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ORIGINAL PAPER

Acidiferrobacter thiooxydans, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur-oxidizer of the family *Ectothiorhodospiraceae*

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Abstract A comprehensive physiological and phylogenetic characterisation was carried out of "*Thiobacillus ferrooxidans*" m-1, an acidophilic iron-oxidizing bacterium first described over 25 years ago. Phylogenetically, strain m-1 is a gammaproteobacterium, most closely related to alkaliphilic *Ectothiorhodospira* spp. and only distantly to iron-oxidizing acidithiobacilli. Physiological examination confirmed that strain m-1 can grow autotrophically not only by ferrous iron oxidation but also, in contrast to previous reports, by oxidation of elemental sulfur, sulfide and tetrathionate, using either oxygen or ferric iron as terminal electron acceptor. The bacterium was also found to be thermo-tolerant, growing optimally at 38°C and up to a maximum of 47°C. Growth in liquid media required an external osmotic potential of >2 bar, and was optimal at ~5 bar, though no growth occurred where the medium osmotic potential was close to that of sea water (~26 bar). From this, it was concluded that strain m-1 is a moderate osmophile. Strain m-1 was also shown to be diazotrophic and tolerant of elevated concentrations of many metals typically found in mine-impacted environments. On the basis of these data, m-1 is proposed as the type strain of a new genus and species of bacteria, *Acidiferrobacter thiooxydans* (DSM 2392, JCM 17358).

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Introduction

Dissimilatory oxidation of iron is carried out by acidophilic prokaryotes that encompass different phyla of bacteria (*Nitrospirae*, *Actinobacteria*, *Firmicutes* and *Proteobacteria*), as well as *Euryarchaeota* and *Crenarchaeota* (Johnson and Hallberg 2008). In one of the first studies of the physiological and genomic diversity among iron-oxidizing acidophiles, Harrison (1982) showed that 23 strains of bacteria identified as "*Thiobacillus ferrooxidans*" fell into seven distinct groups on the basis of their DNA:DNA homologies. One of these groups was represented by a single strain (m-1) that had been isolated from coal spoil refuse in Missouri, USA. Isolate m-1 was also reported to differ from other "*T. ferrooxidans*" strains in its much higher chromosomal G+C content (65, compared with 55–58 mol%) and in apparently being unable to oxidize elemental sulfur, which is a key characteristic of bacteria of the genus *Thiobacillus*. Analysis of partial sequences of 16S rRNA revealed that strain m-1 was a gammaproteobacterium and that it was very distantly related to other acidophilic thiobacilli (Lane et al. 1992). The latter were reclassified as *Acidithiobacillus* spp. by Kelly and Wood (2000), but no classification was ascribed to strain m-1.

Hitherto, there has been little information published on the physiology and phylogeny of strain m-1. Harrison (1984) reported that it grew as non-motile, rod-shaped cells that did not form endospores. While it could be subcultured in organic-free media, the question whether it was able to utilize organic carbon was not ascertained, though Harrison

(1984) found that its relatively slow growth in liquid media could be enhanced by the addition of 0.01% (w/v) cysteine. Also, in contrast to *At. ferrooxidans*, strain m-1 did not grow on the solid media available at that time.

Here, we provide a comprehensive description of the physiological and phylogenetic characteristics of strain m-1. Our investigations have confirmed that it is an obligately autotrophic acidophile that can grow using ferrous iron as sole electron donor. However, in contrast to earlier reports, strain m-1 was also found to be able to oxidize elemental sulfur and under both aerobic and anaerobic conditions and is thus a facultative anaerobe. Other notable characteristics are its thermo-tolerance and requirement of an external osmotic potential of >2.3 bar for reliable growth. Phylogenetically, it is distantly related to known species of acidophilic bacteria, but more closely related to the purple sulfur bacteria of the family *Ectothiorhodospiraceae*, which includes bacteria that are typically alkaliphilic and halophilic (Imhoff 2006).

Materials and methods

Cultivation of isolate m-1 in liquid and solid media

Strain m-1 was obtained as a freshly cultivated liquid culture from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany), where it is currently listed as a strain of *Acidithiobacillus ferrooxidans* (DSM 2392). Initial attempts at cultivating this bacterium in liquid media used routinely to cultivate various species of iron-oxidizing acidophiles (Hallberg and Johnson 2007; Johnson and Hallberg 2007) met with only limited success, with poor rates and incomplete oxidation of ferrous iron recorded in liquid media. However, after the discovery of enhanced growth of the strain in magnesium sulfate- and potassium tetrathionate-amended media (described below), the growth medium comprising 20 mM ferrous sulfate as energy source in basal salts and trace elements (Wakeman et al. 2008) at pH 1.9 was supplemented with 100 mM magnesium sulfate and 0.5 mM tetrathionate unless otherwise noted, which facilitated routine culturing of this acidophile. Growth of strain m-1 on solid media was tested with a variety of overlay media used to cultivate different species of iron- and sulfur-oxidizing acidophiles (Johnson and Hallberg 2007). For long-term storage, bacteria were grown on pyrite before freezing concentrated cell suspensions at −80°C in 7% (v/v) DMSO (Hallberg and Johnson 2007).

Osmotolerance and metal tolerance

To overcome the sporadic and poor growth of strain m-1 with ferrous iron as substrate, media supplemented with 10–100 mM of NaCl, Na_2SO_4, KCl, K_2SO_4 or $MgSO_4$ were tested to determine if these could support better growth and routine culturing of this acidophile. Quantitative effects of elevated osmotic pressure on growth of m-1 was tested in shake flask cultures containing 5 mM tetrathionate as energy source and basal salts/trace elements solution, supplemented with varying concentrations (0–500 mM) of magnesium sulfate. The ability of isolate m-1 to grow in the presence of 100 mM NaCl (with or without 50 mM magnesium sulfate) was also tested in shake flask cultures containing tetrathionate as energy source.

To evaluate its tolerance to metals frequently present at elevated concentrations in mine waters, bacteria were grown in 20 mM ferrous sulfate liquid medium (pH 1.9), supplemented with varying concentrations of metals (Table 2). Cultures were set up in duplicate and incubated at 30°C for 10 days. Growth was determined by measuring residual ferrous iron relative to control cultures.

Effects of pH and temperature on growth

Strain m-1 was grown in batch culture in a pH- and temperature-controlled 2 l bioreactor (1 l working volume; Electrolab, UK), which was aerated (1 l min^{-1}) and stirred (100 rpm). The temperature was varied between 21 and 45°C at a pH set-point of 1.9, or the pH was varied from between 1.25 and 2.4 at a constant temperature of 30°C. Ferrous iron concentrations were determined at regular intervals and growth rates were calculated from semi-logarithmic plots of iron oxidized against time. Growth at pH 1.0 and 5, and between 45 and 50°C was tested in shake flask cultures.

Chemolithotrophic growth of strain m-1

Cultures of strain m-1 grown on ferrous iron, supplemented or not with pyrite (FeS_2; Strem Chemicals, Newburyport, MA, USA), were inoculated into liquid medium (with ferrous iron added to ~100 μM as a trace element) containing either ~1% (w/v) elemental sulfur or ~2 mM potassium tetrathionate. The shake flasks were incubated aerobically at 37°C and bacterial growth assessed by measuring optical densities at 440 nm, culture pH and concentrations of sulfate or tetrathionate, respectively. Growth with sulfide as electron donor was assessed by inoculating gradient tubes of semi-solid (0.1% w/v agarose) HBS + 100 mM $MgSO_4$, pH 4, overlaying an agarose plug (2% agarose) containing 0.025% Na_2S. Growth was apparent as a visible halo of bacteria within the gradient tube, and oxidation of sulfide to sulfuric acid was assessed by adding bromophenol blue to the gradients and observing a color change from blue (pH > 4) to yellow (pH < 3).

The ability of strain m-1 to catalyze the oxidative dissolution of pyrite was tested by inoculating medium (pH 2.5) containing 1% (w/v) pyrite in duplicate and incubating at 37°C. Samples were withdrawn over a period of 4 weeks to determine total iron concentrations and pH, relative to negative (non-inoculated) and positive (inoculated with the type strain of *Acidithiobacillus ferrooxidans*, NCIMB 11820^T = ATCC 23270^T), incubated at 30°C and with no additional magnesium sulfate) controls.

To assess whether strain m-1 could oxidize hydrogen, standard liquid medium containing 100 μM ferrous iron and iFeo plates (Johnson and Hallberg 2007) were inoculated with actively growing cultures and incubated in an anaerobic jar (Oxoid, UK) with an activated H_2/CO_2 generating kit (Oxoid). No palladium catalyst was included to ensure that the atmosphere inside the jar contained oxygen. Liquid and plate cultures of *At. ferrooxidans*^{T} were included as positive controls.

Ferric iron reduction and anaerobic growth of strain m-1

To ascertain if strain m-1 could reduce ferric iron, bacteria grown aerobically on elemental sulfur were inoculated into flasks containing the same medium, but with ferrous sulfate replaced by soluble ferric sulfate (10 mM) or solid ferric iron (produced by adding an aliquot of ferric sulfate solution to a basal salt solution initially at pH 7). These were incubated anaerobically at 37°C (AnaeroGen system; Oxoid) and concentrations of ferrous iron determined after 1 week. Anaerobic growth with sulfur as electron donor and soluble ferric iron as electron acceptor was tested by incubating cultures containing varying (and limiting) amounts of ferric sulfate anaerobically at 37°C and relating final cell counts to the concentrations of ferrous iron produced.

Specific rates of ferrous iron oxidation

The specific rates of ferrous iron oxidation were compared for cultures of strain m-1 grown with ferrous iron, elemental sulfur or tetrathionate as electron donor. Cells were harvested, washed and resuspended in basal salts (pH 1.9), and the protein concentrations of the cell suspensions determined. Aliquots of cell suspensions were added to 5 ml of liquid medium containing 1 mM ferrous sulfate in 25 ml universal bottles, which was continuously aerated and held at 38°C. Samples were withdrawn at regular intervals to determine ferrous iron concentrations. Specific rates of ferrous iron oxidation were calculated as mg of ferrous iron oxidized per minute per mg of protein. Specific rates of iron oxidation were compared with those of *At. ferrooxidans*^{T} and *At. ferrivorans* strain CF27 (Hallberg et al. 2010) determined at 30°C.

Carbon assimilation

Isolate m-1 was grown routinely in liquid media that contained no added organic carbon. To assess whether the acidophile could, like some other species, grow mixotrophically using ferrous iron as an energy source and organic carbon as a carbon source, media at pH 1.9 containing 20 mM ferrous sulfate were supplemented with one of the following: yeast extract or peptone (both at 0.01%, w/v), glucose, fructose, glutamate or cysteine (at 1 mM, final concentrations), citrate or glycerol (at 2 mM) and ethanol (at 3 mM). Cultures were incubated at 30°C for 1 week, and examined for total cell numbers. To identify the biochemical mechanism used by strain m-1 to fix CO_2, PCR was carried out to amplify genes coding for RuBisCO as described below.

Assessment of dinitrogen fixation by strain m-1

The ability of strain m-1 to fix dinitrogen was assessed by determining whether the acidophile could be routinely subcultured in nitrogen-free liquid media, combined with biomolecular analysis (PCR amplification of the dinitrogenase reductase (*nifH*) gene, described below). Growth in ammonium-free basal salts containing either ferrous iron (20 mM) or tetrathionate (2 mM) as electron donor was tested in shake flask cultures incubated in a sealed jar containing a 50% (v/v) solution of H_2SO_4 (a sink for atmospheric ammonium) as described previously (Hallberg et al. 2010). When the electron donor had been completely oxidized, growth was determined as total cell counts when using ferrous iron as electron donor, and as optical density at 440 nm for tetrathionate-grown cultures.

Biomolecular analyses

DNA was extracted as previously described (Okibe et al. 2003) from 2 ml of a pyrite-grown culture to serve as template for PCR reactions. The 16S rRNA gene was amplified and sequenced as described by Okibe et al. (2003), the *nifH* gene was amplified according to Ueda et al. (1995) and PCR targeting *cbbL* and *cbbM* genes was carried as described by Johnson et al. (2009), but with 1.5 mM Mg^{2+} in the PCR reaction. As the primers for the *cbbM* gene used were not degenerate, a second primer pair was designed based on an alignment of *cbbM* genes from a phylogenetically diverse range of bacteria, including many that were obtained from genome sequencing projects. The new primers tested were cbbM500F (CGGIACIATCATC AAICC, where I = inosine) that targeted the gene from nucleotide positions (based on the *cbbM* gene from the *Acidithiobacillus ferrooxidans* ATCC 23270 genome) 483–500 and cbbM975R (TCCATCTTGCCIIAICCC) targeting positions 992–975. A range of annealing temperatures from

45 to 57°C was tested, along with a range of Mg^{2+} concentrations in the PCR reaction from 1.5 to 3.5 mM using DNA extracted from *At. ferrooxidans*^{T}, *At. ferrivorans*^{T}, and *At. thiooxidans*^{T} as positive controls. The best yield of PCR products from these DNAs was obtained at $T_a = 49$°C and [Mg^{2+}] of 3 mM. The purity of cultures grown with S and tetrathionate was assessed by terminal restriction fragment length polymorphism (Hallberg et al. 2006), and was judged to be pure (and strain m-1) by the presence of a single terminal restriction fragment when using four restriction enzymes that have tetrameric recognition sites in individual digestion reactions.

Accession numbers for gene sequences determined in this study are AF387301 (16S rRNA gene), HQ234763 (*cbbL*) and HQ234764 (*nifH*).

The classification of strain m-1 based on 16S rRNA gene sequence was carried out using a naïve Bayesian classifier (Wang et al. 2007) and the closest known type strain of a species determined using the EZTaxon server (Chun et al. 2007). 16S rRNA genes of strain m-1 and selected gammaproteobacteria were aligned using ClustalX (Larkin et al. 2007), followed by manual editing to remove gaps and positions of ambiguous nucleotides, which resulted in a final alignment of 1218 nucleotides. This alignment was used to make phylogenetic trees by DNA parsimony, neighbor-joining and maximum-likelihood analyses. In all cases, general tree topology and clusters were stable, and reliability of the tree topologies was confirmed by bootstrap analysis using 1000 replicate alignments. As the topologies of trees generated by all three methods were nearly identical, only the neighbor-joining tree is presented.

The G+C content of chromosomal DNA purified from a 100-ml culture grown with pyrite as electron donor was determined by melting point analysis as described previously (Okibe et al. 2003).

Miscellaneous techniques

Concentrations of ferrous iron in experiments to determine effects of temperature and culture medium pH on growth of strain m-1 were determined by titration of culture aliquots with 1 mM potassium permanganate. Otherwise, ferrous iron concentrations were determined colorimetrically using the ferrozine assay (Lovley and Phillips 1987). Protein concentrations were measured using the method described by Bradford (1976). Total iron was determined by ion chromatography (Ñancucheo and Johnson 2010). Tetrathionate was assayed by cyanolysis as described by Kelly et al. (1969) and sulfate concentrations were measured using a turbidimetric technique (Kolmert et al. 2000). Culture pH was measured using a pHase combined electrode (VWR, United Kingdom) coupled to an Accumet 50 pH meter (Cole-Palmer, Vernon Hills, IL, USA). Cell counts were obtained using a Helber counting chamber marked with Thoma ruling (Hawksley, United Kingdom) and viewed with a Leitz Labolux phase-contrast microscope at a magnification of 400×.

Results and discussion

Phylogeny of strain m-1 and closely related bacteria

The iron-oxidizing acidophile, strain m-1, was first described over 25 years ago, but until now has remained something of an anomaly in that its physiological and phylogenetic traits were largely unknown. One reason for this is that there have been very few reports in the literature of this or closely related acidophiles other than the original strain and one brief report on the isolation of a strain with >99% 16S rRNA gene sequence identity to strain m-1 from mine drainage in Cartagena, Spain (Mitchell et al. 2004). However, BLAST searches using the 16S rRNA gene sequence from the DSM 2392 culture have revealed that bacteria with >99% gene sequence identity (Fig. 1) have been detected by gene cloning in macroscopic streamer growths in the Rio Tinto in Spain (Garcia-Moyano et al. 2007), mine waters at a copper mine in China (He et al. 2007) and lead/zinc mine tailings in Arizona in the USA (Mendez et al. 2008). The latter two sites have both been described as being located in semi-arid climates. One closely related (>99% identity) cloned 16S rRNA gene and one closely related isolate have, within the past decade, been obtained in the authors' laboratory, the clone from a bioreactor in South Africa processing copper/nickel concentrate and operated at 37°C and pH ~1.4, and the isolate from leach liquor, temperature of 35°C and a pH of 2.7, from a 50000-ton black schist ore heap in Finland. The m-1-like bacterium in the bioreactor could not be isolated, and viable cultures of the heap isolate could not be maintained. Interestingly, the Finnish isolate was obtained from plates that were incubated at 43°C, which suggests that the thermo-tolerance observed in strain m-1 (see below) is a general trait of this species. The detection of strain m-1 in locations worldwide suggests a global distribution of this bacterium in acidic, metal-rich environments, including metal bioprocessing reactors.

Analysis based on the 16S rRNA gene revealed that isolate m-1 belongs to the family *Ectothiorhodospiraceae* and not the *Acidithiobacillaceae*, as other, characterized, Gram-negative iron- and sulfur-oxidizing acidophiles. The closest type strain of any species was *Ectothiorhodosinus mongolicus* M9, with which it shared a gene identity of 90.5%. Phylogenetic analysis confirmed the placement of strain m-1 in this family (Fig. 1), clustering with the gene clones from the South African bioreactor and with two

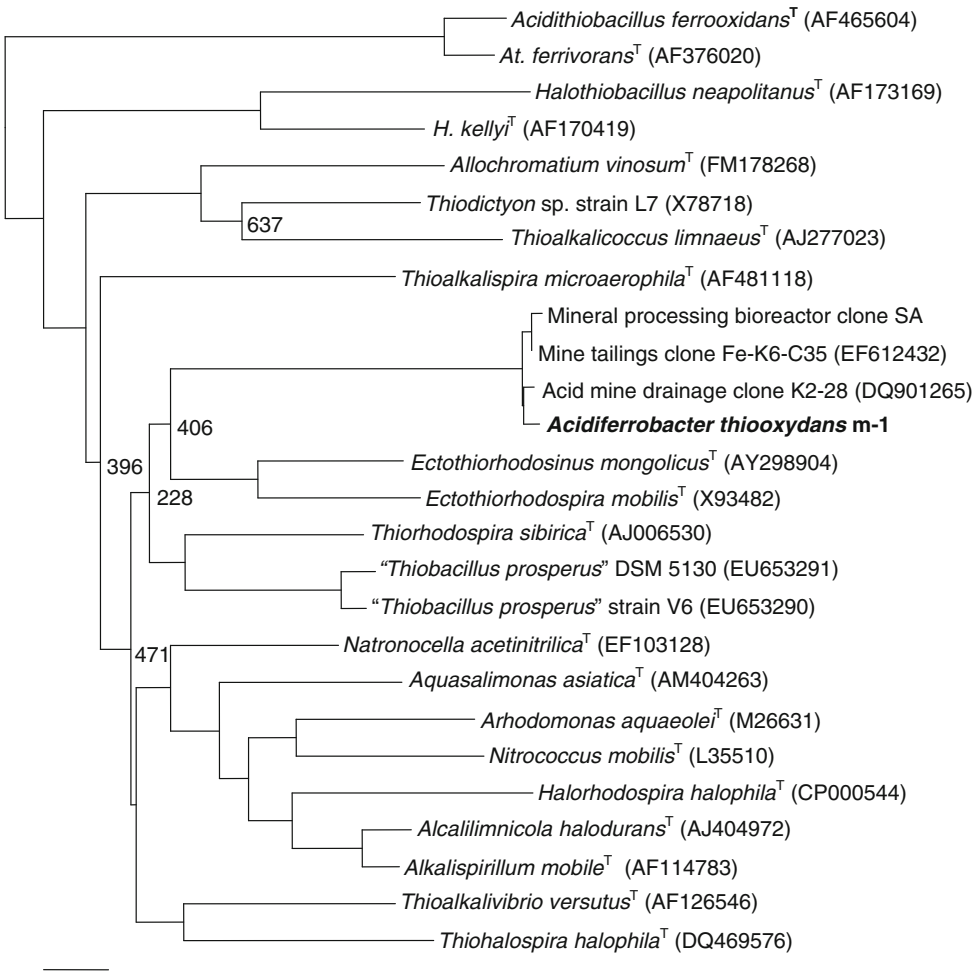

Fig. 1 Phylogenetic tree showing the relationship of strain m-1 (in *bold*), and some closely related (>99% identity) bacteria detected as cloned 16S rRNA genes, to type strains of other species of bacteria of the *Ectothiorhodospiraceae*, and other gammaproteobacteria. This tree is a neighbor-joining tree, which shared similar topologies to trees constructed by DNA parsimony analysis and DNAML analysis. GenBank accession numbers for sequences are given in parenthesis, and numbers at the nodes represent bootstrap values (out of 1000 replicates), which are given only for those that were less than 70%. The *scale bar* represents 10% sequence divergence. The tree was rooted with *Escherichia coli* (not shown)

genes from two mine sites. This group of sequences formed a deep branch from sequences of *Ets. mongolicus* and *Ectothiorhodospira mobilis* DSM 237^T. Few iron-oxidizing bacteria from the order *Chromatiales* have been identified. These include, aside from strain m-1, isolate L7 of the genus *Thiodictyon* (a phototrophic neutrophile) and bacteria that belong to the tentatively named species "*Thiobacillus prosperus*" (Fig. 1), which are also acidophilic and halophilic (Davis-Belmar et al. 2008). Like the latter, growth of m-1 with ferrous iron as electron donor was enhanced by the inclusion of small amounts of tetrathionate (0.5 mM final concentration) in the growth medium (data not shown). The G+C content of the chromosomal DNA of strain m-1 was found to be 63.1 ± 0.4 mol% by melting point analysis, which is similar to that (65.3 mol%) determined by buoyant density analysis (Harrison 1982).

Response of strain m-1 to culture medium osmotic potential

Initial difficulties were encountered in subculturing isolate m-1 in liquid media used routinely at the authors' laboratory to grow acidophilic iron-oxidizing bacteria. This problem was resolved when, following phylogenetic analysis showing that strain m-1 belongs to a group of typically halotolerant or halophilic bacteria, it was discovered that this acidophile is moderately osmophilic. Initial experiments found that growth of strain m-1 in medium containing NaCl at up to 0.5% (w/v, or ~86 mM) was faster than in the absence (data not shown), while no growth occurred in medium containing 1% NaCl. Inclusion of other salts in the medium, including KCl, Na_2SO_4 and K_2SO_4, led to enhanced and reliable growth of this acidophile, suggesting that it was osmophilic rather than halophilic. Subsequently, tests using magnesium sulfate-amended media and tetrathionate as electron donor (at 5 mM, which allowed growth to be determined by measuring optical densities of cultures) showed that very little growth occurred in media containing 0 or 25 mM magnesium sulfate, and that addition of 200 mM of this salt resulted in a lag period before growth was apparent (Fig. 2). Optimum growth was observed in the presence of 100 mM magnesium sulfate (or sodium chloride), and no growth was apparent in cultures containing 500 mM magnesium sulfate, in contrast to *At. ferrooxidans*^{T}

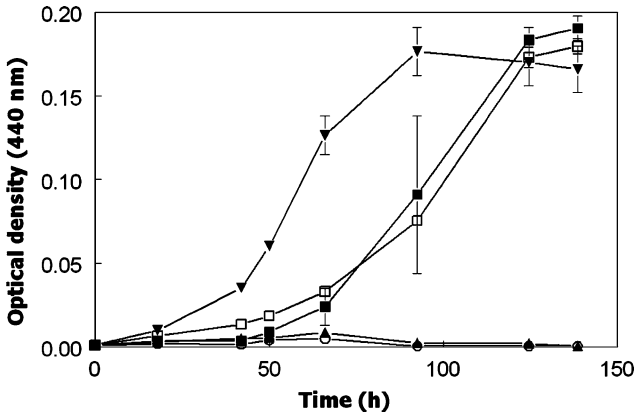

Fig. 2 Growth of isolate m-1 in 5 mM tetrathionate medium in the presence of 0 (*open circles*), 25 (*filled triangles*), 50 (*open squares*), 100 (*filled inverted triangles*) or 200 mM (*filled squares*) magnesium sulfate. The corresponding (calculated) osmotic pressures of these growth media are 1.03, 2.08, 3.14, 5.24 and 9.46 bars, which are equivalent to total dissolved solids concentrations of 3306, 6310, 9322, 15337 and 27367 mg/l, respectively

which grew in tetrathionate media amended with 0–500 mM of this salt. In terms of osmotic pressures (OPs; calculated from summating all dissolved solutes), strain m-1 required media OP of >2 bar, and showed optimum growth at ~5 bar.

Effects of temperature and culture medium pH on growth of strain m-1

The optimum and lowest pH for growth of strain m-1 were found to be ~2 and 1.2 (Fig. 3a), respectively; no growth was observed at pH 1.0. The optimum temperature for growth was ~38°C and maximum ~47°C (Fig. 3b), while no growth was observed at 5 or 50°C. From these data, strain m-1 can be classified as both extremely acidophilic and thermo-tolerant. In contrast to previous reports, isolate m-1 grew on solid medium, though the only variant that was successful was the "iFeo" overlay medium (a double-layer gel containing *Acidiphilium* SJH in the underlayer, and with no organic carbon component apart from the gelling agent, agarose).

Utilization of alternative electron donors by strain m-1

In contrast to earlier reports, strain m-1 was able to grow using elemental sulfur or tetrathionate as sole sources of energy (Fig. 4) and also grew in gradient tubes where it oxidized sulfide to sulfuric acid (supplementary Fig. S1). As when ferrous iron was used as electron donor, growth with these other alternative electron acceptors occurred routinely only when media with elevated osmotic pressure were used. When ferrous iron-grown strain m-1 was used as an inoculum, growth on sulfur was not apparent even after protracted

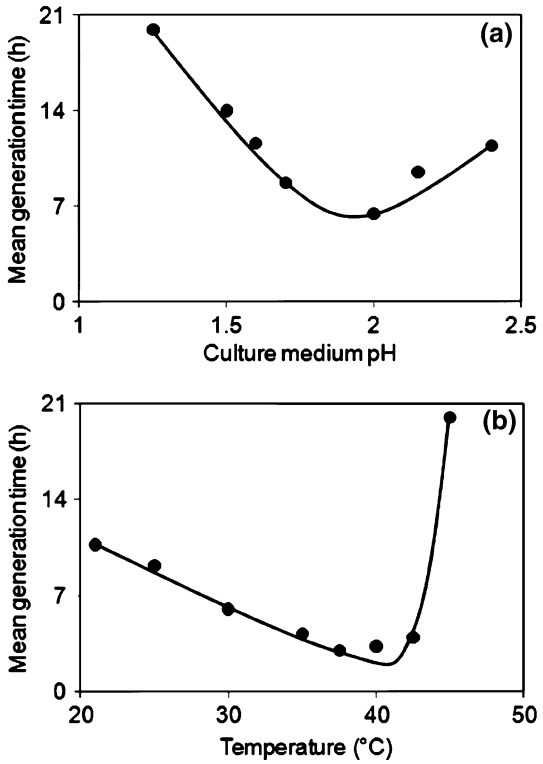

Fig. 3 Growth of strain m-1 in ferrous iron medium at **a** different pH values and temperature maintained at 30°C, and **b** different temperatures at a pH fixed at 1.9

(>2 weeks) incubation. In contrast, bacteria grown on pyrite (supplementary Fig. S2) were successfully sub-cultured in sulfur or tetrathionate media. Soluble sulfur anions and elemental sulfur are generated during the microbiological oxidative dissolution of pyrite as a result of ferric iron attack on the mineral (Schippers and Sand 1999), and it is possible that exposure to these sulfur species switched on genes coding for sulfur oxidation that were strongly repressed when the bacteria were growing on ferrous iron alone. The oxidative dissolution of pyrite by strain m-1 was notably less pronounced than that by *At. ferrooxidans*^{T}, even though both acidophiles were grown at their optimum temperatures (supplementary Fig. S2). Sulfur, tetrathionate and pyrite grown cultures were confirmed to be axenic.

At. ferrooxidans^{T} grew successfully on hydrogen under the experimental protocol used (colonies changed from being ferric iron-encrusted to off-white and much larger with protracted incubation), whereas those of strain m-1 remained small and iron-encrusted throughout. It was therefore concluded that strain m-1 could not oxidize hydrogen.

Specific rates of iron oxidation by strain m-1

The propensity of strain m-1 for iron oxidation was demonstrated from measurements of specific rates of ferrous

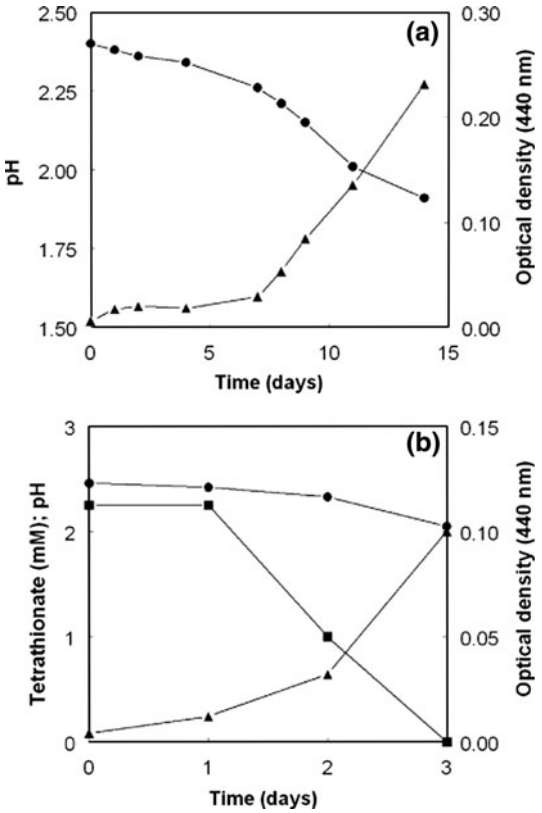

Fig. 4 Growth of strain m-1 on **a** elemental sulfur, and **b** tetrathionate. Key *filled triangles* OD values, *filled circles* pH, *filled squares* tetrathionate concentrations

Table 1 Specific rates of ferrous iron oxidation by strain m-1 grown on ferrous iron, elemental sulfur and tetrathionate, and comparison with *Acidithiobacillus ferrooxidans*^{T} and *At. ferrivorans* strain CF27

Bacterium	Electron donor	Specific rate of Fe^{2+} oxidation (μg/min/mg protein)[a]
m-1	Fe^{2+}	457 ± 20
m-1	S^0	129 ± 21
m-1	$S_4O_6^{2-}$	94 ± 8
At. ferrooxidans^{T}	Fe^{2+}	484 ± 3
At. ferrooxidans^{T}	S^0	44 ± 8
At. ferrooxidans^{T}	$S_4O_6^{2-}$	69 ± 1
At. ferrivorans CF27	Fe^{2+}	312 ± 7
At. ferrivorans CF27	S^0	103 ± 3

[a] Determined at 38°C for strain m-1, and 30°C for *At. ferrooxidans*^{T} and *At. ferrivorans* CF27. No data are available for tetrathionate-grown *At. ferrivorans* CF27

iron oxidation in cultures grown with various electron donors (Table 1). Ferrous iron-grown bacteria oxidized iron at rates that were similar to those of *At. ferrooxidans*^{T}, and faster than those of *At. ferrivorans* strain CF27. However, although rates were slower (~28%) for sulfur-grown m-1, the decrease was much less than corresponding

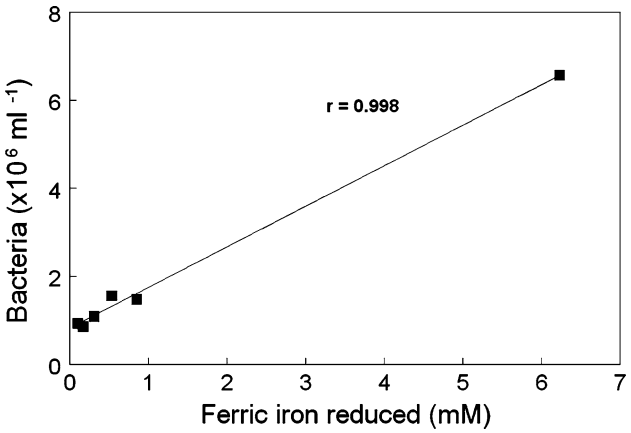

Fig. 5 Correlation between ferric iron reduction and growth of strain m-1 with sulfur as electron donor in the absence of oxygen

data for *At. ferrooxidans*^{T} (9%) and similar to that of *At. ferrivorans* strain CF27 (33%). Similarly, although specific rates of ferrous iron oxidation were lower for strain m-1 grown on tetrathionate than on iron, values were again greater than those of corresponding cultures of *At. ferrooxidans*^{T}.

Iron reduction and anaerobic growth of strain m-1

Ferrous iron was found to accumulate when isolate m-1 was grown under anaerobic conditions in the presence of elemental sulfur and either soluble or solid-phase ferric iron (data not shown). Confirmation that this acidophile can grow by ferric iron respiration came from the observation that cumulative concentrations of ferrous iron and cell numbers were strongly correlated ($r = 0.97$) in cultures incubated under strictly anoxic conditions in the presence of elemental sulfur and ferric sulfate (Fig. 5).

Carbon assimilation and diazotrophy in strain m-1

Strain m-1 was shown to be an obligate autotroph. None of the organic materials added to cultures increased cell numbers beyond those of the organic-free control (data not shown). Two of the organic compounds tested (glycerol and cysteine) were inhibitory at the concentrations used. The result with cysteine was interesting in the light of Harrison's (1984) observation of stimulation of growth when this amino acid was added to cultures of m-1, even though the concentrations used in either case were similar. As with other *Ectothiorhodospiraceae*, strain m-1 fixed inorganic carbon via the Calvin-Benson-Bassham cycle as revealed by PCR amplification of a portion of the *cbbL* gene that codes for the large subunit of form I RuBisCO. In contrast, no *cbbM* (form II RuBisCO large subunit) gene

fragment could be amplified from strain m-1, as is the case for other members of the family (Tourova et al. 2007).

In addition to assimilating a source of carbon from the atmosphere, strain m-1 could also assimilate dinitrogen for growth, using either ferrous iron or tetrathionate as electron donor (data not shown). In both cases, final cell yields were smaller when grown in medium lacking ammonium as nitrogen source, as would be expected. The amplification of a portion of the *nifH* gene from strain m-1 provided genetic evidence to support the assimilatory reduction of dinitrogen.

Metal tolerance

Strain m-1 was shown to tolerate very elevated concentrations of most of the metals tested (Table 2). Aluminum, manganese and iron (ferrous and ferric) are typically found in large concentrations in acidic waters draining coal and metal mines, and zinc, nickel and copper in bioleach liquors and waters draining metal mines (depending on the ore body in question). Interestingly, its sensitivity to copper was much greater than that of the other transition metals tested, differentiating it from *At. ferrooxidans* and *Leptospirillum ferriphilum*, which are known to be highly copper-tolerant, though some other iron-oxidizing acidophiles (e.g. *Leptospirillum ferrooxidans*) are also more sensitive to copper than to zinc and nickel (Galleguillos et al. 2009).

Concluding remarks

Although first isolated over 25 years ago, there is little published data on strain m-1, most likely due to problems in cultivating and maintaining this acidophile in vitro. Phylogenetic analysis placed strain m-1 in the *Ectothiorhodospiraceae*, leading to the discovery that growth was enhanced and could be reproducible by using media of elevated osmotic strength. As the osmotic strength could be elevated using a variety of salts with any cation and either chloride or sulfate as anion, strain m-1 can be considered a moderate osmophile rather than a moderate halophile. This is in contrast to "*Thiobacillus prosperus*", another acidophilic iron-oxidizing member of the family, which has been shown to have a specific requirement for chloride for growth (Davis-Belmar et al. 2008). Other notable traits of strain m-1 include the ability to use dinitrogen as nitrogen source for growth, its thermo-tolerance and its ability to grow by ferric iron reduction. Based on the various traits described above, we propose the new genus and species name of *Acidiferrobacter thiooxydans*, with m-1 as the type strain of the species.

Table 2 Metal tolerance of strain m-1, denoted as minimum inhibitory concentration (MIC) permitting growth

Metal	MIC (mM)
Fe^{2+}	>200
Fe^{3+}	200 (100)
Mn^{2+}	>200
Al^{3+}	>200
Cu^{2+}	20 (10)
Ni^{2+}	>200
Zn^{2+}	>200
MoO_4^{2-}	0.1 (0.05)

Maximum metal concentrations tested were 200 mM. Where growth occurred in these cultures, MIC values are quoted as ">200". Numbers in parentheses are the next lowest concentrations tested where growth was obtained

Description of *Acidiferrobacter* gen. nov. *Acidiferrobacter* (A.ci.di.fer.ro.bac'ter. N.L. n. *acidum* (from L. adj. acidus, sour), an acid; L. n. *ferrum*, iron; N.L. masc. n. *bacter*, rod; N.L. masc. n. *Acidiferrobacter*, an acid-loving, ferrous iron-oxidizing rod).

Sequence analysis of the 16S rRNA gene places the genus within the subclass of *Ectothiorhodospiraceae* in the *Gammaproteobacteria*. The type species is *Acidiferrobacter thiooxydans*, and the genus description is based on that of the type species, currently the only species ascribed to this genus.

Description of *Acidiferrobacter thiooxydans* sp. nov. *Acidiferrobacter thiooxydans* (thi.o.o.xy'dans. Gr. n. *theion* (Latin transliteration thium), sulfur; Gr. adj. *oxus*, sharp, acid; N.L. v. oxydo, to make acid, to oxidize; N.L. part. adj. *thiooxydans*, sulfur-oxidizing).

Cells are non-motile slender straight rods (ca. 2 μm long and ca. 0.3 μm wide). Gram-negative; endospores are not formed. Forms small, ferric iron-stained colonies of ca. 1–2 mm diameter on ferrous iron overlay plates that lack soluble organic carbon. Acidophilic (pH optimum ~2 and minimum 1.2) and thermotolerant (optimum and maximum temperatures for growth 38 and 47°C, respectively). Moderately osmophilic, displaying poor growth in media with osmotic pressures <2 bar, and optimum growth at ~5 bar. A variety of inorganic salts, such as magnesium sulfate and sodium chloride can act as suitable external solutes for solution-phase osmotic potential. Grows by oxidation of ferrous iron, pyrite, sulfide, sulfur or tetrathionate. Facultative anaerobe that can use either molecular oxygen or ferric iron as terminal electron acceptor. Strictly autotrophic and facultative diazotroph. Chromosomal DNA base composition is 63.1 ± 0.4 mol% G+C. Sequence of 16S rRNA gene (GenBank accession number AF387301) places *Acidiferrobacter thiooxydans* in the family *Ectothiorhodospiraceae* of the *Gammaproteobacteria*. Found in acidic, iron-rich environments associated with the

oxidation of sulfide minerals such as acid mine drainage waters and bioleachate liquors. The type strain is m-1^T and has been deposited within the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSM 2392) and the *Japan Collection of Microorganisms* (JCM 17358).

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