environments and acetate may also be produced via abiotic processes in nearby environments that are too hot for micro-organisms to inhabit. Long-chain fatty acids represent another component of the complex assemblage of organic matter found in many sedimentary environments. To our knowledge, strain 234^T is the first hyperthermophile found to oxidize long-chain fatty acids anaerobically with any electron acceptor. The only previously described Fe(III)-reducing micro-organism known to oxidize long-chain fatty acids is the mesophile *Desulfuromonas palmitatus* (Coates *et al.*, 1995). Strain 234^T grows much faster on long-chain fatty acids than does *D. palmitatus*. This is probably due to the fact that long-chain fatty acids are more soluble at 85 °C than at 30 °C. While *D. palmitatus* grew much faster on acetate than on long-chain fatty acids, the reverse was true for strain 234^T.

Autotrophic growth with hydrogen

In hydrothermal environments, in which the primary source of electron donors is geothermal hydrogen, there may be little organic carbon available either as an electron donor or as a carbon source. For example, Fe(II), which is abundant in marine hydrothermal fluids, is oxidized upon exposure to aerobic seawater and the poorly crystalline Fe(III) oxides that are produced can precipitate to form Fe(III)-rich, presumably organic-poor, deposits in nearby hot zones that are bathed in lower flows of geothermal, presumably hydrogen-rich, anaerobic waters (Jannasch, 1995; Karl, 1995; Pichler & Veizer, 1999; Pichler *et al.*, 1999). An organism such as strain 234^T that can grow autotrophically on hydrogen and Fe(III) oxide should be metabolically adapted for such environments. The autotrophic growth of strain 234^T on hydrogen and Fe(III) contrasts with all previously described hydrogen-oxidizing, Fe(III)-reducing micro-organisms, which require a carbon source for growth on hydrogen and Fe(III). These include a diversity of mesophilic and moderately thermophilic micro-organisms (Lovley, 2000a) as well as *T. maritima* and *P. islandicum*, the two hyperthermophiles previously found to conserve energy to support growth from hydrogen oxidation coupled to Fe(III) reduction (Kashefi & Lovley, 2000; Vargas *et al.*, 1998).

In addition to providing a model for how micro-organisms may grow on hydrogen and Fe(III) in modern hydrothermal environments, the finding that strain 234^T can conserve energy to support growth without the need for an organic carbon source may have important implications for the evolution of early forms of life. It has been proposed that the protometabolism that eventually led to modern life was carried out by inorganic iron-sulfur membranes that coupled the oxidation of hydrogen to the reduction of Fe(III) (Russell *et al.*, 1998). Geochemical models suggest that Fe(III) and hydrogen to support such a protometabolism were abundant on prebiotic Earth. For example, it has been suggested that large quantities of Fe(III) and hydrogen were produced from UV radiation hydrolysing the abundant Fe(II) in Archaean seas (Cairns-Smith *et al.*, 1992). The geochemical literature suggests that other sources of hydrogen and Fe(III) are also likely to have been available on prebiotic Earth (Lovley, 2000b). Strain 234^T demonstrates not only that it is possible to gain enough energy to support growth from hydrogen oxidation coupled to Fe(III) reduction, but also that enough energy can be derived from this form of respiration to provide the cell's entire requirement for organic carbon from carbon dioxide fixation. Such carbon fixation could have been important in the evolution of Fe(III) reduction from an inorganic- to an organic-based life form.

Placement of strain 234^T in a new genus, *Geoglobus* gen. nov.

The 16S rDNA sequence of strain 234^T indicates that it is most closely related to species of *Archaeoglobus* and *Ferroglobus*, but different enough to warrant the establishment of a new genus if the phenotype is also significantly different. *Archaeoglobus* and *Ferroglobus* species have been placed in separate genera even though they are similar in morphology and temperature and salinity optima, and both have fluorescent co-factors. The primary justification for placing *F. placidus* in a genus separate from *Archaeoglobus* species appears to be the differences in the form of respiration that these organisms use to conserve energy to support growth (Hafenbradl *et al.*, 1996). *F. placidus* has been shown to grow with Fe(II) or sulfide as the electron donor and nitrate as the electron acceptor (Hafenbradl *et al.*, 1996). It can also grow with hydrogen as the electron donor and sulfite as the electron acceptor (Hafenbradl *et al.*, 1996). In contrast, *Archaeoglobus* species do not use nitrate as an electron acceptor, but reduce sulfate, thiosulfate or sulfite with organic electron donors or hydrogen (Huber *et al.*, 1997; Stetter, 1988; Stetter *et al.*, 1987).

Although analysis of its 16S rDNA sequence suggests that strain 234^T is most closely related to *Archaeoglobus* species, its respiratory mode is completely different. Unlike the *Archaeoglobus* species, strain 234^T is unable to use sulfur forms as electron acceptors, it lacks a dissimilatory sulfite reductase (a property shared by all *Archaeoglobus* species) and it grows exclusively with Fe(III) oxide as the electron acceptor. Cell suspensions of *A. fulgidus* reduced Fe(III) with hydrogen as the electron donor (Vargas *et al.*, 1998), but attempts to grow this organism or *A. profundus* or *A. veneficus* with hydrogen or organic electron donors and Fe(III) as the electron acceptor have been unsuccessful. Another indication of the differences in modes of respiration between strain 234^T and *Archaeoglobus* species is the finding that both *A. fulgidus* and *A. profundus* possess *c*-type cytochromes that resemble the *Desulfovibrio* c_3-cytochromes in spectral properties and molecular mass (K. Kashefi and P. L. Hartzell, unpublished results), whereas strain 234^T lacks *c*-type cytochromes (S. Childers, personal communication). In contrast to *A. fulgidus* and *A. veneficus*, which produce small amounts of methane during growth (Huber *et al.*, 1997; Stetter, 1988; Zellner *et al.*, 1989), no methane was detected in cultures of strain 234^T.

In a similar manner, although it has been recognized recently that *F. placidus* can reduce Fe(III) (Tor *et al.*, 2001), strain 234^T does not have the hallmark respiratory characteristic of *F. placidus*, the ability to oxidize Fe(II) with the reduction of nitrate. Furthermore, strain 234^T lacks the fluorescent co-factors found not only in *F. placidus*, but also in *A. fulgidus*, *A. profundus* and *A. veneficus* (Hafenbradl *et al.*, 1996; Huber *et al.*, 1997; Stetter, 1988; Zellner *et al.*, 1989). Finally, the 16S rDNA sequence similarity of isolate 234^T to *F. placidus* is 95·1 % (1230 alignment positions considered). Under current taxonomic practice, an evolutionary distance in this range is considered justification for the establishment of a new genus. Just as the criteria for differences in mode of respiration justify the separation of *Archaeoglobus* and *Ferroglobus* species into separate genera, equally significant respiratory differences warrant the placement of strain 234^T in a genus separate from *Archaeoglobus* and *Ferroglobus*. The name *Geoglobus ahangari* gen. nov., sp. nov. is proposed.

Description of *Geoglobus* gen. nov.

Geoglobus (Ge.o.glo′bus. Gr. n. *Ge* the Earth; L. masc. n. *globus* ball; N.L. masc. n. *Geoglobus* a ball from the Earth).

Cells are regular to irregular, lobe-shaped cocci, 0·3–0·5 μm in diameter, occurring singly or in pairs. Motile by a monopolar flagellum. Strictly anaerobic chemo-organotrophs that grow by oxidizing acetate, pyruvate, palmitate and stearate coupled to reduction of Fe(III). Can also grow autotrophically with H_2 as the electron donor and poorly crystalline Fe(III) oxide as the electron acceptor. Reduction of poorly crystalline Fe(III) oxide results in the accumulation of what appears to be extracellular, ultrafine-grained magnetite. G+C content of the DNA of the type species is 58·7 mol %. The type species is *Geoglobus ahangari*.

Description of *Geoglobus ahangari* sp. nov.

Geoglobus ahangari [a.han.ga′ri. N.L. n. *ahangari* from Farsi masc. n. *ahangar* a blacksmith or a smith who works with iron, named after Kaveh Ahangar, the mythical Persian hero who was also a blacksmith by trade; arbitrary name referring to the ability to use Fe(III) as an electron acceptor].

The type strain was enriched from a deep-sea hydrothermal vent (chimney) sample from Guaymas Basin, Gulf of California (at a depth of 2000 m) with pyruvate as the electron donor and poorly crystalline Fe(III) oxide as the electron acceptor. Grows by oxidizing H_2, acetate, pyruvate, succinate, fumarate, formate, malate, glycerol, methanol, ethanol, yeast extract, peptone, isoleucine, aspartic acid, glutamic acid, arginine, L-cysteine, serine, glutamine, asparagine, propionate, butyrate, valerate, palmitate or stearate coupled to the reduction of Fe(III). Only poorly crystalline Fe(III) oxide and Fe(III) citrate are used as electron acceptors. Other electron acceptors such as $S_2O_3^{2-}$, SO_4^{2-}, SO_3^{2-}, S^0, NO_3^-, NO_2^-, O_2, Mn(IV), AQDS, malate and fumarate are not utilized as electron acceptors (Table 1). Growth occurs at temperatures between 65 and 90 °C (optimum temperature, approximately 88 °C), in the presence of NaCl concentrations of 9·0–38·0 g l^{-1} (optimum 19 g NaCl l^{-1}) and at near-neutral pH (pH 6·8–7·0). Sensitive to ampicillin, chloramphenicol, penicillin G, phosphomycin, rifampicin and trimethoprim, but resistant to cycloheximide, kanamycin, neomycin sulfate, novobiocin, puromycin, streptomycin and vancomycin hydrochloride.

The type strain is strain 234^T (= ATCC BAA-425^T). The 16S rDNA sequence has been deposited in GenBank under accession number AF220165.

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