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# Schwertmannite formation at cell junctions by a new filament-forming Fe(II)-oxidizing isolate affiliated with the novel genus *Acidithrix*

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A new acidophilic iron-oxidizing strain (C25) belonging to the novel genus *Acidithrix* was isolated from pelagic iron-rich aggregates ('iron snow') collected below the redoxcline of an acidic lignite mine lake. Strain C25 catalysed the oxidation of ferrous iron [Fe(II)] under oxic conditions at 25 °C at a rate of 3.8 mM Fe(II) day<sup>−1</sup> in synthetic medium and 3.0 mM Fe(II) day<sup>−1</sup> in sterilized lake water in the presence of yeast extract, producing the rust-coloured, poorly crystalline mineral schwertmannite [Fe(III) oxyhydroxylsulfate]. During growth, rod-shaped cells of strain C25 formed long filaments, and then aggregated and degraded into shorter fragments, building large cell–mineral aggregates in the late stationary phase. Scanning electron microscopy analysis of cells during the early growth phase revealed that Fe(III)-minerals were formed as single needles on the cell surface, whereas the typical pincushion-like schwertmannite was observed during later growth phases at junctions between the cells, leaving major parts of the cell not encrusted. This directed mechanism of biomineralization at specific locations on the cell surface has not been reported from other acidophilic iron-oxidizing bacteria. Strain C25 was also capable of reducing Fe(III) under micro-oxic conditions which led to a dissolution of the Fe(III)-minerals. Thus, strain C25 appeared to have ecological relevance both for the formation and transformation of the pelagic iron-rich aggregates at oxic/anoxic transition zones in the acidic lignite mine lake.

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**Abbreviations:** ARDRA, amplified rDNA restriction analysis; EPS, extracellular polymeric substance; FeOB, iron-oxidizing bacteria; SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of *Acidithrix ferrooxidans* strain C25 is LN866582.

One supplementary figure is available with the online Supplementary Material.

## INTRODUCTION

Iron-oxidizing bacteria (FeOB) mediate the oxidation of ferrous iron [Fe(II)] to ferric iron [Fe(III)] to conserve energy for growth (Colmer & Hinkle, 1947). Biogenic Fe(III) subsequently hydrolyses and precipitates from solution forming various Fe(III) oxides when the pH exceeds 2 (Kappler & Straub, 2005). Due to the low rates of chemical Fe(II) oxidation under acidic conditions, acidophilic aerobic FeOB are the main drivers for Fe(II) oxidation, especially in acid mine drainage impacted sites (e.g. Hallberg *et al.*, 2006; Leduc & Ferroni, 1994; López-Archilla *et al.*, 2001; Tyson *et al.*, 2004). When the pH increases, FeOB face the problem of disposing their poorly soluble

metabolic products. Some acidophilic FeOB, such as *Acidithiobacillus* spp., *Ferroplasma* spp. and *Leptospirillum* spp., form macroscopic biofilms composed of cells and extracellular polymeric substances (EPSs) that can serve as nucleation sites for mineral precipitation (Bond *et al.*, 2000; Hallberg *et al.*, 2006; Kay *et al.*, 2013; Wakao *et al.*, 1985). This mechanism also prevents the bacterial cells from being encrusted with insoluble Fe(III) oxides which can ultimately lead to cell death due to the inhibition of nutrient supply and metabolite efflux (Hedrich *et al.*, 2011; Neubauer *et al.*, 2002). Thus, EPS formation allows many acidophilic FeOB to contact Fe(II) in the water phase and to proceed with Fe(II) oxidation (Hedrich *et al.*, 2011).

Microbially mediated iron precipitation is the dominant process in acidic lakes located in areas with extensive surface lignite mining activities (Falagán *et al.*, 2014; Reiche *et al.*, 2011). These mine lakes were formed by groundwater inflow, drainage and rainfall accumulation after closing open cast mines (Geller *et al.*, 1998; Klapper & Schultze, 1995; Nixdorf *et al.*, 2001). In these mine lakes, poorly crystalline Fe(III) oxyhydroxysulfate minerals precipitate due to the high concentrations of sulfate released during the oxidation of pyrite in the surrounding mine tailings (Küsel, 2003). The preferred location for mineral formation in these mine lakes is the redoxcline where opposing gradients of oxygen and Fe(II) provide optimal conditions for both aerobic and microaerophilic acidophilic FeOB (Reiche *et al.*, 2011). Schwertmannite (ideal formula:  $\text{Fe}_8\text{O}_8\text{SO}_4(\text{OH})_{6.7}\text{H}_2\text{O}$ ) is characterized by its pincushion-like morphology with a large specific surface area; thus, schwertmannite appears to be the nucleus for pelagic aggregate formation by adsorption and co-precipitation of organic matter, rapid microbial colonization, and EPS formation (Hedrich *et al.*, 2011; Paikaray *et al.*, 2011). These structures have been called 'iron snow' (Reiche *et al.*, 2011) as an analogue to the more organically rich snow-like aggregates known to form in marine and freshwater habitats (Grosart & Ploug, 2000; Luef *et al.*, 2007; Ploug *et al.*, 1999).

Molecular microbiological analyses of the iron snow revealed a predominance of chemoautotrophic FeOB, such as *Acidimicrobium*-related group, *Ferroplasma*, *Acidithiobacillus*, and *Thiobacillus*, and heterotrophic Fe(III)-reducing bacteria, such as *Acidiphilium*, *Albidiferax*-like and *Geobacter*-like groups (Lu *et al.*, 2013). Furthermore, RNA-based quantitative PCR and metaproteomic analysis have shown that the actinobacterial *Acidimicrobium*-like group is an abundant metabolically active group in acidic, iron snow-rich lakes (Lu *et al.*, 2013). *Acidimicrobium*-related bacteria have also been detected in many other acidic environments (Brown *et al.*, 2011; García-Moyano *et al.*, 2012; Kay *et al.*, 2013; López-Archilla *et al.*, 2004; Méndez-García *et al.*, 2014; Tyson *et al.*, 2004) where they may contribute to primary production and Fe(II) oxidization. Some isolates have been studied in detail and one isolate obtained from an abandoned copper mine in north Wales was shown to be the

representative of a novel genus (Hallberg *et al.*, 2006; Jones & Johnson, 2015). This new type strain, named *Acidithrix ferrooxidans* strain PY-F3<sup>T</sup>, forms long filaments, and also is able to reduce Fe(III) under micro-oxic and anoxic conditions.

To better understand microbially mediated processes during the formation and transformation of iron snow, we aimed to isolate representatives from the dominant groups and sampled iron snow below the redoxcline of a mine lake. In this study, we present the isolation and characterization of an *Acidithrix*-like strain that is able to oxidize Fe(II) at pH 2.5 in lake water. We elucidated the mechanisms of biomineralization using different microscopic and spectroscopic techniques that suggest a novel approach of acidophilic FeOB to initiate mineral formation at specific locations on the cell surface.

## METHODS

**Sampling site and isolation procedure.** The acidic lignite mine lake 77 is located in the Lusatian mining area in Germany. The central basin (maximum depth 7 m, 51° 31' 8.2" N, 13° 41' 34.7" E) shows a dimictic stratification pattern with typical spring and fall mixes. High amounts of pelagic iron-rich aggregates (iron snow) form within the redoxcline due to the steep opposing gradients of oxygen and Fe(II). The iron snow is highly colonized by microbial cells (Lu *et al.*, 2013; Reiche *et al.*, 2011) and contains a high iron fraction, primarily in the form of schwertmannite (>91% of mineral content) (Ciobotă *et al.*, 2013). In September 2012, 4 l of water samples were collected right below the redoxcline at 5 m depth using a water sampler (55 cm length) based on the Ruttner design and transported to the laboratory at 4 °C. The position of the redoxcline was determined by depth-dependent measurements of temperature, pH and oxygen content using a multiparameter U-10 water quality checker (Horiba). For microcosm incubation experiments, lake water was filter-sterilized (pore size 0.2 µm; Pall Corporation).

Lake water was sequentially diluted ( $10^0$  to  $10^{-3}$ ) with filter-sterilized lake water and 100 µl of each dilution was plated onto double-layer 'FeO' medium (final pH 2.5) which contained 25 mM  $\text{FeSO}_4$  and 0.25% Tryptone Soy Broth (TSB; Oxoid) as carbon source (Johnson & Hallberg, 2007; Lu *et al.*, 2010). Cycloheximide (Carl Roth) was added into the top-layer medium at  $50 \mu\text{g ml}^{-1}$  to inhibit fungal growth. All plates were incubated at ~20 °C in the dark, and checked frequently for colony formation and appearance of an iron rust colour indicative for  $\text{FeSO}_4$  oxidation. Colonies were transferred at least five times, and purity was controlled both by colony morphology assessment and microscopic examination (Axiovert 25; Carl Zeiss). Purified colonies were also transferred into FeO liquid medium, and tested for growth at 25 °C and pH 2.5 in artificial pilot-plant water (APPW) medium that contained 25 mM  $\text{FeSO}_4$ , 0.022 g  $\text{Na}_2\text{SO}_4 \text{ l}^{-1}$ , 0.024 g  $\text{K}_2\text{SO}_4 \text{ l}^{-1}$ , 3.24 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O l}^{-1}$ , 0.515 g  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O l}^{-1}$ , 0.058 g  $\text{NaHCO}_3 \text{ l}^{-1}$ , 0.010 g  $\text{NH}_4\text{Cl l}^{-1}$ , 0.014 g  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O l}^{-1}$ , 0.023 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O l}^{-1}$  and 0.0004 g  $\text{ZnCl}_2 \text{ l}^{-1}$  (Tischler *et al.*, 2013), and supplemented with yeast extract (YE; 0.2 g  $\text{l}^{-1}$ ).

The genomic DNA was purified and used for phylogenetic analysis of all isolates. In brief, cell colonies were suspended into 20 µl lysis solution containing 0.05 M NaOH and 0.25% SDS, and then boiled for 15 min at 95 °C, followed by centrifugation at 4000 g for 5 min. An aliquot of 1 µl supernatant was used for bacterial 16S rRNA gene PCR using primer set 27F/1492R (Lane, 1991). The amplicons were purified using a spin column (NucleoSpin Gel and PCR Clean-up;



Macherey-Nagel) and were screened by amplified rDNA restriction analysis (ARDRA) with restriction enzymes *Hha*I and *Bsu*RI (*Hae*III) (Fermentas). Representative sequences were chosen for sequencing (Macrogen). The raw sequences were processed in Geneious 4.6.1 (Biomatters) for trimming and assembling, followed by BLAST homology search (Johnson *et al.*, 2008) and phylogenetic characterization by ARB 6.0.2 (Ludwig *et al.*, 2004).

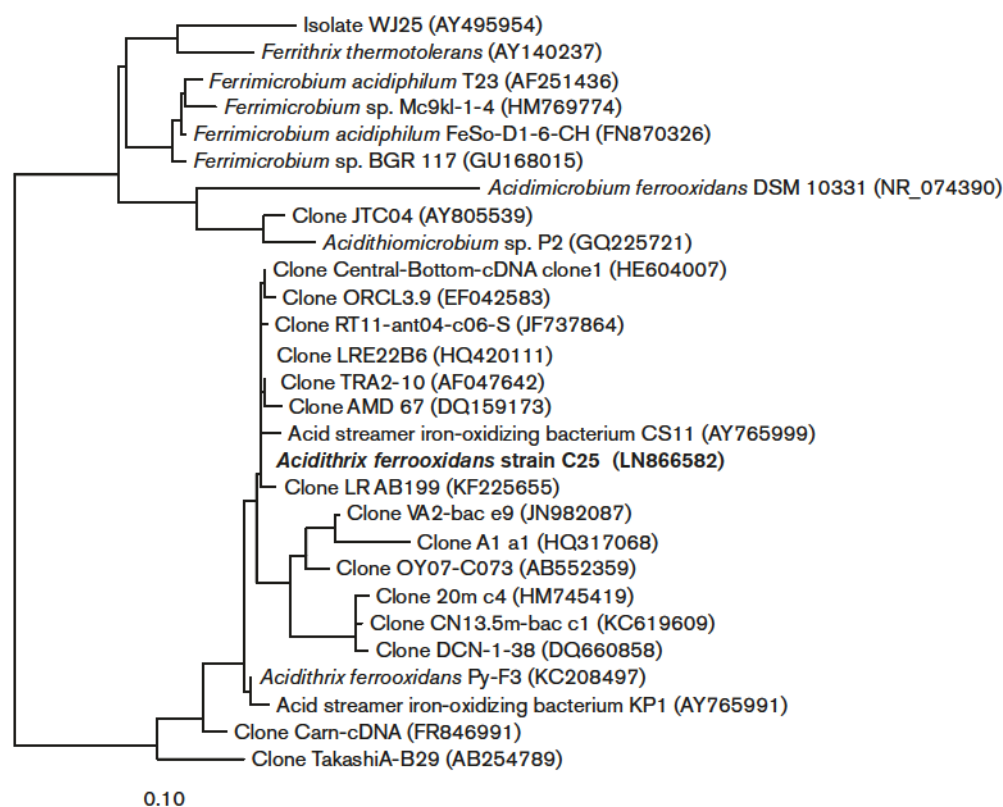
**Characterization of strain C25.** Growth of strain C25 was tested in APPW + YE medium at different temperatures and initial pH values. Rates of Fe(II) oxidation were determined both in APPW + YE liquid medium and in filter-sterilized lake water collected in June 2014 from ~50 cm depth. FeSO<sub>4</sub> was added to APPW + YE medium and to lake water [ $<0.2$  mM Fe(II)] as Fe(II) source to reach a final concentration of 25 mM. Aliquots of 5 ml pre-cultures growing in APPW + YE medium were added at the same time to either 100 ml medium or lake water in glass bottles closed with a sterile cotton stopper and incubated at 15 or 25 °C for 30 days. Potential chemical Fe(II) oxidation was determined in abiotic controls and in inoculated medium amended with 0.1 mM sodium azide. All treatments were prepared in triplicates. Aliquots of 0.2 ml were obtained with sterile pipettes every 2–3 days for measurements of pH (pH 330+ SenTix; WTW) and Fe(II) using the phenanthroline method (Tamura *et al.*, 1974). After 10 days of incubation, 0.2 g YE was added to each bottle of lake water as additional carbon source. Rates were calculated using the linear decrease of the Fe(II) concentration.

The capacity for dissimilatory Fe(III) reduction of strain C25 was tested in FeSO<sub>4</sub>-supplemented APPW + YE medium at 25 °C after Fe(II) was depleted and rust-coloured precipitates were formed. Then glucose was added to reach a final concentration of 5 mM. When no de-colouration was detected after 2 days, rubber stoppers were removed for ~2 min every other day to maintain micro-oxic conditions. Reduction of Fe(III) was identified by de-colouration of the medium and increase of Fe(II) concentration.

Attempts were also made to amplify form I (*cbbL*) and form II (*cbbM*) (large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase, found in autotrophic bacteria) genes for evidence of the ability of strain C25 to fix carbon dioxide using primer sets described by Alfreider *et al.* (2003, 2009).

**Microscopic observation of cell–mineral aggregation.** The morphology of the isolate and its association with the Fe(III)-minerals was observed under light and fluorescent microscopy (Axioplan<sub>1</sub>). Bacterial cells taken from liquid culture were stained with SYTO 13 (Life Technologies) on glass slides and visualized. The light and fluorescent images were taken by a mono-colour camera (Axio Cam MRm; Carl Zeiss).

The bacterial aggregates were examined by CLSM using a TCS SP5X (Leica) equipped with a white laser source, an upright microscope and  $\times 63/1.2$  water immersion lens. The bacterial aggregates were stained with SYTO 9 dye (Molecular Probes) for nucleic acid staining. Samples were analysed by CLSM using the laser line at



**Fig. 1.** 16S rRNA gene phylogenetic tree showing the close relationship of strain C25 (bold) with other closely related bacterial isolates and clones. The tree was reconstructed using the neighbour-joining method. GenBank accession numbers for sequences are given in parentheses. *Rubrobacter radiotolerans* (GenBank accession number U65647) was used as out-group (not shown). Bar, 0.1 change per nucleotide position.

483 nm. Emission signals were detected from 478 to 488 nm (reflection signals from inorganic and mineral compounds) and 500 to 550 nm (SYTO 9). Optical sections were collected in the z-direction with a step size of 0.5 µm. Images were subjected to blind deconvolution using Huygens 15.05 (SVI) and projected in Imaris 8.1.2 (Bitplane).

**Biogenic Fe(III)-mineral identification and visualization of cell–mineral associations.** Raman spectroscopy was used to characterize the air-dried Fe(III)-mineral phases produced by strain C25 grown in APPW + YE medium. The Raman spectra were recorded with a Jobin-Yvon Labram Raman Confocal Microscope (Horiba). Whilst performing the measurements the microscope was equipped with a  $\times 50/0.5$  microscope objective and a 300 lines  $\text{mm}^{-1}$  grating spectrometer with a spectral resolution of  $\sim 12 \text{ cm}^{-1}$ . When recording of the spectra, the 532 nm laser line with a power of 50 µW was employed. The samples were left out to dry at room temperature and then Raman spectra were recorded. The measurement time of a Raman spectrum varied from 250 to 300 s.

Scanning electron microscopy (SEM) was used to visualize the cell–mineral associations. Droplets of the sample suspensions were put on silicon wafers and subjected to air drying. High-resolution secondary electron images were recorded with an ULTRA PLUS field emission scanning electron microscope (Carl Zeiss).

## RESULTS AND DISCUSSION

### Placing strain C25 into the novel genus *Acidithrix*

From a total of 61 isolates obtained from iron snow collected in the central basin of the lake, eight isolates were 100% identical based on ARDRA and sequencing results. One representative strain, strain C25, formed rust-coloured colonies within 1 week on FeO solid medium containing TSB. Colonies were circular, raised and produced rusty precipitates on the plate with a slightly brown centre. Cells of strain C25 were rod-shaped with a length of 1.5–2 µm and a width of 0.5 µm, stained Gram-positive, and were not observed to form

**Table 1.** List of bacterial isolates and clones closely related to strain C25

| Sampling site                                                          | Site pH | Site temperature (°C) | Isolate/clone designation                                    | GenBank accession no. | Similarity to strain C25 (%) | Reference                                        |
|------------------------------------------------------------------------|---------|-----------------------|--------------------------------------------------------------|-----------------------|------------------------------|--------------------------------------------------|
| Acidic lignite mine lake 77, Germany                                   | 2.8     | 11.5–13               | <i>Acidithrix ferrooxidans</i> C25                           | LN866582              | NA                           | This study                                       |
| Acid copper mine Mynydd Parys, UK                                      | 2.5     | 11.3                  | <i>Acidithrix ferrooxidans</i> Py-F3, DSM 28176 <sup>T</sup> | KC208497              | 100                          | Jones & Johnson (2015); Kay <i>et al.</i> (2013) |
| Acidic, iron-rich spa water in Trefriw Well Spa, UK                    | 2.7     | 10.1                  | Acid streamer iron-oxidizing bacterium CS11                  | AY765999              | 100                          | Hallberg <i>et al.</i> (2006)                    |
| Acid copper mine Mynydd Parys, UK                                      | 2.4–2.7 | 8.8–12.4              | Acid streamer iron-oxidizing bacterium KP1                   | AY765991              | 100                          | Hallberg <i>et al.</i> (2006)                    |
| Acidic lignite mine lake 77, Germany                                   | 4.0     | 9.8                   | Clone Central-Bottom-cDNA 1                                  | HE604007              | 100                          | Lu <i>et al.</i> (2013)                          |
| Acidic river, Rio Tinto, Spain                                         | 3.2     | NA                    | Clone RT11-ant04-c06-S                                       | JF737864              | 100                          | García-Moyano <i>et al.</i> (2012)               |
| Acidic coal mine drainage in the Lower Red Eyes spring, USA            | 3.0     | 14–24                 | Clone LRE22B6                                                | HQ420111              | 100                          | Brown <i>et al.</i> (2011)                       |
| Acid mine drainage from Iron Mountain, USA                             | 2.5     | 20                    | Clone TRA2-10                                                | AF047642              | 99.9                         | Edwards <i>et al.</i> (1999)                     |
| Copper mine drainage in the Iberian Pyrite Belt, Spain                 | 2.7–2.8 | NA                    | Clone ORCL3.9                                                | EF042583              | 99.7                         | Rowe <i>et al.</i> (2007)                        |
| Acid mine Los Ruedos, Spain                                            | <2      | <13                   | Clone LR AB 199                                              | KF225655              | 99.6                         | Méndez-García <i>et al.</i> (2014)               |
| Carbondale constructed wetland system treating acid mine drainage, USA | 2.0–3.9 | NA                    | Clone AMD 67                                                 | DQ159173              | 98.3                         | Nicomrat <i>et al.</i> (2008)                    |
| Volcanic ash deposit from Miyake-jima, Japan                           | 3.4     | 12.7                  | Clone OY07-C073                                              | AB552359              | 98.1                         | Fujimura <i>et al.</i> (2012)                    |
| Acidic river, Rio Agrio, Argentina                                     | 1.0     | 59                    | Clone VA2-bac e9                                             | JN982087              | 98.0                         | Urbietta <i>et al.</i> (2012)                    |
| Acidic pit lake, Lake Concepcion, Spain                                | 2.5–3.5 | 12–13                 | Clone CN13.5m-bac c1                                         | KC619609              | 97.2                         | Santofimia <i>et al.</i> (2013)                  |
| Acid mine effluent from La Zarza-Perun, Spain                          | 3.1     | 25.6                  | Clone 20m c4                                                 | HM745419              | 97.1                         | González-Toril <i>et al.</i> (2011)              |
| Acidic hot springs, Iceland                                            | 2       | 45–50                 | <i>Acidimicrobium ferrooxidans</i> DSM 10331 <sup>T</sup>    | NR_074390             | 91.9                         | Clark & Norris (1996)                            |

NA, not available in references.



**Table 2.** Growth experiments of strain C25 in APPW + YE liquid medium at different cultivation temperatures and with different initial pH values

| Temperature (pH 2.5) |       |       |       |       | Starting pH of medium (25 °C) |              |              |              |
|----------------------|-------|-------|-------|-------|-------------------------------|--------------|--------------|--------------|
| 15 °C                | 25 °C | 30 °C | 33 °C | 37 °C | 2.0                           | 2.5          | 4.5          | 6.5          |
| +                    | ++    | ++    | +/-   | -     | -(2.0)*                       | ++(2.2-2.3)* | ++(2.2-2.3)* | ++(2.2-2.3)* |

\*Final pH of media at day 14 of incubation in triplicates.

endospores. No growth was observed in liquid FeO medium without YE. However, better growth was observed in APPW + YE medium supplemented with FeSO<sub>4</sub>. Cells could grow in iron-free medium with a reduced growth rate.

The 16S rRNA gene of strain C25 places it within the family *Acidimicrobiaceae* in the phylum *Actinobacteria* (Fig. 1). The 16S rRNA gene of strain C25 was only 91.9% identical to the moderate thermophile *Acidimicrobium ferrooxidans* DSM 10331<sup>T</sup> (GenBank accession number NR\_074390) (Clark & Norris, 1996), but 100% identical to a high fraction of clones obtained from RNA extracted from samples of the same site in 2011 (Clone Central-Bottom-cDNA 1 in Table 1) (Lu *et al.*, 2013). Strain C25 also shared 100% 16S rRNA gene identity with the FeOB strain CS11 (GenBank accession number AY765999), strain KP1 (GenBank accession number AY765991) and *Acidithrix ferrooxidans* strain PY-F3<sup>T</sup> (GenBank accession number KC208497) (Fig. 1, Table 1) that was recently isolated from an acidic, metal-rich water in north Wales in the UK (Jones & Johnson, 2015).

Strain C25 was able to grow and oxidize Fe(II) at pH 2.5–6.5 in the temperature range from 15 to 33 °C, but not at 37 °C, with an optimum rate of oxidation observed between 25 and 30 °C (Table 2). However, during growth the medium pH subsequently declined due to the release of protons that occurs when ferric iron hydrolyses water and produces Fe(III) oxide hydroxides. The final pH remained stable between 2.2 and 2.3 after 14 days of incubation regardless of initial pH values (Table 2).

During the initial phase of Fe(II) oxidation by strain C25, a lag phase of ~5 days was observed prior to the onset of Fe(II) oxidation when grown at 25 °C (Fig. 2a). During this 5 day period, the medium pH increased slightly by ~0.3 pH units before Fe(II) was consumed at a rate of  $2.94 \pm 0.27$  mM day<sup>-1</sup> and rust-coloured precipitates formed. However, no second lag phase was observed when this culture was resupplemented with FeSO<sub>4</sub> after Fe(II) depletion. Following resupplementation with FeSO<sub>4</sub>, the rates of Fe(II) oxidation increased to  $3.77 \pm 0.25$  mM day<sup>-1</sup> (Fig. 2a). Cells cultivated at 15 °C showed a slower rate of Fe(II) oxidation ( $1.02 \pm 0.07$  mM day<sup>-1</sup>) with a 3 day lag phase (Fig. 2b). When strain C25 was cultivated in sterile-filtered lake water with a pH of 2.6 supplemented with FeSO<sub>4</sub> and YE, the observed rates of Fe(II) oxidation were approximately  $3.01 \pm 0.40$  and  $0.83 \pm 0.11$  mM day<sup>-1</sup> at 25 and 15 °C, respectively

(Fig. 2c, d). Neither cell growth nor Fe(II) oxidation was observed in any control cultures.

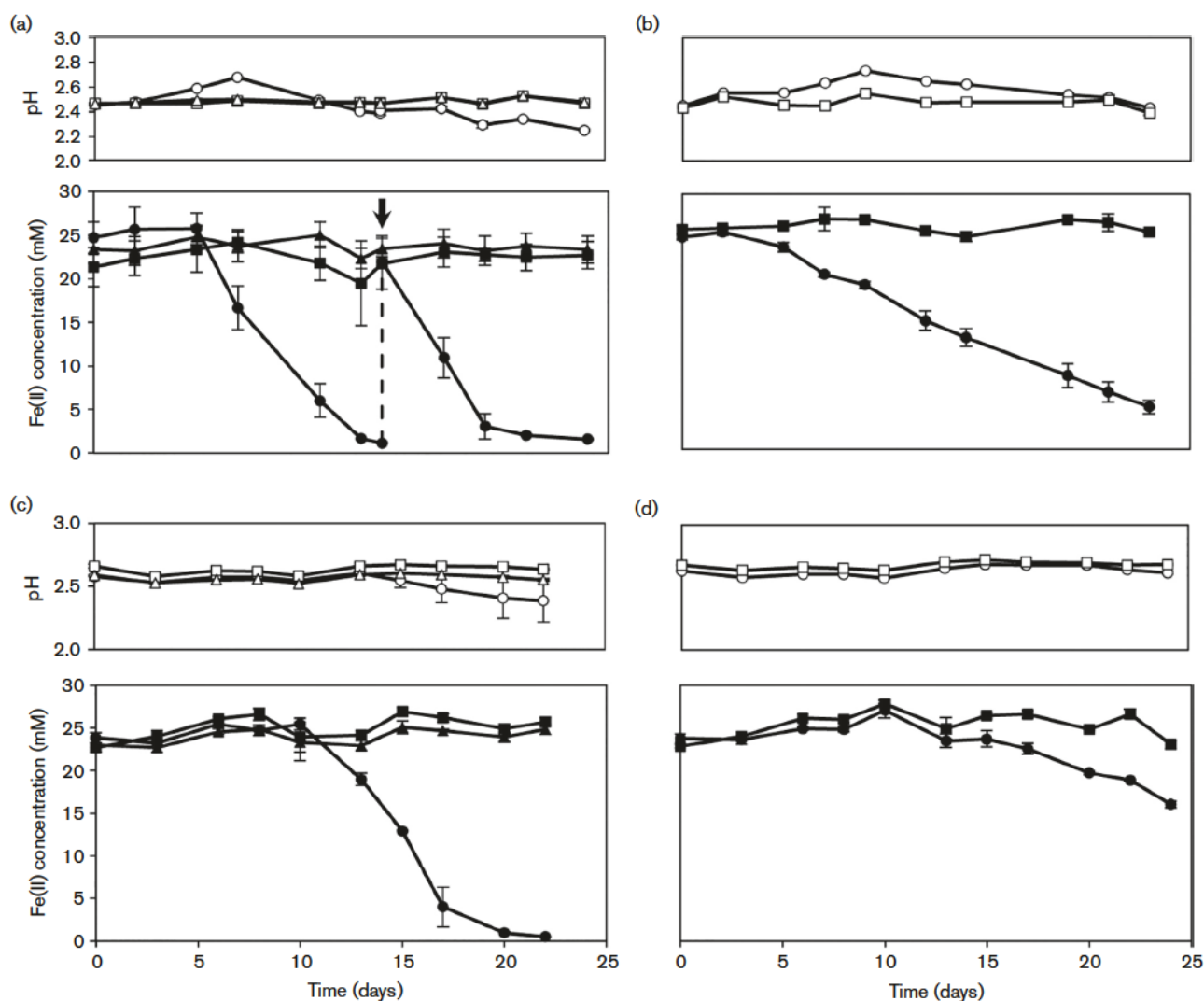
Dissimilatory Fe(III) reduction was observed on strain C25 within 5 days under micro-oxic condition after an adjustment of the headspace and the addition of 5 mM glucose. All Fe(III)-minerals generated under previous oxic conditions disappeared until no rust colour could be observed. Bacterial cells regenerated filaments during Fe(III) reduction (Fig. S1, available in the online Supplementary Material). Similarly, strain Py-F3<sup>T</sup> can catalyse the dissimilatory reduction of solid-phase Fe(III) under micro-oxic conditions (Jones & Johnson, 2015) as well as many other acidophilic *Actinobacteria* (Bridge & Johnson, 1998; Itoh *et al.*, 2011; Johnson *et al.*, 2003, 2009).

Strain C25 showed high morphological and physiological similarities to the strain Py-F3<sup>T</sup>. However, strain C25 has different physiological characteristics compared with the new *Acidithrix*-type strain. Strain C25 could not grow at pH 2.0, but was shown to tolerate higher pH values than strain Py-F3<sup>T</sup>. In addition, strain C25 was unable to reduce solid-phase Fe(III) under strictly anoxic conditions, in contrast to strain Py-F3<sup>T</sup>. The genome sequence of strain Py-F3<sup>T</sup> encodes two subunits for a type I ribulose 1,5-bisphosphate carboxylase/oxygenase and several enzymes required for carbon fixation via the Calvin–Benson–Bassham cycle (Eisen *et al.*, 2015). However, PCR products of the *cbhL* gene in strain C25 were only detected as a very weak band, thus we could not confirm the presence of genes encoding ribulose 1,5-bisphosphate carboxylase/oxygenase in strain C25 (data not shown).

### Novel iron precipitation approach of strain C25

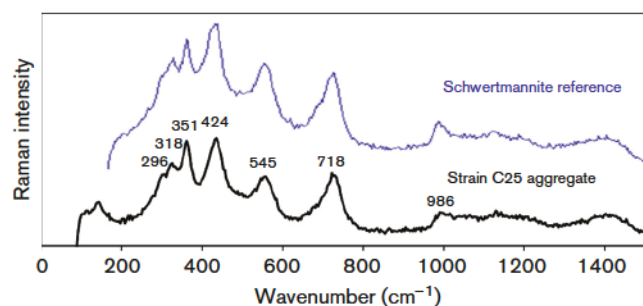
A novel iron precipitation approach was identified with cells of strain C25. Schwertmannite was identified as the sole product of Fe(II) oxidation by strain C25 using Raman spectroscopy (Fig. 3). The peaks exhibiting at 296, 318, 351, 424, 545, 718, and 986 cm<sup>-1</sup> were close to the reference peak pattern of schwertmannite (294, 318, 350, 421, 544, 715 and 981 cm<sup>-1</sup>).

Fluorescence microscopic examination showed that single cells of strain C25 formed long filaments (up to 400 µm) during the active phase of Fe(II) oxidation (Fig. 4a). No EPS-like matrix was confirmed by SEM or fluorescence



**Fig. 2.** Ferrous iron oxidation by strain C25 in (a, b) synthetic medium APPW + YE and (c, d) lake water at 25 (a, c) and 15 °C (b, d), respectively. Additional ferrous iron was added at day 14 of incubation in APPW + YE at 25 °C (a, arrowed point). Strain C25 cultured in APPW + YE medium (●) or lake water (○). APPW + YE medium (■) or lake water (□) without bacterial inoculation. Strain C25 cultured in APPW + YE medium (▲) or lake water (△) with 0.1 mM sodium azide. Data represent mean  $\pm$  SD;  $n=3$ .

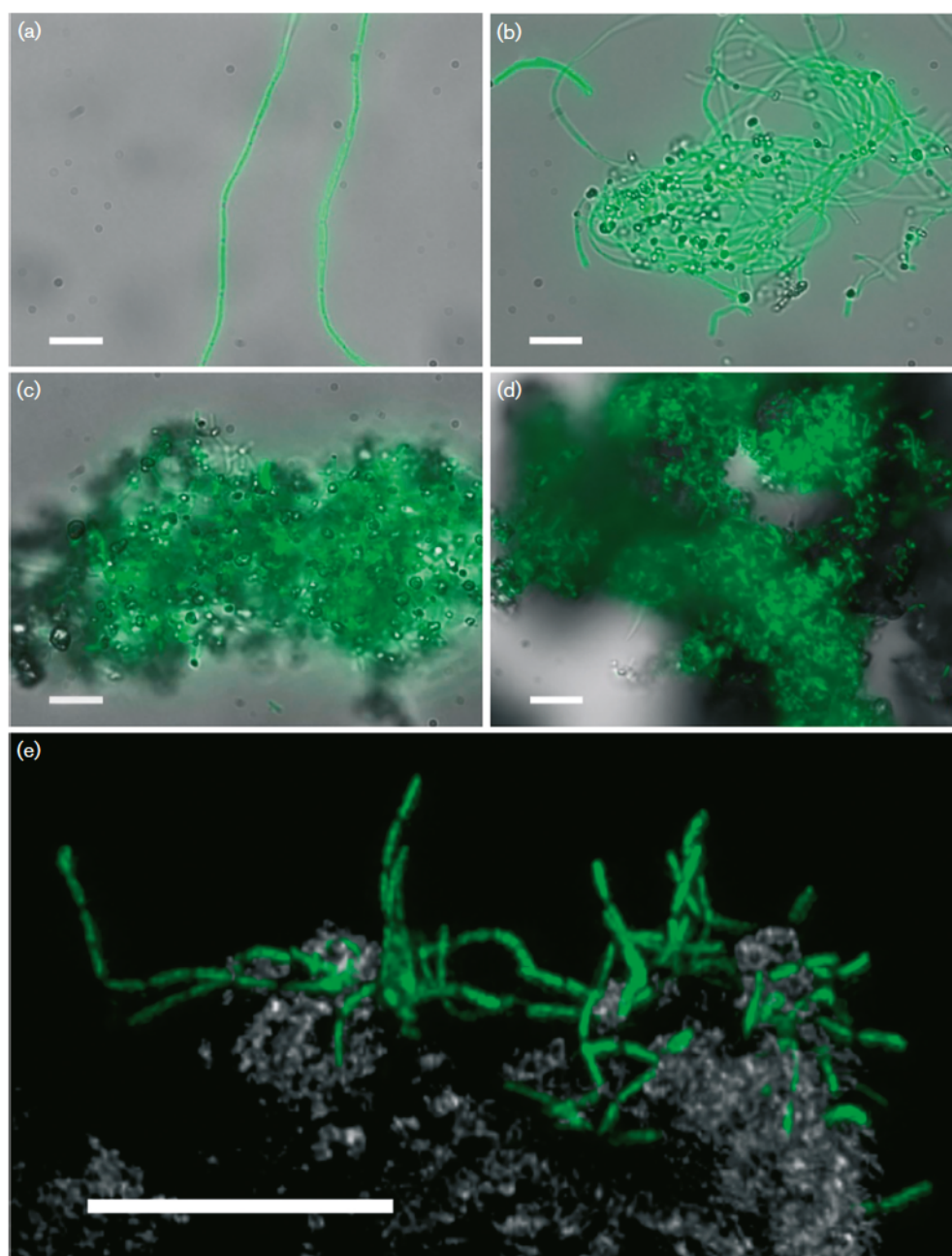
2



**Fig. 3.** Raman spectra of the Fe(III)-mineral produced by strain C25 in APPW + YE medium (black line) and authentic schwertmannite standard (blue line).

microscopy. Eventually the entangled filaments were associated with rust-coloured schwertmannite (Fig. 4b). In the later stationary phase, long filaments broke into smaller fragments and single cells leading to the formation of greater cell-mineral aggregates (Fig. 4c–e). Filaments were also observed when cells were inoculated in Fe(II)-free medium. Interestingly, different stages of schwertmannite aggregation could be clearly differentiated using SEM (Fig. 5). Initially, cells growing for 3–4 days displayed needle-shaped whiskers formed on the cell surface, which implies all cell-oxidized iron is cell wall-associated (Fig. 5b). After 1 week, filamentous cells featured ‘matured’ spheres with numerous peripheral needles attached to the cell surface only at junctions between the cells, leaving major parts of the cell surface free of encrustation



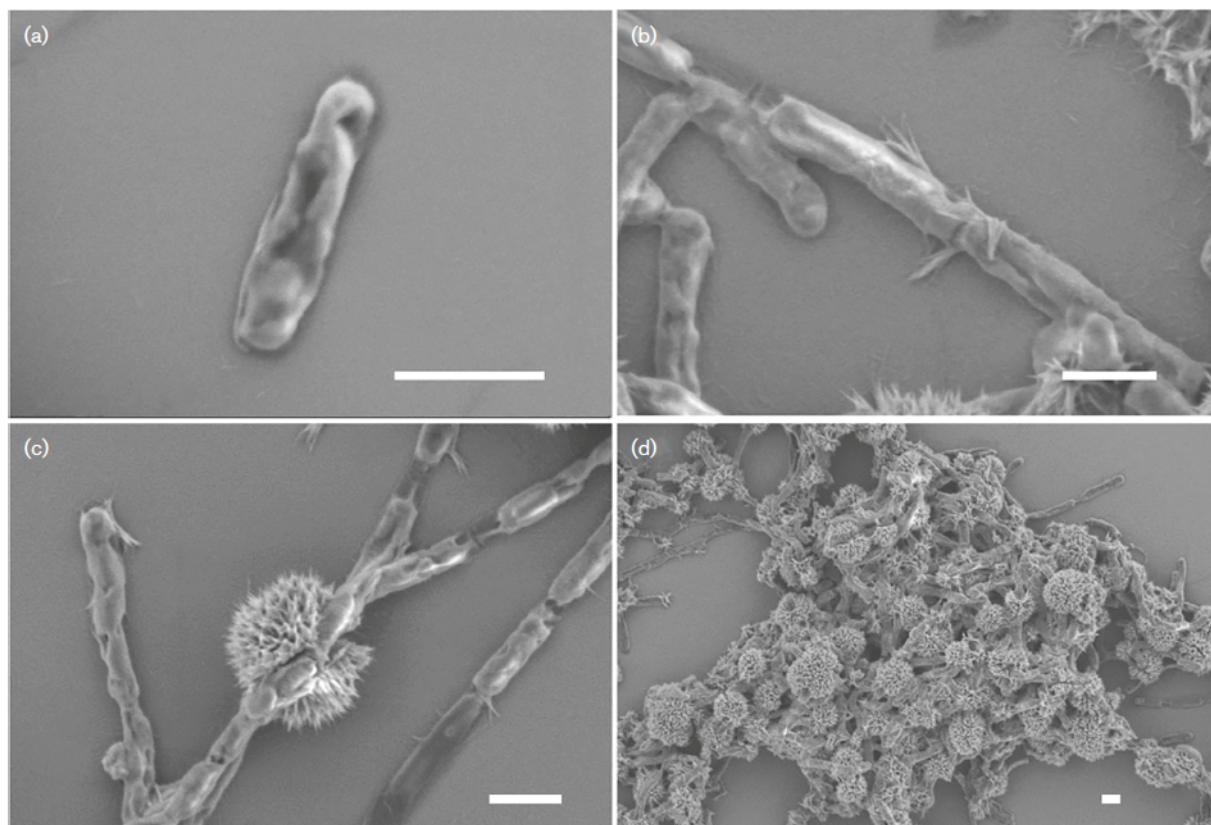


**Fig. 4.** Formation of long filaments of strain C25 during different growth phases. (a–d) Cells of strain C25 in APPW + YE medium were stained with SYTO 13 (green, nucleic acids). Images were taken at day 4 (a), 7 (b), 12 (c) and 30 (d) of cultivation. (e) Three-dimensional volume view of strain C25 cells which form iron-rich aggregate, stained with SYTO 9 (green, nucleic acid; grey, reflection). Bar, 10 µm.

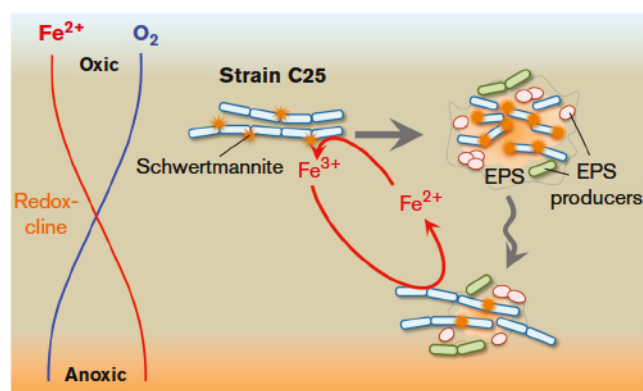
(Fig. 5c). Finally, ‘matured’ schwertmannite clumped together with other neighbouring minerals attached to cell surfaces, leading to the formation of coiled-cell filaments. During the formation of these large cell–mineral assemblages, the long cell filaments appeared to break into shorter fragments. This directed mineral formation allowed parts of the cell surface to remain free of encrustation, enabling them to retain access to dissolved Fe(II) and

other nutrients (Fig. 5d). This well-coordinated mechanism for biomineralization controlled by strain C25 was distinct from other known Fe(II) oxidation approaches under low pH conditions. Acidophilic bacteria have to maintain their cytoplasm at neutral pH by reversing their membrane potential. Some researchers suggest a positive charge on the cell surfaces (Hedrich *et al.*, 2011; Norris *et al.*, 1992), whilst others suggest a negative surface charge





**Fig. 5.** Scanning electron micrographs of strain C25 and Fe(III)-minerals formed by the oxidation of Fe(II) by stain C25. (a) A single cell of strain C25. (b) The initial phase of Fe(III)-mineral precipitation on the cell filaments of strain C25. (c) Precipitation of pincushion-like Fe(III)-mineral spheres at the joint part of cell filaments. (d) A cell-Fe(III)-mineral aggregate formed by strain C25 cells and pincushion-like Fe(III)-mineral spheres in the later stationary growth phase. Bar, 1  $\mu\text{m}$ .



**Fig. 6.** Putative schematic model depicting the significant role of strain C25 in iron snow formation in the redoxcline of acidic iron-rich lakes.

(Baker-Austin & Dopson, 2007). Schwertmannite at the given pH has a positive charge and schwertmannite needle formation was observed at the sites of cell-to-cell connections of strain C25, suggesting a heterogeneous

distribution of surface charge or an accumulation of specific anionic compounds at the polar end of the cells might be involved in the directed localization of the Fe(III)-minerals at this specific position. The biomineralization approaches of other *Actinobacteria* species have not been well characterized. Strain Py-F3<sup>T</sup> has only been shown to initiate schwertmannite precipitation (Jones & Johnson, 2015) without further details. Acidophiles, including *Leptospirillum* and *Ferroplasma*, capable of growing in extremely acidic (pH < 2) environments, are not susceptible to iron encrustation (Druschel *et al.*, 2004; Tyson *et al.*, 2004). Other acidophilic FeOB, such as *Ferroplasma myxofaciens* and *Acidithiobacillus ferrooxidans*, which are known to grow in less extreme environments, are able to deposit Fe(III)-minerals into their EPS as a mechanism to avoid encrustation by the Fe(III)-minerals (Frankel & Bazylinski, 2003; Hedrich *et al.*, 2011).

### Ecological significances of strain C25

Filamentous bacteria with a similar morphology to strain C25 are present in iron snow as revealed by CLSM imaging in a previous study (Lu *et al.*, 2013). The oxic/anoxic

transition in the redoxcline is ideally suited for micro-organisms with the ability to oxidize Fe(II) and reduce Fe(III). Strain C25 and other related species make up the largest fraction of the metabolically active bacteria in the iron snow, followed by the EPS-forming *Ferrovum* species (Lu *et al.*, 2013). Thus, we suggest that strain C25 is involved in early stages of iron snow formation via Fe(II) oxidation and subsequent large cell aggregate formation associated with the pincushion-like mineral schwertmannite (Fig. 6). EPS released by *Ferrovum* species should favour the cohesiveness and additional growth of these pelagic aggregates, attract heterotrophic micro-organisms, and aid in the prevention of complete iron encrustation. These pelagic aggregates, reaching a mean size between 60 and ~240 µm (Reiche *et al.*, 2011), will begin to sink through the redoxcline. When the oxygen concentration declines, strain C25 could exploit the adsorbed organic compounds as a carbon source under micro-oxic conditions and trigger the utilization of schwertmannite as an alternative electron acceptor. This reductive dissolution ultimately results in a reduced size that would enable the iron snow to remain in the redoxcline for an extended period of time prior to sinking to the sediment. The capacity of strain C25 to serve both halves of the iron cycle provides a unique insight into the ecological relevance and importance in linking the redoxcline with the sediment by providing a mechanism for removal of iron, microbial cells and organic carbon from the water column, and subsequent accumulation in the sediment.

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