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Ferroglobus placidus gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe^{2+} at neutral pH under anoxic conditions

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Abstract A novel coccoid, anaerobic, Fe^{2+}-oxidizing archaeum was isolated from a shallow submarine hydrothermal system at Vulcano, Italy. In addition to ferrous iron, H_2 and sulfide served as electron donors. NO_3^- was used as electron acceptor. In the presence of H_2, also $S_2O_3^{2-}$ could serve as electron acceptor. The isolate was a neutrophilic hyperthermophile that grew between 65° C and 95° C. It represents a novel genus among the Archaeoglobales that we name *Ferroglobus*. The type species is *Ferroglobus placidus* (DSM 10642).

Key words Archaea · Hyperthermophiles · *Ferroglobus placidus* · Neutrophilic anaerobic Fe^{2+}-oxidizer

Introduction

Hydrothermal vent systems harbor communities of anaerobic hyperthermophiles consisting of primary producers and decomposers of organic matter (Blöchl et al. 1994; Stetter 1995). The primary producers are chemolithoautotrophs that use various inorganic compounds present in the hot vents (Stetter 1992a). Hydrothermal fluids usually contain high concentrations of ferrous iron that form iron sulfide precipitates in the presence of hydrogen sulfide (Ehrlich 1990). To date, ferrous iron oxidation at high temperatures has been observed only in members of *Acidianus brierleyi* and *Sulfolobus* sp. from oxygen-rich, acidic solfataric fields (Brierley and Brierley 1973; Brock 1978; Segerer et al. 1986). With this electron donor, growth has been reported to be poor. In addition, it has been difficult to observe because of vigorous autoxidation of Fe^{2+} (Brock 1978; Stetter 1986). At neutral pH, only mesophilic oxidizers of ferrous iron have been described (Hanert 1989; Hallbeck and Pederson 1991).

Anaerobic Fe^{2+}-oxidizing mesophilic bacteria that are either photosynthetic or nitrate-reducers have recently been obtained for the first time (Widdel et al. 1993; Ehrenreich and Widdel 1994; Straub et al. 1996). Here we describe the isolation and characterization of a novel hyperthermophilic archaeum capable of oxidizing ferrous iron, H_2, and sulfide anaerobically at neutral pH.

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Materials and methods

Sources of organisms and isolation of strain AEDII12DO

A sample of sand-water mixture was taken from a shallow beach (depth: 1 m) situated at the base of the reef close to Porto di Levante, Vulcano, Italy. Sample AEDII12 consisted of a mixture of hot, grey sediment and water with an original temperature of approximately 95° C. The pH was 7.0. *Archaeoglobus fulgidus* (DSM 4304) and *Archaeoglobus profundus* (DSM 5631) were obtained from the culture collection of our institute. They were grown as described previously (Burggraf et al. 1990; Stetter 1992b). Strain AEDII12DO was isolated using an "optical tweezer" (Huber et al. 1995).

Culture conditions

Strain AEDII12DO was cultivated under strictly anoxic conditions using the anaerobic technique according to Balch and Wolfe (1976). Isolate AEDII12DO was grown in FM medium ("Ferroglobus" medium) containing per liter 0.34 g KCl, 4.3 g $MgCl_2 \times 6\ H_2O$, 0.24 g NH_4Cl, 0.14 g $CaCl_2 \times 2\ H_2O$, 18 g NaCl, 5 g $NaHCO_3$, 0.14 g $K_2HPO_4 \times 3\ H_2O$, 1 g KNO_3, 2 mM FeS, 10 ml trace vitamins (Balch et al. 1979), 10 ml trace minerals (Balch et al. 1979) and 1 mg resazurin. Prior to autoclaving, FM medium was reduced by addition of 0.5% $Na_2S \times 9H_2O$. The pH was adjusted to 7.0 (H_2SO_4). The new isolate was cultured in 120-ml serum bottles (type III glass; SGD Glashüttenwerke, Kipfenberg, Germany) containing 10 ml medium. The gas phase consisted of 300 kPa N_2/CO_2 (80:20,v/v). Incubation was carried out at 85° C with shaking (200 rpm).

Amorphous FeS was prepared in an anaerobic chamber by adding a solution of 0.6 M $Na_2S \times 9H_2O$ to 0.6 M $FeSO_4 \times 7\ H_2O$, filtering the precipitate, washing it with H_2O, and drying it under N_2/H_2 (95:5, v/v).

For the cultivation of isolate AEDII12DO with H_2 as electron donor, FM medium was prepared without the addition of FeS. The gas phase consisted of 300 kPa H_2/CO_2 (80:20). Modified FM medium was used for the cultivation of isolate AEDII12DO in the absence of sulfide. Modified FM medium was supplemented with 0.25 g $FeCl_2$ per liter instead of with FeS. The medium was boiled and gassed with nitrogen during cooling. Afterwards, the pH was adjusted to 7.0 with H_2SO_4. Serum bottles (120 ml) were filled with 10 ml medium under a protective CO_2 atmosphere and tightly stoppered. K_2CO_3 was added to each bottle from a stock solution to a final concentration of 0.27 g/l. Before autoclaving, the gas phase was exchanged by mixtures of N_2/CO_2 (300 kPa; 80:20). $FeCO_3$ was formed as a colorless precipitate after autoclaving. Unless stated otherwise, the media contained either 4 mM $Na_2S_2O_3$, 4 mM $MgSO_4$, 1 mM Na_2SO_3, or 0.1% (w/v) elemental sulfur.

To determine which organic substrates supported growth, the following compounds were tested as electron donors: peptone, meat extract, yeast extract, casamino acids (each 0.05%, final concentration), L(+)-lactate (2 mM), sodium acetate (6 mM), pyruvate, propionate, isovalerate, succinate, fumarate, citrate, glucose, and fructose (each 10 mM). Incubations were carried out under strictly anoxic conditions and with 0.1% KNO_3 as electron acceptor (gas phase: N_2/CO_2; 80:20, v/v). The same concentrations of organic compounds were tested as carbon source under strictly anoxic conditions with 0.1% KNO_3 as electron acceptor and H_2 as electron donor. Batch cultures were grown at 85° C in a 50-l, enamel-protected fermentor (HTE Bioengineering, Wald, Switzerland) with stirring (200 rpm) and continuous gassing (H_2/CO_2; 80:20; 2.5 l/min).

Light and electron microscopy

Light microscopy and photography were carried out as described previously (Huber et al. 1989). Bacterial growth was determined by direct cell count using a Thoma chamber (depth: 0.02 mm). Electron microscopy was performed as described previously (Völkl et al. 1993). Micrographs were taken on a Philips CM 12 at 100 kV. FeS was analyzed with a Joel JSM/840, Edax PV 9100 scanning electron microscope and the ZAF program.

Analysis of metabolic products

Nitrate and nitrite were quantified concomitantly by HPLC on a Spherisorb 5-µm ODS 2 column and were detected at 205 nm. The running buffer was 10 mM *n*-octyl amine (pH 6.0; adjusted with 0.5% H_2SO_4) at a flow rate of 2 ml/min (pump 420, UV detector 432; Kontron, Neufahrn, Germany). For qualitative analysis of ammonia, 0.5 ml of an ammonia-free culture medium was added to a freshly prepared mixture of 0.5 ml 27% NaOH and 0.5 ml potassium tetraiodomercurate(II) solution ("Neßler's" reagent). A brown precipitate indicated the presence of ammonia. NO and NO_2 were measured with a gas detection system (Dräger, Lübeck, Germany). On a Hewlett Packard 5890 gas chromatograph, N_2 and N_2O were quantified using a column packed with Porapack QS 100/120 mesh and were detected by a thermal conductivity detector (injector temperature 70° C; oven temperature 60° C; detector temperature 220° C) using argon as carrier gas. H_2S was analyzed quantitatively using the methylene blue method (Fonselius 1983). SO_3^{2-} and SO_4^{2-} were determined by ion chromatography with a BT I AN column and a conductivity detector (Biotronik, Maintal, Germany). The running buffer contained 1.8 mM $NaHCO_3$ and 1.0 mM Na_2CO_3. Methane was determined by gas chromatography with a Carbosieve (Supelco, Bellefonte, Pa., USA) S2 column and was detected using a flame ionization detector (oven temperature, 190° C; detector and injector temperature, 220° C). Degradation products of low molecular weight formed from organic compounds were analyzed by HPLC (gradient former 425, pump 420, UV detector 432; Kontron, Neufahrn, Germany) using an Aminex HPX 87 H column (300 × 7.8 mm; BioRad, Munich, Germany) equipped with a precolumn (cation H refill cartridges, 30 × 4.6 mm; BioRad). The running buffer was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min; the column temperature was 30° C. Organic acids were detected at 210 nm. Ferrous iron was quantified photometrically at 510 nm after chelation with 2 mM *o*-phenanthroline in 0.7 M sodium acetate buffer (pH 5) in a test volume of 1 ml (Ehrenreich and Widdel 1994). Anoxic samples were withdrawn with syringes from the culture bottles, acidified with concentrated HCl (final concentration: 1 M), and heated to 100° C for 10 min. Ferric iron was determined by the same method after reduction with 0.28 M hydroxylammonium chloride in the acidified sample; the ferrous iron concentration determined before reduction was substracted.

Isolation of DNA, DNA base composition, and 16S rRNA analysis

DNA was isolated by extraction with phenol/chloroform as described previously (Lauerer et al. 1986). The G+C content of DNA was determined by direct analysis after digestion of the DNA with nuclease P1 and separation by HPLC (Völkl et al. 1993).

A 16S rRNA gene fragment corresponding to base 23–1390 in *Escherichia coli* 16S rRNA (Brosius et al. 1978) was amplified using the polymerase chain reaction (PCR; Saiki et al. 1988). The PCR product was cloned using the CLONEAMP system (Gibco-BRL, Bethesda, Md., USA) and partially sequenced using a sequenase version 2.0 DNA sequencing kit (U.S. Biochemical, Cleveland, Ohio, USA). The phylogenetic tree was calculated by using the phylogenetic program ARB kindly provided by W. Ludwig (Technische Universität München, Germany), using parsimony calculation.

DNA fingerprinting

The PCR experiment was performed essentially as described by Welsh and McClelland (1990). A 50-µl reaction volume contained: 10 × Taq buffer (5 µl), forward primer (5.39 µl, approximately 50 pmol), nucleotides at a 1:1:1:1 ratio (4 µl), H_2O (30.36 µl), Taq polymerase (0.25 µl, 5 U/µl), and DNA (5 µl at a concentration of 10 ng/ml). The primer used was 5′-GTAA AACGACGGCCAGT-3′. Two cycles of reaction were conducted for 5 min at 94° C, 5 min at 40° C, and 5 min at 72° C. Forty cycles of reaction were conducted for 1 min at 94° C, 1 min at 40° C, and 2 min at 72° C. The reaction was stopped by reducing the temperature of the mixture to 4° C, and the products were separated on a conventional 1.5% agarose gel.

Lipid composition

Lipids were extracted according to Nishihara and Koga (1987) and Hafenbradl et al. (1993). Polar lipids were separated by two-dimensional thin layer chromatography with solvent A in the vertical direction and solvent B in the horizontal direction (solvent A: chloroform/methanol/7 M ammonia, 7:3.5:0.8, by vol.; solvent B: chloroform/methanol/acetic acid/water, 20:6:3:1, by vol.). The total lipid extract was hydrolyzed in 1 M methanolic HCl to cleave the polar headgroups. Core lipids and the nonpolar lipids were separated by thin layer chromatography using the solvent *n*-hexane/ethyl acetate, 4:1 (v/v). All compounds were detected by spraying with anisaldehyde, followed by heating at 150° C for 5 min. The Dittmer and Lester reagent was used for phospholipids, α-naphthol/H_2SO_4 for glycolipids, and ninhydrin for amino lipids (Kates 1991).

Results

Enrichment and isolation

In order to enrich anaerobic iron-oxidizing hyperthermophiles FM medium containing FeS as electron donor

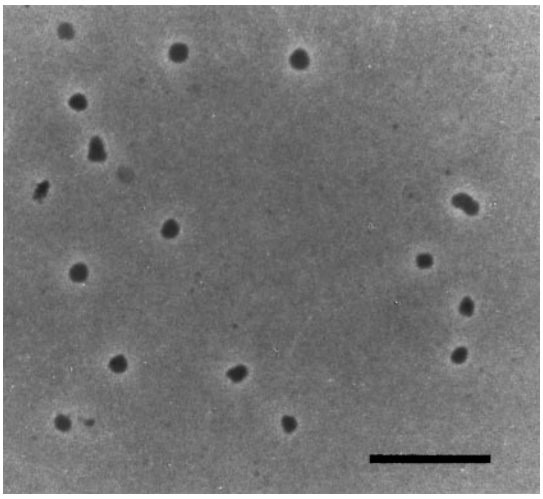


Fig. 1 Phase-contrast micrograph of the cells of strain AEDII12DO in the mid-exponential growth phase. *Bar* 10 μm

was inoculated with approximately 1 ml of the sample and incubated with shaking (50 rpm) at 85° C. After approximately 4 days, growth of irregular cocci was observed in the enrichment culture from sample AEDII12. The new isolate was named AEDII12DO. *Thermococcus alcaliphilus* was isolated from the same sample and has been described previously by Keller et al. (1995). Strain AEDII12DO was purified from the enrichment culture using the "optical tweezer" technique (Huber et al. 1995).

Morphology

By phase-contrast microscopy, highly irregular coccoid cells occurring singly and in pairs were visible (Fig. 1). Cells were triangularly-shaped with one or two flagella (not shown) and appeared to be flat at the broader base. The width of the cells was approximately 0.7–1.3 μm. Freeze etching showed that cells were covered by a regular surface layer protein with orthogonal symmetry (either p2 or p4) and revealed a lattice constant of approximately 23 nm (Fig. 2). Under UV microscopy at 420 nm, cells showed a blue-green fluorescence that faded rapidly under UV radiation.

Fig. 2a, b Electron micrographs of strain AEDII12DO: **a** freeze-fractured, **b** freeze-etched. *Bar* (for **a** and **b**) 0.5 μm

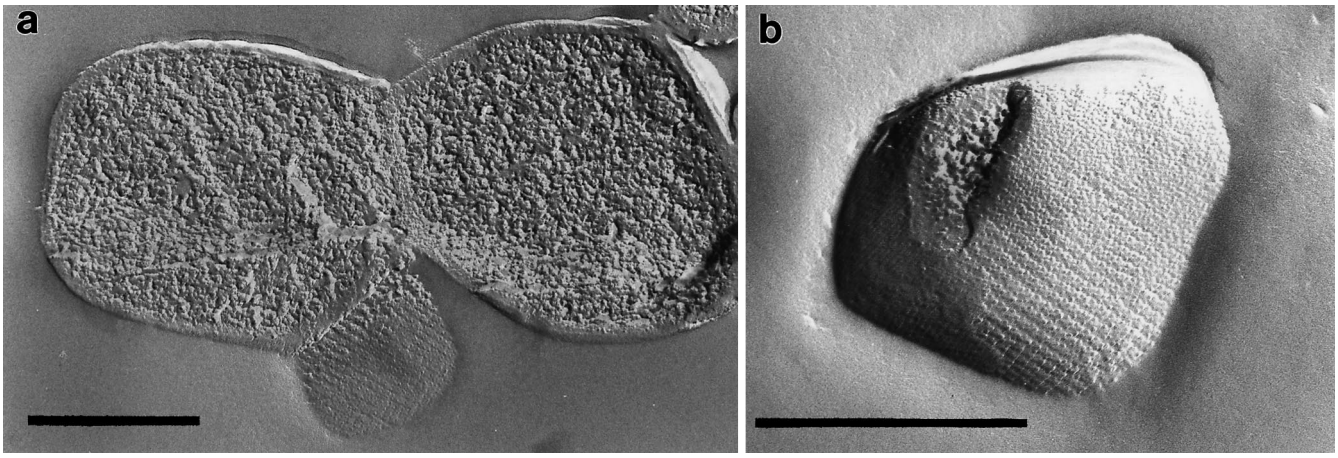
Temperature, pH, and salt optima

Growth of isolate AEDII12DO was observed between 65°C and 95° C, with an optimum at 85° C (doubling time: 2.8 h; Fig. 3). No growth was detected at 63° C and at 96° C. The pH range for growth was 6.0–8.5, with an optimum around 7.0. At pH values below 6.0 or above 8.5, cells lysed within 2 h. The isolate AEDII12DO grew in media containing 0.5–4.5% NaCl, with an optimum around 2%. Below 0.5% or above 4.5% NaCl, cells lysed within 3 h. All growth experiments were done in FM media containing nitrate, H_2, and pyruvate.

Metabolism

The new isolate AEDII12DO gained energy by nitrate reduction. Molecular hydrogen, sulfide, and Fe^{2+} served as electron donors. The isolate was not able to grow on various organic acids or sugars in the presence of nitrate. With sulfide as electron donor, elemental sulfur that deposited extracellularly during the stationary phase was formed. In the presence of $FeCO_3$, the colorless precipitate became rust-brown; with FeS as electron donor, black FeS became green-grey (not shown). Iron oxidation also occurred to a lesser extent in uninoculated (nonreduced) medium with $FeCO_3$ (Fig. 4). In the FeS-containing uninoculated medium, all acid-soluble iron remained in the ferrous state (data not shown). In the lithotrophic enrichment culture, the molar ratio of formed iron(III) to reduced nitrate was about 2:0.9, which is in agreement with the expected stoichiometry of ferrous oxidation according to the following equation:

$$NO_3^- + 2\ FeCO_3 + 6\ H_2O \rightarrow NO_2^- + 2\ Fe(OH)_3 + 2\ HCO_3^- + 2\ H^+ + H_2O$$



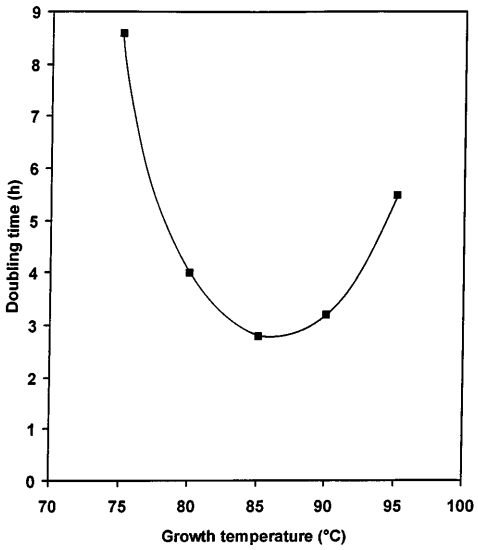


Fig. 3 Effect of temperature on the growth of *Ferroglobus placidus* with H_2 as electron donor and nitrate as electron acceptor. Doubling times were calculated from the slopes of the growth curves (not shown) at pH 7.0 by using a Thoma chamber

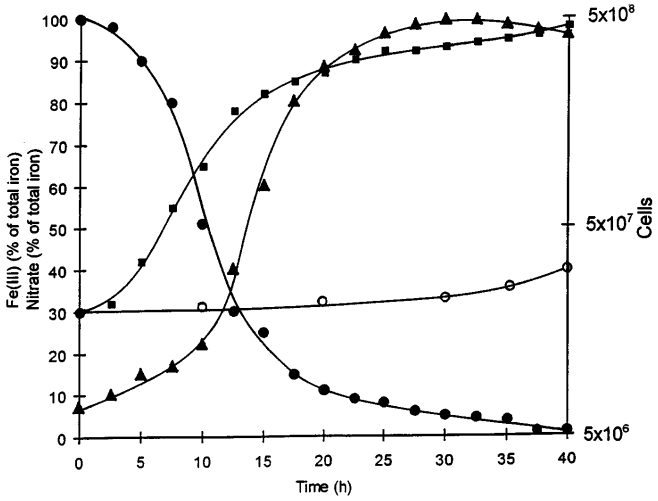


Fig. 4 Anaerobic oxidation of ferrous iron (2 mmol of $FeCO_3$ was added per liter) with nitrate (0.64 mmol KNO_3 was added per liter) under chemolithoautotrophic conditions. ■ Ferric iron in growing culture, ○ autoxidation in uninoculated culture medium at 85° C, ● nitrate in growing culture, ▲ cells/ml

During growth on nitrate and hydrogen, nitrite (up to approximately 0.55 mM) accumulated in the culture medium and turned into NO and NO_2 during further incubation. No N_2, N_2O, or ammonia was detected. Alternatively, the new isolate was able to grow by reduction of $S_2O_3^{2-}$ to H_2S using H_2 as electron donor. In addition, the cells unexpectedly showed significant growth in the presence of $S_2O_3^{2-}$ and Fe^{2+}; SO_3^{2-} and FeS were detected as products. No SO_4^{2-}, a product of the disproportionation of $S_2O_3^{2-}$ was found. In this experiment, therefore, the energy-yielding reaction remains unclear. Growth was not obtained with nitrite, SO_4^{2-}, SO_3^{2-}, or S° as electron acceptor in combination with H_2 as electron donor. The new isolate was not able to grow on oxygen and did not tolerate oxygen, not even at very low concentrations (0.2–1%). Cultures of isolate AEDII12DO (incubation for 2 days in FM medium with nitrate as electron acceptor and H_2 as electron donor; final cell concentration: 2×10^8/ml) did not form detectable amounts of methane. Organic compounds were not essential for growth. However, casamino acids, yeast extract, acetate, and pyruvate increased final cell yields about threefold when added in addition to the electron donor and acceptor. Small amounts of pyruvate were degraded (analysed by HPLC); however, no end products such as acetate or other low-molecular-weight products were detected.

Lipid analysis

Membrane core lipids of isolate AEDII12DO consisted of C_{40} tetraethers and C_{20}, C_{20} diethers. Analysis of the complex lipids showed amino-, glycophospho- and phospholipids.

DNA base composition and phylogenetic analyses

The DNA base composition of isolate AEDII12DO was 43 mol% G+C. Analysis of the 16S rRNA sequence of isolate AEDII12DO showed that strain AEDII12DO is a member of the Archaeoglobales. The new isolate differed from *Archaeoglobus fulgidus* by an estimated exchange of 4.2 bases per 100 nucleotides (Fig. 5). The PCR profile of an arbitrarily primed PCR reaction obtained on a 1.5% agarose gel showed a pattern clearly different from that of *Archaeoglobus profundus* and *A. fulgidus* (not shown).

Discussion

The novel marine isolate AEDII12DO represents the first hyperthermophilic, anaerobic iron oxidizer. Based on the phytanyl ether lipids, the S-layer envelope, and its 16S rRNA sequence, isolate AEDII12DO is a member of the archaeal domain (De Rosa and Gambacorta 1988; Woese et al. 1990; Baumeister and Lembcke 1992). With its energy-yielding metabolism, it is a variable neutrophilic lithotroph: molecular hydrogen, ferrous iron, and sulfide serve as electron donors, while nitrate and thiosulfate (in the presence of H_2) are used as electron acceptors. Within the archaea, nitrate reduction has only been found among the mesophilic extreme halophiles (Zumft 1992) and in the hyperthermophilic *Pyrobaculum aerophilum* (Völkl et al. 1993). Growth by thiosulfate reduction is known in a variety of hyperthermophiles of the genera *Pyrodictium*, *Archaeoglobus*, and *Pyrobaculum* (Stetter et al. 1983; Stetter 1992b; Völkl et al. 1993). The blue-green fluorescence of isolate AEDII12DO is characteristic of methanogens and Archaeoglobales within the archaea. This is in agreement with the 16S rRNA phylogeny, which places isolate AEDII12DO among the Archaeoglobales (evolu-

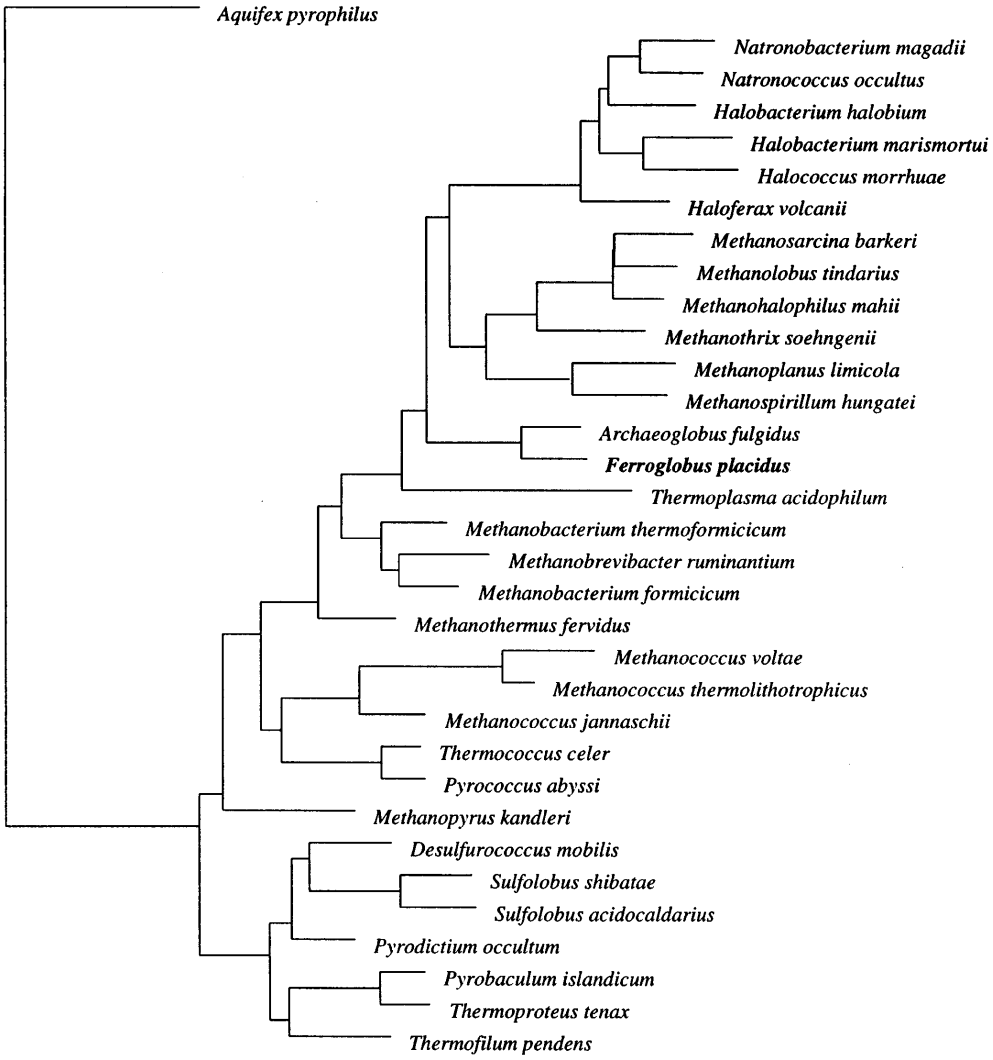


Fig. 5 Phylogenetic relationships of *F. placidus*. The position of *F. placidus* was calculated by transversion analysis according to Woese et al. (1991). *Bar* 10 base exchanges per 100 nucleotides

tionary distance to *Archaeoglobus fulgidus*: 4.2%). Other members of the Archaeoglobales appear unable to grow by nitrate reduction, or by ferrous iron or sulfide oxidation. On the other hand, the new isolate AEDII12DO was unable to reduce sulfate, a property shared by all Archaeoglobales to date (Stetter 1992b). In its unique symmetry and lattice constant, the S-layer envelope of AEDII12DO differed from that of all Euryarchaeota. Based on metabolic properties, we named the new isolate *Ferroglobus placidus*.

It has been hypothesized that nitrate formed early in the history of the Earth and was present in a hot Archaean ocean (Mancinelli and McKay 1988; Lowe 1993). On the early Earth, CO_2 and N_2 were the major atmospheric constituents (Walker 1977). Lightning in such an atmosphere could have been an important source of NO (Walker 1977). The NO produced in the lightning channel would almost certainly have undergone further reactions in the atmosphere and in the oceans. These reactions would have resulted in the formation of NO_3^- and NO_2^- (Mancinelli and McKay 1988). Ferrous iron has also been thought to be a constituent of this environment (Ehrlich 1990; Ehrlich et al. 1991) and, in combination with nitrate, could have fueled the metabolism of an organism similar to *Ferroglobus placidus*. This scenario is consistent with the phylogenetic position of *F. placidus* within the domain Archaea and would offer a biological mechanism for the anaerobic formation of Fe^{3+}, one of the major components of banded iron formations (BIFs), which are thought to have been formed up to 3.8×10^9 years ago (Appel 1980). Thus, the unique metabolism of *F. placidus* may offer insight into ancient processes thought to have been operating under the anoxic, high-temperature conditions of the early history of the Earth.

Description of a new genus and one new species

Ferroglobus, gen. nov.; Fer. ro. glo' bus. L. n. *ferrum*, iron; L. masc. n. *globus*, ball; L. masc. n. *Ferroglobus*, the iron ball.

Cells irregular coccoid, 0.7–1.3 μm in diameter. Weak, blue-green fluorescence at 420 nm. Optimal growth at approximately 85°C, pH 7.0, and 2% NaCl. Motile by

monopolar flagella. Strictly anaerobic. Chemolithoautotroph or heterotroph. Growth on Fe(II), S^{2-} or H_2 as electron donor and nitrate as electron acceptor, and in the presence of H_2, also with $S_2O_3^{2-}$ as electron acceptor. S-layer envelope. Membrane contains phytanyl ether lipids. G+C content approximately 43 mol%. By phylogenetic analysis of the 16S rRNA sequence, a member of the *Archaeoglobales*. Habitat: marine hydrothermal systems.

Type species: *Ferroglobus placidus*

Ferroglobus placidus, sp. nov. pla. ci. dus. L. masc. adj. *placidus*, peace-loving (because of reduction of nitrate, a component of gun powder).

Cells are irregular coccoid, about 0.7–1.3 μm in diameter, occurring singly and in pairs, sometimes in aggregates. Strictly lithotrophic, strictly anaerobic. Fe(II), H_2, and sulfide serve as electron donors. Reduction of nitrate leads to formation of nitrite and nitrous gases. Reduction of thiosulfate in the presence of H_2 leads to H_2S. S° inhibitory to growth. Pyruvate, acetate, and yeast extract do not serve as electron donors for nitrate reduction, but stimulate growth. Core lipids consist of 2,3-di-*O*-phytanyl-*sn*-glycerol and glycerol-dialkyl-glycerol. Growth between 65°C and 95° C (optimum: 85° C), 0.5% and 4.5% NaCl (optimum: 1.8–2.0% NaCl), and pH 6.0 and 8.5 (optimum: pH 7.0). DNA base composition 43 mol% G+C.

Type strain: *Ferrogolobus placidus*, AEDII12DO (DSM 10642), Braunschweig, Germany, isolated from a shallow marine hydrothermal system at Vulcano Island, Italy.

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