

# *Ferrimicrobium acidiphilum* gen. nov., sp. nov. and *Ferrithrix thermotolerans* gen. nov., sp. nov.: heterotrophic, iron-oxidizing, extremely acidophilic actinobacteria

D. Barrie Johnson, Paula Bacelar-Nicolau,† Naoko Okibe,‡ Angharad Thomas and Kevin B. Hallberg

School of Biological Sciences, College of Natural Sciences, Bangor University, Bangor LL57 2UW, UK

## Correspondence

D. Barrie Johnson

d.b.johnson@bangor.ac.uk

Two novel extremely acidophilic, iron-oxidizing actinobacteria were isolated, one from a mine site in North Wales, UK (isolate T23<sup>T</sup>), and the other from a geothermal site in Yellowstone National Park, Wyoming, USA (Y005<sup>T</sup>). These new actinobacteria belong to the subclass *Acidimicrobidae*, and in contrast to the only other classified member of the subclass (*Acidimicrobium ferrooxidans*), both isolates were obligate heterotrophs. The mine site isolate was mesophilic and grew as small rods, while the Yellowstone isolate was a moderate thermophile and grew as long filaments, forming macroscopic flocs in liquid media. Both isolates accelerated the oxidative dissolution of pyrite in yeast extract-amended cultures, but neither was able to oxidize reduced forms of sulfur. Ferrous iron oxidation enhanced growth yields of the novel mesophilic actinobacterium T23<sup>T</sup>, though this was not confirmed for the Yellowstone isolate. Both isolates catalysed the dissimilatory reduction of ferric iron, using glycerol as electron donor, in oxygen-free medium. Based on comparative analyses of base compositions of their chromosomal DNA and of their 16S rRNA gene sequences, the isolates are both distinct from each other and from *Acidimicrobium ferrooxidans*, and are representatives of two novel genera. The names *Ferrimicrobium acidiphilum* gen. nov., sp. nov. and *Ferrithrix thermotolerans* gen. nov., sp. nov. are proposed for the mesophilic and moderately thermophilic isolates, respectively, with the respective type strains T23<sup>T</sup> (=DSM 19497<sup>T</sup>=ATCC BAA-1647<sup>T</sup>) and Y005<sup>T</sup> (=DSM 19514<sup>T</sup>=ATCC BAA-1645<sup>T</sup>).

## INTRODUCTION

Actinobacteria are high-G + C, Gram-positive bacteria, comprising a wide range of physiologically diverse species. The *Acidimicrobidae*, a subclass within the actinobacteria (Stackebrandt *et al.*, 1997), is currently represented by only a single genus (*Acidimicrobium*) and a single species (*Acidimicrobium ferrooxidans*). *Acidimicrobium ferrooxidans* is a moderately thermophilic, iron-oxidizing acidophile (Clark & Norris, 1996), and was first isolated (strain

TH3) from a copper leaching operation in New Mexico, USA (Brierley, 1978). A second strain (ICP<sup>T</sup>), isolated from a geothermal site in Iceland by enriching in a pyrite-containing medium, was selected as the type strain of the species by Clark & Norris (1996). Since then, there have been few reports of the isolation of *Acidimicrobium*-like bacteria. Interestingly, novel *Acidimicrobidae* were shown, by 16S rRNA gene community analysis, to be major components of the bacterial community associated with two species of sponge (*Xestospongia* spp.) and were hypothesized to form symbiotic associations with the animals (Montalvo *et al.*, 2005).

Initially *Acidimicrobium ferrooxidans* was considered to be an obligate heterotroph, but later Clark & Norris (1996) showed unequivocally that it could fix carbon dioxide when grown in organic carbon-free media. Mesophilic iron-oxidizing acidophilic bacteria that have an absolute requirement for organic carbon have also been described (Johnson & Roberto, 1997). These bacteria were shown to use organic carbon (such as yeast extract) included in growth media when grown in pure culture, or to use that

†Present address: Universidade Aberta, 1269-001-Lisboa, Portugal.

‡Present address: Research Institute for Innovative Technology for the Earth (RITE), Kyoto, Japan.

Abbreviation: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Ferrimicrobium acidiphilum* and *Ferrithrix thermotolerans* are AF251436 and AY140237, respectively.

Supplementary figures showing pyrite oxidation and reduction of ferric iron by isolates T23<sup>T</sup> and Y005<sup>T</sup> are available with the online version of this paper.

derived from chemolithotrophic primary producers when grown as mixed cultures with other iron and sulfur oxidizers (Bacelar-Nicolau & Johnson, 1999). More recently, a variety of moderately thermophilic acidophiles (including a strain of *Acidimicrobium ferrooxidans*) were isolated from acidic geothermal sites in Yellowstone National Park, Wyoming, USA (Johnson *et al.*, 2003). One of these isolates (Y005<sup>T</sup>) was originally described (on the basis of erroneous 16S rRNA gene sequencing) as being related to methylophilic betaproteobacteria. However, resequencing of the isolate confirmed that it too (like isolate T23<sup>T</sup>) was an actinobacterium.

Here we describe the physiological and phylogenetic characteristics of obligately heterotrophic, iron-oxidizing actinobacteria, for which two novel genera are proposed.

## METHODS

**Isolation, purification and cultivation of bacteria.** Bacterium T23<sup>T</sup> was isolated from pyrite enrichment cultures inoculated with mine water from the abandoned Cae Coch sulfur mine, North Wales, UK (Jenkins & Johnson, 1993), as described previously (Bacelar-Nicolau & Johnson, 1999) and Y005<sup>T</sup> from an iron/yeast extract enrichment culture inoculated with mineral sample (pH 2.7; 31 °C) taken in the Beryl Spring/Gibbon river area of Yellowstone National Park (Johnson *et al.*, 2003). In both cases, isolates were obtained by streaking enrichment cultures onto overlaid solid media designed to cultivate acidophilic prokaryotes (Johnson & Hallberg, 2007), and were purified by repeated plating and selection of single colonies. The solid media used contained (i) ferrous iron ('iFeo'); (ii) ferrous iron and tryptone soya broth ('Feo'); (iii) ferrous iron, tryptone soya broth and potassium tetrathionate ('FeSo'). Both bacteria were routinely subcultured in liquid media containing 10 mM ferrous sulfate and 0.02% (w/v) yeast extract at pH 2.0. Isolate T23<sup>T</sup> was grown routinely at 30 °C and Y005<sup>T</sup> at 45 °C. Cultures of *Acidimicrobium ferrooxidans* (the type strain, ICP<sup>T</sup>, and strain TH3) were kindly provided by Dr Paul Norris of the University of Warwick, UK. Other bacteria used were maintained in the Acidophile Culture Collection at the authors' laboratory.

**Microscopy.** Bacteria were viewed using a phase-contrast microscope (Leitz Labolux, ×400) and an Hitachi S-520 scanning electron microscope. Sample preparation for the latter was as described by Johnson *et al.* (1992).

**Oxidation of ferrous iron, sulfur and pyrite.** Isolates T23<sup>T</sup> and Y005<sup>T</sup> were grown in 10 mM ferrous iron/0.02% (w/v) yeast extract liquid medium in shake flask cultures, incubated at either 30 °C (T23<sup>T</sup>) or 45 °C (Y005<sup>T</sup>). Cells were harvested and suspended in mineral salts (at pH 2.0). To confirm ferrous iron oxidation, cultures containing 25 mM ferrous sulfate (pH 1.9) were inoculated, and ferrous iron concentrations measured at regular intervals using the ferrozine assay (Lovley & Phillips, 1987). For sulfur oxidation, the cultures contained 0.5% (w/v) of finely ground elemental sulfur in place of iron, while the oxidation of pyrite (FeS<sub>2</sub>) was assessed in media containing 1% (w/v) of the mineral, pre-treated with hydrochloric acid to remove any oxidized surface layers. Sulfur oxidation was monitored by measuring changes in culture pH (oxidation of sulfur to sulfate is an acid-generating reaction), and pyrite oxidation by recording changes in concentrations of soluble total iron, either by atomic absorption spectrophotometry or using ion chromatography. Duplicate cultures, amended, or not, with 0.02% yeast extract were set up in each case.

**Determination of pH and temperature optima.** Growth of isolates T23<sup>T</sup> and Y005<sup>T</sup> at different temperatures and pH values was tested in a 2 l bioreactor run in batch mode. The growth medium for both bacteria contained ferrous iron (25 mM) and yeast extract (0.02%). Growth rates at a specific temperature and pH were determined from semi-logarithmic plots of iron oxidized against time.

**Utilization of organic substrates.** Bacteria were grown in a liquid medium containing 10 mM ferrous sulfate and 0.01% yeast extract. Various organic materials were added to this medium, at concentrations of between 5 and 15 mM (in order to provide similar amounts of organic carbon). The organic compounds tested were: (i) sugars (glucose, fructose, ribose, glucosamine and glucuronic acid); (ii) alcohols (ethanol and glycerol); (iii) acids (citric and acetic); and (iv) amino acids (glycine and glutamic acid). Bacterial growth was determined by measuring optical densities at 600 nm (isolate T23<sup>T</sup>) or protein concentrations [isolate Y005<sup>T</sup>, using the method described by Bradford (1976)] and comparing them to control cultures containing only ferrous iron and yeast extract. Subsequently, growth was tested in yeast extract-free glycerol medium in order to ascertain whether the isolates required specific organic growth factors. The toxicity of acetic acid to the bacteria was tested by adding this organic acid at concentrations of between 100 μM and 10 mM to a liquid medium containing 10 mM ferrous iron and 0.02% yeast extract, and recording bacterial growth and iron oxidation.

**Reduction of ferric iron.** Bacteria were grown in 10 mM ferrous sulfate/0.01% yeast extract (pH 2.0) liquid medium in foam-stoppered universal bottles, incubated (shaken) at 30° or 45 °C under aerobic conditions. When all of the ferrous iron had been oxidized to ferric iron, glycerol was added (to a final concentration of 5 mM) and the bottles transferred to an anaerobic jar and incubated under anaerobic conditions (unshaken) using the AnaeroGen system (Oxoid). Samples were taken from the cultures at regular intervals and ferrous iron concentrations measured. Non-inoculated cultures were set up as controls.

**Effect of ferrous and ferric iron concentrations on biomass yields and growth rates.** In order to ascertain whether ferrous iron oxidation by isolates T23<sup>T</sup> and Y005<sup>T</sup> was an energy-generating reaction, cultures were set up (at pH 1.9, in triplicate) containing 0.02% (w/v) yeast extract and different concentrations of either ferrous sulfate or ferric sulfate (0.1–50 mM). These were incubated under aerobic conditions (shaken at 30 or 45 °C) and, when all of the iron in the 50 mM cultures had been oxidized, cells were harvested, inorganic precipitates dissolved (by addition of sulfuric acid) and total protein yields determined using the Bradford assay. A second experiment, set up with T23<sup>T</sup> only, measured the effect of different concentrations of ferrous and ferric iron on the growth rate of this isolate. Ferrous (or ferric) sulfate was added to final concentrations of 1, 10, 25 or 50 mM to 0.02% yeast extract/mineral salts medium in 100 ml shake flasks. Cultures were incubated at 30 °C and growth rates determined by measuring changes in optical densities (at 600 nm) and ferrous iron concentrations.

**Heavy metal tolerance.** Isolates T23<sup>T</sup> and Y005<sup>T</sup> were tested for the abilities to grow and oxidize iron in the presence of elevated concentrations of copper, zinc and ferrous and ferric iron. Concentrations of between 10 and 200 mM of each of these metals were added to standard ferrous iron/yeast extract medium, and bacterial numbers (cell counts using a Thoma counting chamber) and ferrous iron concentrations (using ferrozine) were recorded.

**Autotrophic growth and screening of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) genes.** Isolates T23<sup>T</sup> and Y005<sup>T</sup> were tested for their abilities to grow in 'inorganic' ferrous sulfate medium (i.e. without any added organic material). In

addition, CO<sub>2</sub> assimilation by isolate T23<sup>T</sup> was examined using two variants of a method described by Ghauri & Johnson (1991). In the first of these, isolate T23<sup>T</sup> and the type strain of the autotrophic iron oxidizer *Acidithiobacillus ferrooxidans* (ATCC 23270<sup>T</sup>) as a positive control, were grown in 20 mM ferrous sulfate medium supplemented or not with 0.02 % yeast extract in sealed flasks containing <sup>14</sup>CO<sub>2</sub>, and measurements were made of the amounts of labelled carbon assimilated. In the second experiment, isolate T23<sup>T</sup> was grown to late exponential phase in ferrous sulfate/yeast extract medium, and cells were harvested, washed and resuspended in 20 mM ferrous sulfate medium containing <sup>14</sup>CO<sub>2</sub>. A corresponding culture of *Acidithiobacillus ferrooxidans* grown in 20 mM ferrous sulfate medium was used as a control. The cell numbers in the inoculated cultures were 4.1 × 10<sup>7</sup> ml<sup>-1</sup> (isolate T23<sup>T</sup>) and 1.8 × 10<sup>7</sup> ml<sup>-1</sup> (*Acidithiobacillus ferrooxidans*).

Isolates T23<sup>T</sup> and Y005<sup>T</sup> were screened by PCR for the presence of the RuBisCO large subunit-encoding genes *cbbL* and *cbbM*. Cell lysates, prepared for 16S rRNA gene sequence analysis (see below), were used as templates, and the primers used were those described by Alfreider *et al.* (2003). PCR was carried out using PCR Master Mix (Promega), to which MgCl<sub>2</sub> was added to a final concentration of 3.5 mM for both genes, for 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s followed by 10 min at 72 °C before a final hold at 4 °C. Positive and negative control cultures (*Acidithiobacillus caldus* strain KU<sup>T</sup> and *Acidocella facilis* strain PW2<sup>T</sup>, respectively) and *Acidimicrobium ferrooxidans* (strains ICP<sup>T</sup> and TH3) were tested concurrently.

**Analysis of G+C content of chromosomal DNA.** Cultures grown in 10 mM ferrous iron/0.02 % yeast extract medium were harvested by centrifugation and washed twice with 100 mM oxalic acid to remove ferric iron precipitates. Chromosomal DNA was purified by the technique described by Wilson (1987), and incorporated a caesium chloride gradient centrifugation stage. The DNA extracted from the gradients was dialysed against three changes of 0.1 × SSC (15 mM NaCl plus 1.5 mM Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>), and the ethidium bromide was extracted from the DNA with 0.1 × SSC-saturated butanol. G+C contents were determined from melting temperatures of the DNA, which were determined with an HP 8453A UV-visible spectrophotometer connected to an HP 89090A Peltier temperature controller. The base composition of DNA was determined from its melting point by using the equation  $G + C = 2.44[(T_m - 81.5) - (16.6 \log M)]$ , where  $T_m$  is the melting temperature of the DNA and  $M$  is the molar concentration of the cations in the DNA suspension (Marmur & Doty, 1962).

**Phylogenetic analysis.** Lysates of T23<sup>T</sup> and Y005<sup>T</sup> were prepared by resuspending bacterial pellets in lysis solution (0.05 M NaOH and 0.25 % SDS) and heating to 95 °C for 15 min before diluting tenfold with sterile MilliQ water. The 16S rRNA genes were amplified by PCR using these templates as described previously (Okibe *et al.*, 2003). The resulting products were purified using QIAquick spin columns (Qiagen) and sequenced using a Beckman Coulter dye terminator cycle sequencing kit and a CEQ8000 Genetic Analysis System. These 16S rRNA gene sequences (GenBank accession number AF251436 for T23<sup>T</sup> and AY140237 for Y005<sup>T</sup>) were compared with others in the GenBank database with the BLAST program (Altschul *et al.*, 1997). Sequences from selected micro-organisms were obtained from GenBank and aligned with the T23<sup>T</sup> and Y005<sup>T</sup> gene sequences using ARB (Ludwig *et al.*, 2004). This alignment was corrected manually and phylogenetic trees were constructed by neighbour-joining, DNA parsimony and maximum-likelihood algorithms in ARB. Each of the three methods gave trees of similar topology. The reliability of the phylogenetic relationships inferred was tested by bootstrap analysis on 1000 samples (Felsenstein, 1985), which yielded values better than 70 % in all cases.

**Chemotaxonomic analysis.** Cultures of T23<sup>T</sup> and Y005<sup>T</sup> were grown at 30 or 37 °C, respectively, in a liquid medium at pH 2

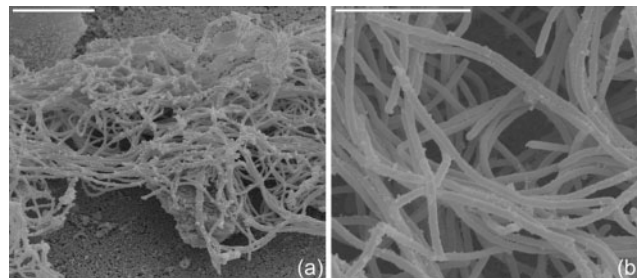
containing 10 mM ferrous sulfate, 5 mM glycerol and 0.02 % yeast extract. Biomass was obtained by centrifugation and was sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany, for determination of fatty acids and structural analysis of the peptidoglycan of these bacteria. In addition, respiratory quinones were extracted from freeze-dried biomass following method three described by DiSpirito *et al.* (1983). The extracted quinones were subjected to HPLC analysis on a Dionex Acclaim 120 C18 reverse phase column (150 × 4.6 mm) with a solvent of 80 parts methanol and 20 parts 2-propanol at a flow rate of 1 ml min<sup>-1</sup>. Quinones were detected by UV absorption at 248 nm for menaquinones and 275 nm for ubiquinones. Standards included menaquinone MK-6 and ubiquinone Q10 from Sigma-Aldrich, as well as those extracted from well-characterized bacterial strains. To confirm the identity of the menaquinones, the extracts were also subjected to mass spectrometry analysis using a Bruker high-resolution time of flight spectrometer.

## RESULTS

### Growth on solid and in liquid media

Colonies of T23<sup>T</sup> displayed a 'fried egg'-like morphology on Feo solid medium, and grew poorly on both FeSo and iFeo solid media. Cells were rod-shaped, 1–3 µm long by 0.5 µm wide and motile, and did not produce endospores. Chains of three to five cells were common during exponential growth. Colonies of Y005<sup>T</sup> on Feo solid medium contrasted with T23<sup>T</sup> in being small, iron-encrusted but not gelatinous, and having a distinct rhizoidal morphology. Similar morphologies of Y005<sup>T</sup> colonies were observed on FeSo medium and on iFeo medium, though they were much smaller on the latter.

In ferrous iron-containing liquid media, cultures of T23<sup>T</sup> became increasingly turbid and orange-coloured (due to the accumulation of ferric iron) during incubation. In contrast, although colour change could also be used to indicate iron oxidation in cultures of Y005<sup>T</sup>, the cultures did not become turbid, as the bacteria grew as visible flocs approx. 1–3 mm in diameter. Microscopic examination showed that isolate Y005<sup>T</sup> grew as long entangled filaments (Fig. 1). Occasionally single cells were observed; these were 3–4 µm long and approx. 0.5 µm wide. Cells were non-motile and were not observed to form endospores.



**Fig. 1.** Scanning electron micrographs of (a) a Y005<sup>T</sup> floc and (b) Y005<sup>T</sup> filaments. Bars, 10 µm (a); 5 µm (b).

The pH optimum and minimum for growth of isolate T23<sup>T</sup> were 2.0 and 1.4, respectively, and its temperature optimum was 35 °C. Isolate T23<sup>T</sup> grew at 37 °C, but not at 45 °C. In the case of isolate Y005<sup>T</sup>, the pH growth optimum was 1.8 and minimum pH 1.6. Its temperature optimum and maxima were 43 °C and 50 °C, respectively. Under optimum conditions of pH and temperature, culture doubling times of isolate T23<sup>T</sup> and Y005<sup>T</sup> were 6.3 and 3.0 h, respectively.

### Oxidation of iron, sulfur and pyrite

Both isolates T23<sup>T</sup> and Y005<sup>T</sup> catalysed the dissimilatory oxidation of ferrous iron, but not of elemental sulfur (data not shown). Pyrite was oxidized by both isolates, but only in media amended with yeast extract (Supplementary Fig. S1, available in IJSEM Online). Even in yeast extract-containing medium pyrite oxidation by T23<sup>T</sup> was much slower than that by the chemolithotrophic acidophile *Acidithiobacillus ferrooxidans* (Supplementary Fig. S1a).

### Utilization of organic substrates

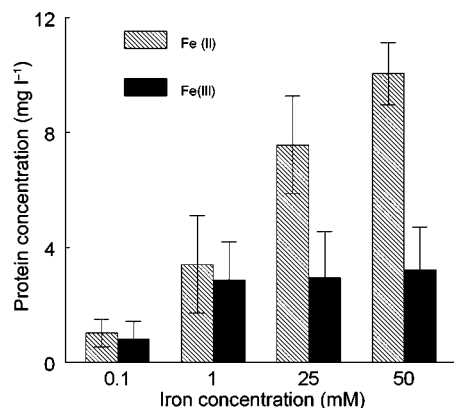
Both isolates T23<sup>T</sup> and Y005<sup>T</sup> displayed limited growth on single organic substrates. In both cases, none of the sugars tested were utilized by the bacteria. Growth yields of isolate T23<sup>T</sup> were greater in media containing glycerol, citric acid and glutamic acid than in control cultures, while only glycerol and ethanol, among the potential substrates tested, were used by isolate Y005<sup>T</sup>. Acetic acid was highly toxic to both bacteria, inhibiting the growth of T23<sup>T</sup> at 1 mM and that of Y005<sup>T</sup> at 100 µM. Isolate T23<sup>T</sup> grew in 5 mM glycerol/100 µM ferrous sulfate liquid medium, confirming that it is not auxotrophic. However, attempts to grow isolate Y005<sup>T</sup> in yeast extract-free media were unsuccessful, indicating that it has a requirement for one or more growth factors.

### Ferric iron reduction

Both isolates T23<sup>T</sup> and Y005<sup>T</sup> were able to reduce ferric iron under anaerobic conditions, using glycerol as an electron donor (Supplementary Fig. S2, available in IJSEM Online). No abiotic iron reduction occurred in uninoculated controls.

### Effect of iron concentrations on biomass yields and growth rates

Growth yields of isolate T23<sup>T</sup> in cultures containing the same amount of yeast extract but different concentrations of either ferrous or ferric iron are shown in Fig. 2. Biomass yields were similar, irrespective of the form of iron provided, when iron concentrations were low (0.1 and 1.0 mM), but at higher concentrations (25 and 50 mM) yields were significantly greater ( $P < 0.001$ ) when ferrous rather than ferric iron was added to cultures. No correlations between yields of T23<sup>T</sup> and ferrous iron concentrations were found when yeast extract was present at low (0.001–0.002 %, w/v) concentrations, presumably because cultures were carbon limited rather than energy-



**Fig. 2.** Effect of different concentration of ferrous and ferric iron on the growth yields of isolate T23<sup>T</sup>, grown in the presence of 0.02 % (w/v) yeast extract ( $n=3$  for each treatment).

limited under such conditions (data not shown). Growth rates of isolate T23<sup>T</sup> were much faster (0.081–0.095 h<sup>-1</sup>) in liquid media that contained 10–50 mM ferrous iron than in corresponding cultures that contained either ferric iron (at 1–50 mM) or 1 mM ferrous iron (0.049–0.055 h<sup>-1</sup>). No comparable data were obtained for isolate Y005<sup>T</sup>.

### Heavy metal tolerance

Both Gram-positive isolates were able to grow in media containing elevated concentrations of the heavy metals tested. Isolate T23<sup>T</sup> grew in 200 mM of both ferrous and ferric iron. Growth also occurred in the presence of 150 mM (but not 200 mM) copper, but zinc was more toxic (50 mM inhibited growth). Isolate Y005<sup>T</sup> grew in 200 mM concentrations of all the heavy metals tested, except ferric iron which inhibited growth at 100 mM.

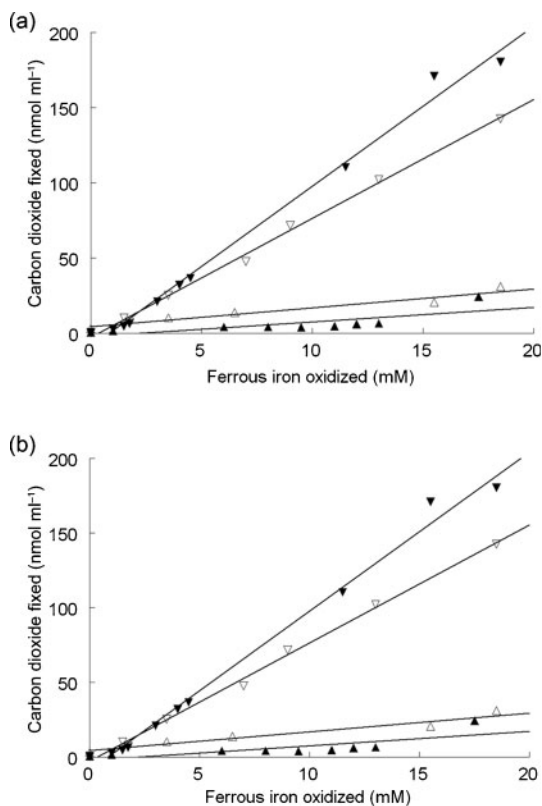
### CO<sub>2</sub> fixation

Neither T23<sup>T</sup> nor Y005<sup>T</sup> was able to grow in organic carbon-free liquid media. While there was limited oxidation of ferrous iron in the first transfer of isolates into ferrous sulfate medium, no increase in cell numbers was recorded. In contrast to *Acidithiobacillus ferrooxidans*, no assimilation of CO<sub>2</sub> was observed in cultures of isolate T23<sup>T</sup> (Fig. 3).

No RuBisCO genes were amplified from cell lysates of either T23<sup>T</sup> or Y005<sup>T</sup>, though both *cbbL* and *cbbM* genes were successfully amplified from *Acidithiobacillus caldus* lysates, and neither from *Acidocella facilis*. Only *cbbL* genes were amplified successfully from both strains of *Acidimicrobium ferrooxidans*.

### Genotypic traits

The G + C contents of genomic DNA were determined as 54.9 ± 0.3 mol% for T23<sup>T</sup> and 50.2 ± 0.8 mol% for Y005<sup>T</sup>. The published value for *Acidimicrobium ferrooxidans* is 67–69 mol% (Clark & Norris, 1996).

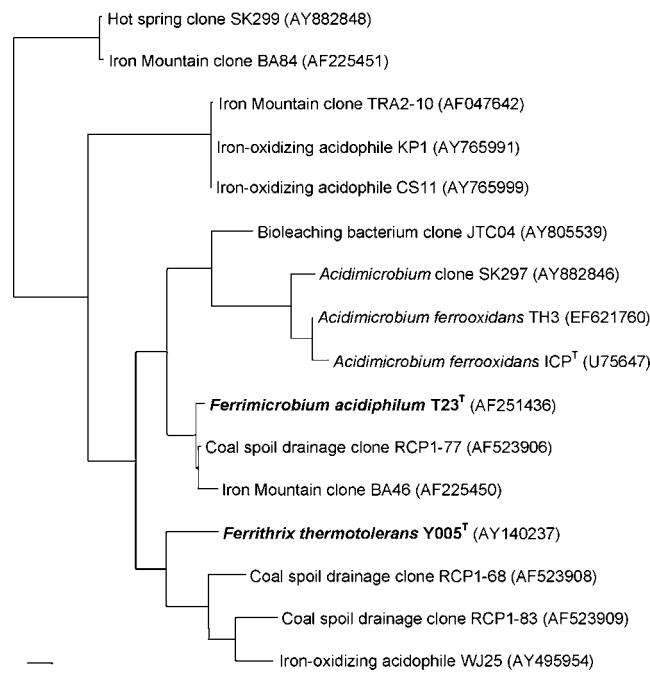


**Fig. 3.** Comparison of CO<sub>2</sub> uptake by isolate T23<sup>T</sup> (▲, △) and the mesophilic chemoautotroph *Acidithiobacillus ferrooxidans* ATCC 23270<sup>T</sup> (▼, ▽). Data shown are from: (a) batch cultures in 20 mM ferrous iron medium (with or without 0.02 % w/v yeast extract); (b) by harvested cells. Solid symbols show data from cultures amended with 0.02 % (w/v) yeast extract, and hollow symbols in yeast extract-free medium.

The 16S rRNA gene sequences of T23<sup>T</sup> and Y005<sup>T</sup> have all the signature nucleotides and nucleotide pairs to indicate that they belong to the family *Acidimicrobiaceae*, as proposed by Stackebrandt *et al.* (1997). The 16S rRNA gene sequences from T23<sup>T</sup> and Y005<sup>T</sup> are 94.2 % identical to each other, and are 92.9 and 90.9 % identical to that from *Acidimicrobium ferrooxidans*, respectively. Phylogenetic analyses placed these two bacteria in a group that includes other iron-oxidizing acidophiles, including the type genus of the family, *Acidimicrobium*, and isolates CS1, KP1 and WJ25 obtained from acid mine drainage sites in the UK (Fig. 4). In addition, this group includes bacteria that have been detected by molecular means in acid mine drainage environments or mineral leaching bioreactors (Fig. 4).

### Chemotaxonomic traits

Both isolates T23<sup>T</sup> and Y005<sup>T</sup> contained *meso*-diaminopimelic acid in the peptidoglycan, and the peptidoglycan type was A1 $\gamma$  (Schleifer & Kandler, 1972). The dominant fatty



**Fig. 4.** Maximum-likelihood tree showing the relationship of *Ferrimicrobium acidiphilum* T23<sup>T</sup> and *Ferrithrix thermotolerans* Y005<sup>T</sup> (in bold) with other actinobacteria, including those detected in acidic sites by molecular means. The tree was rooted with the 16S rRNA gene sequence from *Acidimicrobidae* bacterium Ellin7143 (GenBank accession number AY673309). GenBank accession numbers for other actinobacteria are given in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

acid of isolate T23<sup>T</sup> was *i*-C<sub>16:0</sub> (64.23 %); other fatty acids were *i*-C<sub>14:0</sub> (10.83 %), C<sub>14:0</sub> (0.24 %), *i*-C<sub>15:0</sub> (5.03 %), *ai*-C<sub>15:0</sub> (5.14 %), C<sub>15:0</sub> (0.32 %), *i*-C<sub>14:0</sub>3-OH (1.20 %), *i*-C<sub>16:1</sub>H (2.49 %), C<sub>16:0</sub> (0.48 %), *ai*-C<sub>17:1</sub> $\omega$ 9c (0.19 %), *i*-C<sub>17:0</sub> (0.39 %), *ai*-C<sub>17:0</sub> (3.18 %), C<sub>17:1</sub> $\omega$ 6c (5.97 %), and *i*-C<sub>18:0</sub> (0.30 %). Y005<sup>T</sup> shared the same dominant fatty acid as T23<sup>T</sup>, namely *i*-C<sub>16:0</sub> (90.13 %), but differed in the composition of the minor fatty acids, which included C<sub>12:0</sub> (0.16 %), *i*-C<sub>14:0</sub> (0.54 %), *i*-C<sub>15:0</sub> (1.30 %), *ai*-C<sub>15:0</sub> (0.73 %), C<sub>15:0</sub> (0.88 %), *i*-C<sub>14:0</sub>3-OH (0.51 %), *i*-C<sub>16:1</sub>H (1.45 %), C<sub>16:0</sub> (0.63 %), *ai*-C<sub>17:0</sub> (0.99 %), C<sub>18:1</sub> $\omega$ 9c (0.19 %), C<sub>18:1</sub> $\omega$ 7c (0.25 %), and C<sub>18:0</sub> (0.34 %). The major respiratory quinone of T23<sup>T</sup> was a menaquinone with eight isoprenoid side chains, five of which were saturated [e.g. MK-8(H10)]. In addition, small amounts of MK-8(H10) with one or two methyl groups were also detected, along with a trace of MK-8. Attempts to extract and identify respiratory quinones from Y005<sup>T</sup> by the authors and by staff at the DSMZ were all unsuccessful.

### DISCUSSION

The two bacteria described have many characteristics in common with the current sole cultivated species of the

*Acidimicrobidae* (*Acidimicrobium ferrooxidans*), most notably that they are all obligately acidophilic bacteria that catalyse the dissimilatory oxidation of ferrous iron and reduction of ferric iron. Key differences are that both T23<sup>T</sup> and Y005<sup>T</sup> are obligate heterotrophs, while *Acidimicrobium ferrooxidans* can fix CO<sub>2</sub>, albeit relatively inefficiently compared with other acidophiles such as *Acidithiobacillus ferrooxidans*. Another physiological trait that differentiates T23<sup>T</sup> from both *Acidimicrobium ferrooxidans* and Y005<sup>T</sup> is that the former is mesophilic while the latter are both moderate thermophiles. In terms of nutrition, both T23<sup>T</sup> and Y005<sup>T</sup> can grow using some defined organic carbon compounds, such as glycerol, as sole carbon and energy sources [though the latter requires some unidentified growth factor(s)]; heterotrophic growth of *Acidimicrobium ferrooxidans* on yeast extract has also been reported (Clark & Norris, 1996). Isolate T23<sup>T</sup>, like *Acidimicrobium ferrooxidans*, can utilize the energy from oxidizing ferrous iron, though this was not ascertained in the case of Y005<sup>T</sup>. Given the phenotypic and phylogenetic differences of these two isolates to each other, and also to *Acidimicrobium ferrooxidans*, it is apparent that they represent species of two novel genera, and the names *Ferrimicrobium acidiphilum* gen. nov., sp. nov. and *Ferrithrix thermotolerans* gen. nov., sp. nov. are proposed for isolates T23<sup>T</sup> and Y005<sup>T</sup>, respectively.

Bacteria closely related to *Ferrimicrobium acidiphilum* appear to have worldwide distribution. Besides the isolates referred to by Johnson & Roberto (1997), BLAST searches show that similar clones have been detected in mine waters in China and Iron Mountain, California, USA. DGGE bands of amplified 16S rRNA genes from mine waters draining coal spoil and the Río Tinto, Spain (Gonzalez-Toril *et al.*, 2003) have also been identified as *Ferrimicrobium acidiphilum*-like bacteria. In contrast, *Ferrithrix thermotolerans* has not yet been detected outside of geothermal regions of Yellowstone National Park. The closest (95.4% identity) 16S rRNA gene to that of *Ferrithrix thermotolerans* is of a bacterium (WJ25) isolated from mine water in Cornwall, England, UK (Hallberg & Johnson, 2005).

Other bacterial isolates from acidic, metal-rich environments identified as *Acidimicrobidae* have been described, though not fully characterized (e.g. Hallberg *et al.*, 2006), which appear (on the basis of 16S rRNA gene identities) to be distinct from both novel species described here, and also from *Acidimicrobium ferrooxidans*. Some of these isolates appeared to be morphologically and physiologically similar to isolate 'CCH7' (Johnson *et al.*, 1992), which was not sequenced at the time, and was subsequently lost. Interestingly, one of the characteristics described for CCH7 was its tendency to grow as extended filaments, forming macroscopic flocs and streamers in liquid culture, somewhat similar to *Ferrithrix thermotolerans*. The unclassified isolates described by Hallberg *et al.* (2006) were also acidophilic iron oxidizers. The *Acidimicrobidae* clones described by Montalvo *et al.* (2005) were obtained from

marine sponges, where neither extremely low pH nor elevated ferrous iron would be anticipated. The intriguing prospect of novel *Acidimicrobidae* with very contrasting physiological traits to those described to date awaits the successful isolation of bacteria represented by these clones.

### Description of *Ferrimicrobium* gen. nov.

*Ferrimicrobium* (Fer.ri.mi.cro'bi.um. L. neut. n. *ferrum* iron; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Ferrimicrobium* iron microbe).

Cells are motile rods, Gram-negative, and do not form spores. Acidophilic, mesophilic and obligately heterotrophic. Capable of oxidation and reduction of iron and oxidation of pyrite. Grows with yeast extract or a limited range of organic compounds. Sequence analysis of the 16S rRNA gene places the genus within the subclass of *Acidimicrobidae* in the *Actinobacteria*. The peptidoglycan type is A1 $\gamma$ . The major fatty acids are i-C<sub>16:0</sub>, i-C<sub>14:0</sub>, i-C<sub>15:0</sub>, ai-C<sub>15:0</sub> and C<sub>17:1</sub> $\omega$ 6c. The dominant menaquinone is MK-8(H10), with minor amounts of MK-8 and MK-8(H10) containing one or two methyl groups also detected. The G+C content of the genomic DNA is approx. 55 mol%. The type species is *Ferrimicrobium acidiphilum*.

### Description of *Ferrimicrobium acidiphilum* sp. nov.

*Ferrimicrobium acidiphilum* (a.ci.di'phi.lum. L. adj. *acidus* sour; N.L. neut. n. *acidum* acid; Gr. adj. *philos* loving; N.L. neut. adj. *acidiphilum* acid-loving).

Cells are rod-shaped, 1–3  $\mu$ m in length and 0.5  $\mu$ m in diameter, motile and do not form endospores. Gram-negative. Form gelatinous colonies of approx. 1–3 mm diameter, and with ferric iron deposits in centre on ferrous iron overlay plates. Mesophilic, optimum growth at 35 °C (maximum 37 °C) and acidophilic, optimum growth at pH 2 (minimum pH 1.4). Obligately heterotrophic; growth on yeast extract and a limited range of defined organic substrates including glycerol, citric acid and glutamic acid. Capable of oxidation of ferrous iron and pyrite, and reduction of ferric iron only in the presence of organic carbon. The dominant menaquinone is MK-8 (H10), with minor amounts of MK-8 and MK-8(H10) containing one or two methyl groups also present. The major fatty acids are i-C<sub>16:0</sub>, i-C<sub>14:0</sub>, i-C<sub>15:0</sub>, ai-C<sub>15:0</sub>, and C<sub>17:1</sub> $\omega$ 6c, with other minor fatty acids. The peptidoglycan type is A1 $\gamma$ . Chromosomal DNA base composition is 54.9  $\pm$  0.3 mol% G+C. Sequencing of the 16S rRNA gene (GenBank accession number AF251436) indicates that *Ferrimicrobium acidiphilum* belongs to the class *Actinobacteria* and subclass *Acidimicrobidae*. 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 T23<sup>T</sup> (=DSM 19497<sup>T</sup>=ATCC BAA-1647<sup>T</sup>).

### Description of *Ferrithrix* gen. nov.

*Ferrithrix* (Fer.ri.thr'ix. L. neut. n. *ferrum* iron; Gr. fem. n. *thrix* hair, thread; N.L. fem. n. *Ferrithrix* iron thread).

Grows as filaments (occasionally as single rods), Gram-negative, and does not form spores. Acidophilic, moderately thermophilic, and obligately heterotrophic. Capable of oxidation and reduction of iron. Grows on yeast extract, glycerol or ethanol. A growth factor (or factors), obtained from yeast extract is (are) required for growth. Sequence analysis of the 16S rRNA gene places the genus within the subclass of *Acidimicrobidae* in the *Actinobacteria*. The major fatty acid is i-C<sub>16:0</sub>. The peptidoglycan type is A1 $\gamma$ . The G+C content of the genomic DNA is approx. 50 mol%. The type species is *Ferrithrix thermotolerans*.

### Description of *Ferrithrix thermotolerans* sp. nov.

*Ferrithrix thermotolerans* (ther.mo.to'le.rans. Gr. n. *thermè* heat; L. part. adj. *tolerans* tolerating; N.L. part. adj. *thermo-tolerans* heat-tolerating, able to tolerate high temperatures).

Generally grows as filaments forming visible flocs (approx. 1–3 mm in diameter), though occasionally single, rod-shaped cells are observed (3–4  $\mu$ m in length and 0.5  $\mu$ m in diameter). Non-motile and does not form endospores. Gram-negative. Moderately thermophilic, optimal growth at 43 °C (maximum 50 °C) and acidophilic, optimal growth at pH 1.8 (minimum pH 1.6). Colonies are small (1–2 mm in diameter), rhizoidal and iron-encrusted in ferrous iron, ferrous iron/tetrathionate and inorganic iron solid overlay media. Obligately heterotrophic, capable of growth with yeast extract, glycerol and ethanol. An unknown growth factor(s) supplied by yeast extract is (are) required for growth. Also able to oxidize and reduce iron. The major fatty acid is i-C<sub>16:0</sub>, with other fatty acids present in minor amounts. The peptidoglycan type is A1 $\gamma$ . The G+C content of the chromosomal DNA is 50.2  $\pm$  0.8 mol%. Found in geothermal springs. Sequencing of the 16S rRNA gene (GenBank accession number AY140237) indicates that *Ferrithrix thermotolerans* belongs to the class *Actinobacteria* and subclass *Acidimicrobidae*.

The type strain is strain Y005<sup>T</sup> (=DSM 19514<sup>T</sup>=ATCC BAA-1645<sup>T</sup>).

### ACKNOWLEDGEMENTS

This work was carried out (in part) in the frame of BioMinE (European project contract NMP1-CT-500329-1). The authors acknowledge financial support given to this project by the European Commission under the Sixth Framework Programme for Research and Development. D.B.J. is grateful to the Royal Society (UK) for the provision of an Industrial Fellowship. We would also like to thank Dr Jean Euzéby (École Nationale Vétérinaire, Toulouse, France) for his expert advice on bacterial nomenclature, and Dr Barry

Grail and Mr Denis Williams (Bangor University, Bangor, UK) for their help with mass spectrometry.

### REFERENCES

- Alfreider, A., Vogt, C., Hoffman, D. & Babel, W. (2003). Diversity of ribulose 1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microb Ecol* **45**, 317–328.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bacelar-Nicolau, P. & Johnson, D. B. (1999). Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. *Appl Environ Microbiol* **65**, 585–590.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brierley, J. A. (1978). Thermophilic iron-oxidizing bacteria found in copper leaching dumps. *Appl Environ Microbiol* **36**, 523–525.
- Clark, D. A. & Norris, P. R. (1996). *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology* **142**, 785–790.
- DiSpirito, A. A., Loh, H. T. & Tuovinen, O. H. (1983). A novel method for the isolation of bacterial quinones and its application to appraise the ubiquinone composition of *Thiobacillus ferrooxidans*. *Arch Microbiol* **135**, 77–80.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Ghauri, M. A. & Johnson, D. B. (1991). Physiological diversity amongst some moderately thermophilic iron-oxidising bacteria. *FEMS Microbiol Ecol* **85**, 327–334.
- Gonzalez-Toril, E., Llobet-Brossa, E., Casamayor, E. O., Amann, R. & Amils, R. (2003). Microbial ecology of an extreme acidic environment, the Tinto River. *Appl Environ Microbiol* **69**, 4853–4865.
- Hallberg, K. B. & Johnson, D. B. (2005). Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine. *Sci Total Environ* **338**, 53–66.
- Hallberg, K. B., Coupland, K., Kimura, S. & Johnson, D. B. (2006). Macroscopic “acid streamer” growths in acidic, metal-rich mine waters in north Wales consist of novel and remarkably simple bacterial communities. *Appl Environ Microbiol* **72**, 2022–2030.
- Jenkins, D. A. & Johnson, D. B. (1993). Abandoned metal mines: a unique mineralogical and microbiological resource. *J Russell Soc* **5**, 40–44.
- Johnson, D. B. & Hallberg, K. B. (2007). Techniques for detecting and identifying acidophilic mineral-oxidising microorganisms. In *Bio-mining*, pp. 237–261. Edited by D. E. Rawlings & D. B. Johnson. Heidelberg: Springer.
- Johnson, D. B. & Roberto, F. F. (1997). Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals. In *Bio-mining: Theory, Microbes and Industrial Processes*, pp. 259–280. Edited by D. E. Rawlings. Berlin, New York: Springer.
- Johnson, D. B., Ghauri, M. A. & Said, M. F. (1992). Isolation and characterisation of an acidophilic heterotrophic bacterium capable of oxidizing ferrous iron. *Appl Environ Microbiol* **58**, 1423–1428.
- Johnson, D. B., Okibe, N. & Roberto, F. F. (2003). Novel thermo-acidophiles isolated from geothermal sites in Yellowstone National Park: physiological and phylogenetic characteristics. *Arch Microbiol* **180**, 60–68.

- Lovley, D. R. & Phillips, E. J. P. (1987).** Rapid assay for microbially reducible ferric iron in aquatic sediments. *Appl Environ Microbiol* **53**, 1536–1540.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004).** ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Marmur, J. & Doty, P. (1962).** Determination of base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Montalvo, N. F., Mohamed, M. N., Enticknap, J. J. & Hill, R. T. (2005).** Novel actinobacteria from marine sponges. *Antonie van Leeuwenhoek* **87**, 29–36.
- Okibe, N., Gericke, M., Hallberg, K. B. & Johnson, D. B. (2003).** Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred tank bioleaching operation. *Appl Environ Microbiol* **69**, 1936–1943.
- Schleifer, K. H. & Kandler, O. (1972).** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997).** Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Wilson, K. (1987).** Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Green Publishing & Wiley-Interscience.