FEMS Microbiology Ecology, 93, 2017, fix054

doi: 10.1093/femsec/fix054
Advance Access Publication Date: 18 April 2017
Research Article

RESEARCH ARTICLE

Physiological characterization of a halotolerant anoxygenic phototrophic Fe(II)-oxidizing green-sulfur bacterium isolated from a marine sediment

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One sentence summary: This study presents the first green-sulfur photoautotrophic Fe(II)-oxidizer isolated from a marine coastal sediment.
Editor: Alfons Stams

ABSTRACT

Anoxygenic photoautotrophic bacteria which use light energy and electrons from Fe(II) for growth, so-called photoferrotrophs, are suggested to have been amongst the first phototrophic microorganisms on Earth and to have contributed to the deposition of sedimentary iron mineral deposits, i.e. banded iron formations. To date only two isolates of marine photoferrotrophic bacteria exist, both of which are closely related purple non-sulfur bacteria. Here we present a novel green-sulfur photoautotrophic Fe(II) oxidizer isolated from a marine coastal sediment, *Chlorobium* sp. strain N1, which is closely related to the freshwater green-sulfur bacterium *Chlorobium luteolum* DSM273 that is incapable of Fe(II) oxidation. Besides Fe(II), our isolated strain grew phototrophically with other inorganic and organic substrates such as sulfide, hydrogen, lactate or yeast extract. Highest Fe(II) oxidation rates were measured at pH 7.0–7.3, the temperature optimum was 25°C. Mössbauer spectroscopy identified ferrihydrite as the main Fe(III) mineral and fluorescence and helium-ion microscopy revealed cell-mineral aggregates without obvious cell encrustation. In summary, our study showed that the new isolate is physiologically adapted to the conditions of its natural habitat but also to conditions as proposed for early Earth and is thus a suitable model organism for further studies addressing phototrophic Fe(II) oxidation on early Earth.

Keywords: geomicrobiology; phototrophic Fe(II) oxidation; early Earth biogeochemical processes; green-sulfur bacteria

INTRODUCTION

Iron is an abundant redox-active element in the environment (Taylor and McLennan 1985; Raiswell and Canfield 2012). The main redox states of Fe in the environment are Fe(II) and Fe(III). In the biogeochemical Fe cycle, Fe is transformed between these two redox states by different biotic and abiotic processes (Melton *et al.* 2014b). At circumneutral pH, Fe(II) is rapidly oxidized abiotically by oxygen; the formed Fe(III) quickly undergoes hydrolysis and precipitates as Fe(III) minerals (Cornell and Schwertmann 2003; Majzlan, Navrotsky and Schwertmann 2004).

Some neutrophilic Fe(II)-oxidizing bacteria can oxidize Fe(II) under microoxic conditions, where the abiotic oxidation by O_2 is slower so that microaerophilic microorganisms can outcompete the abiotic reaction (Emerson and Moyer 1997; Druschel *et al.* 2008). Other neutrophilic Fe(II) oxidizers live under anoxic

Received: 2 February 2017; **Accepted:** 17 April 2017

conditions and couple Fe(II) oxidation either to nitrate reduction or use Fe(II) as electron donor in anoxygenic photosynthesis (Widdel *et al.* 1993; Straub *et al.* 1996). Anoxygenic photosynthesis with Fe(II) as electron donor (photoferrotrophy) is believed to be one of the most ancient metabolisms and is thought to have evolved before oxygenic photosynthesis (Canfield, Rosing and Bjerrum 2006; Xiong 2006). The first isolates that were able to perform photoferrotrophy according to Equation 1 were obtained in 1993 (Widdel *et al.* 1993).

$$HCO_3^- + 4Fe^{2+} + 10H_2O + h\nu \rightarrow 4Fe(OH)_3 + CH_2O + 7H^+ \quad (1)$$

During the Precambrian eon, photoferrotrophy could have been one important mechanism that led to the formation of massive Fe-Si rich sedimentary deposits, the banded iron formations (BIFs) (Beukes *et al.* 1992; Konhauser *et al.* 2002; Kappler *et al.* 2005; Posth *et al.* 2008; Bekker *et al.* 2010; Posth, Konhauser and Kappler 2013). BIFs were formed between 3.85 and 1.8 billion years ago and represent the largest iron ore deposits on Earth (Bekker *et al.* 2010; Mloszewska *et al.* 2012). To which extent photoferrotrophs have actually contributed to the formation of BIFs is still a matter of debate. To determine more precisely how much photoferrotrophs could have potentially contributed to BIF formation, and to identify possible biomarkers of photoferrotrophy (e.g. C or Fe isotope fractionation, mineralogical, organic or morphological markers), it is necessary to understand the metabolic potentials and the physiology of these organisms (Eickhoff *et al.* 2013; Swanner *et al.* 2015).

Known photoferrotrophs belong to different groups of anoxygenic phototrophic bacteria: the green-sulfur, the purple-sulfur and purple non-sulfur bacteria (Ehrenreich and Widdel 1994; Heising *et al.* 1999; Croal *et al.* 2004; Jiao *et al.* 2005). Of the currently existing isolates most are freshwater organisms (Ehrenreich and Widdel 1994; Heising *et al.* 1999; Jiao *et al.* 2005; Crowe *et al.* 2008; Llirós *et al.* 2015). By now only two isolates of marine anoxygenic phototrophic Fe(II) oxidizers exist. These are the closely related purple non-sulfur bacteria *Rhodovulum iodosum* and *R. robiginosum* (Straub, Rainey and Widdel 1999). Consequently, only a limited number of physiological studies with marine photoferrotrophs exist (Straub, Rainey and Widdel 1999; Wu *et al.* 2014). Instead, most physiological studies from which also conclusions on photoferrotrophy in ancient oceans were drawn were performed with freshwater photoferrotrophs (Kappler and Newman 2004; Hegler *et al.* 2008; Schädler *et al.* 2009). For these freshwater photoferrotrophs, there are even some genes known that are involved in phototrophic Fe(II) oxidation. These genes are, however, not homologous among different isolates, what indicates that the different bacteria use different biochemical pathways (Croal, Jiao and Newman 2007).

Recent studies in modern ferruginous lakes highlighted the potential importance of green-sulfur bacteria for Fe(II) oxidation under conditions that are comparable to conditions on early Earth (Crowe *et al.* 2008; Walter *et al.* 2014; Llirós *et al.* 2015). Green-sulfur bacteria are especially adapted to thrive under low light intensities (Overmann 2006). Furthermore, green-sulfur bacteria are metabolically more restricted than other anoxygenic phototrophs. Green-sulfur bacteria belonging to the family *Chlorobiaceae* are strict anaerobes and obligate phototrophs. Besides hydrogen and reduced sulfur compounds, bacteria within the *Chlorobiaceae* can utilize only a limited number of organic substrates (Overmann 2006; Imhoff 2014). Besides the two marine isolates, which are both purple non-sulfur bacteria, there is virtually nothing known about marine photoferrotrophs and no isolate of a marine photoferrotrophic green-sulfur bacteria has been described and characterized so far. This illustrates the need for more studies and novel isolates of marine photoferrotrophs and most importantly photoferrotrophic green-sulfur bacteria.

In a previous study, we reported abundances and identities of photoferrotrophs at two coastal field sites in the Aarhus Bay area, Denmark (Laufer *et al.* 2016). In this study, we characterize a newly isolated marine green-sulfur Fe(II)-oxidizing strain. We determined which organic and inorganic substrates other than Fe(II) can be used by the isolate for growth and quantified the influence of light intensity, pH, temperature and salinity on phototrophic Fe(II) oxidation rates. Moreover, the Fe(III) minerals that are produced by the isolate during Fe(II) oxidation were identified by Mössbauer spectroscopy and the cell–mineral associations were characterized by fluorescence microscopy and helium-ion microscopy (HIM).

MATERIAL AND METHODS

Isolation and cultivation of photoferrotrophs

The photoferrotrophic culture was isolated and cultivated in Hungate tubes (15 ml) containing 9 ml of anoxic artificial seawater (ASW) medium amended with 10 mM $FeCl_2$. For standard cultivation, 10% inoculum was used. Incubation was done at 20°C in an incubator that was illuminated by a fluorescent light tube (15 W, 5500 K). The ASW medium for isolation and standard cultivation had a salinity of 23. Per liter it contained the following salts: 17.3 g NaCl, 8.6 g $MgCl_2 \times 6\ H_2O$, 0.025 g $MgSO_4 \times 7\ H_2O$, 0.99 g $CaCl_2 \times 2\ H_2O$, 0.39 g KCl, 0.059 g KBr, 0.25 g NH_4Cl and 0.4 g KH_2PO_4. The medium was prepared with a headspace of N_2/CO_2 (90/10) and was buffered with 22 mM bicarbonate buffer. Additionally, per liter, 1 ml of a vitamin solution (Widdel and Pfennig 1981), 1 ml of a trace element solution (Tschech and Pfennig 1984) and 1 ml of a selenite-tungstate solution (Widdel 1980) were added. The pH of the medium was adjusted to 6.8–7.0 with either anoxic 1 M HCl or anoxic 0.5 M Na_2CO_3. If not stated otherwise, the above-described standard cultivation conditions were applied in all experiments. The enrichment technique and the field site from which the sediment that was used for inoculation was sampled are described in detail in a previous publication (Laufer *et al.* 2016). In short, the isolate described here originates from Norsminde Fjord, Denmark. The fjord is a shallow estuary with a narrow opening to the Baltic Sea. The sediment is muddy and organic rich. For enrichment, ca. 1 g of the sediment from Norsminde Fjord was added to a Hungate tube containing 9 ml ASW and a serial 10-fold dilution series was prepared up to a dilution of 10^{-6}. When Fe(II) oxidation was visible (indicated by the formation of orange-rusty Fe(III) precipitates), growth was confirmed by fluorescence microscopy and LIVE/DEAD staining (Molecular Probes, Carlsbad, CA, USA). The highest positive dilution (indicated by presence of cells and active Fe(II) oxidation) was transferred and a new dilution series was prepared. The new dilution series was always prepared at least two dilution steps higher than the previous dilution series, to achieve isolation of a pure culture by the dilution-to-extinction method.

Phylogenetic analysis

DNA from a culture of the isolate that was grown under standard conditions was extracted with the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the instructions of the manufacturer. A PCR to obtain 16S rRNA gene fragments was performed with the

general bacterial 16S rRNA gene-specific primers GM3-F (5′-AGAGTTTGATCMTGGCTCAG-3′) (Muyzer *et al.* 1995) and 1392-R (5′-ACGGGCGGTGTGTRC-3′) (Lane *et al.* 1985). The resulting PCR products were loaded on a 1% agarose gel, and the bands were excised and cleaned with the Wizard PCR clean-up system (Promega Laboratories, WI, USA). The cleaned DNA was cloned into a plasmid vector using a PCR cloning kit (Qiagen, Germany). Vectors were transformed into competent cells (*Escherichia coli* DH5α) that were afterwards plated onto LB medium containing ampicillin, IPTG and XGal for blue/white screening. White colonies were picked and tested for their correct insert size. Overnight cultures were prepared from colonies with the correct inserts in 5 ml liquid LB medium containing ampicillin. Plasmid DNA was isolated from these cultures and sent to GATC Biotech (Konstanz, Germany) for sequencing. Three clones were picked and sequenced. The quality testing of the resulting sequences and trimming of the sequences were performed with the software Geneious R6 (Biomatters, http:// www.geneious.com). The sequences were then assigned to bacterial phyla using the online Ribosomal Database Project (RDP) naive Bayesian classifier version 2.2 (http://rdp.cme.msu.edu/classifier/classifier.jsp). Good quality sequences were uploaded to the EMBL Genbank database (accession number LT575234). For constructing a phylogenetic tree, the 16S rRNA gene sequences from the closest relative of the isolate and sequences of more distantly related anoxygenic phototrophic Fe(II)-oxidizing bacteria were downloaded from the EMBL database. Bacterial 16S rRNA gene sequences from three species belonging to different phyla than the Fe(II) oxidizer sequences were chosen as outgroups. ClustalW (Thompson, Higgins and Gibson 1994) was used for alignment of the 16S rRNA gene sequences. Phylogenetic trees were constructed by the maximum-likelihood method with the software package MEGA7 (Kumar, Stecher and Tamura 2016).

Physiological characterization

Standard growth curve

To determine Fe(II) oxidation rates and cellular growth rates, the concentrations of Fe(II) and Fe(III), as well as cell numbers were quantified over time in cultures that were grown under standard conditions (as defined above). All measurements were performed for triplicate cultures. Samples for both Fe quantification and cell counts were taken every second day under anoxic (N_2-flushed syringes) and sterile conditions. Fe samples were immediately stabilized in 1 M HCl (100 μl sample in 900 μl HCl). Samples for cell counts were fixed with paraformaldehyde (PFA, 4% final concentration). All samples were stored at 4°C in the dark until analysis.

Alternative growth substrates

To test if the isolate uses other substrates than Fe(II) for phototrophic growth, duplicate cultures were prepared containing different organic and inorganic electron donors (Table 1). The different organic substrates were all added at a final concentration of 4 mM, except yeast extract, which was added to a concentration of 10 mg l^{-1}. Inorganic sulfur compounds were added to a final concentration of 2 mM. H_2 was supplied by flushing the headspace every second day with H_2/CO_2 (80/20). Furthermore, the ability of the isolate to grow under anoxic conditions in the dark on organic compounds with several different electron acceptors was tested. Cultures were supplied either with a mixture of acetate and lactate (each 4 mM final concentration) or with yeast extract (10 mg l^{-1}) and with one of the following electron acceptors: SO_4^{2-} (5 mM), NO_3^- (5 mM), or Fe(III) (5 mM in the form of ferrihydrite, prepared according to Schwertmann and Cornell (2008)). Furthermore, autotrophic growth by NO_3^--reducing Fe(II) oxidation (4 mM NO_3^- and 10 mM Fe(II)) as well as mixotrophic growth by NO_3^--reducing Fe(II) oxidation with the addition of a mixture of acetate and lactate (2 mM each) was tested. Moreover, aerobic growth was tested on different substrates: on agar plates containing either LB-ASW (per liter: 10 g tryptone, 5 g yeast extract, ASW salts, 15 g agar) or R2A-ASW (per liter: 18.1 g R2A agar (Merck), ASW salts) and on liquid LB-ASW medium (same as LB but without agar). Growth was evaluated visually by changes in colors and turbidity of the culture medium and by fluorescence microscopy. Cultures where growth occurred were transferred for at least one more time on the same substrate to confirm growth. In cultures grown under phototrophic conditions, the purity of the cultures was checked by comparing the total cell number (cells stained with SYTO 9 (Molecular Probes)) to the number of autofluorescent cells. Therefore, a sample of each culture that showed growth was fixed with PFA (4% final concentration) and stored at 4°C in the dark until further analysis.

Table 1. Growth tests of the photoferrotrophic Norsminde Fjord isolate on alternative substrates under anoxic, phototrophic conditions.

Substrate	Growth +/–	Substrate	Growth +/–
Organic acids		*Amino acids*	
Acetate	–	Cysteine	–
Lactate	+	Peptone	–
Propionate	–		
Fumarate	–	*Sugars*	
Succinate	–	Sucrose	+
Butyrate	–	Glucose	+
Formic acid	–		
Pyruvate	+	*Complex substrates*	
Citrate	+	Yeast extract	+
Benzoic acid	–		
		Inorganic e^- donors	
Alcohols		Sulfide	+
Glycerol	+	Sulfur	+
Mannitol	+	DMSO	–
Ethanol	–	FeS	–
		H_2	+

All organic compounds were added at a final concentration of 4 mM, except yeast extract, which was added at a concentration of 10 mg l^{-1}. Inorganic sulfur compounds were added at a final concentration of 2 mM. H_2 was supplied as H_2/CO_2 (80/20) in the headspace. Positive growth was confirmed microscopically by at least one further transfer on the respective substrate.
+ growth; – no growth

Light dependence of Fe(II) oxidation rates

To quantify the dependence of Fe(II) oxidation rates on light intensity, incubations with the photoferrotrophic isolate from Norsminde Fjord were made at different light intensities. Therefore, cultures were incubated at different distances from a fluorescent light tube (15 W, 5500 K). The resulting light intensities were as follows: 46, 470 and 1270 lux. All incubations were performed in triplicates. A negative control was incubated in the dark (wrapped in tinfoil). Samples for Fe quantification were taken every second day, as described above.

pH dependence of Fe(II) oxidation rates

For quantifying the effect of pH on Fe(II) oxidation rates, the isolate from Norsminde Fjord was incubated in medium with different pH values, ranging from pH 6.0 to 9.1. The incubations

were performed in 50 ml serum vials containing 22.5 ml ASW medium and 2.5 ml inoculum. The pH of the medium was adjusted by either adding 1 M HCl or 0.5 M Na_2CO_3. The tested pH values were as follows: 6.0, 6.3, 6.5, 6.7, 7.0, 7.2, 7.3, 7.6, 7.9, 8.4 and 9.1. Incubations for all pH values were carried out in triplicates. Samples for Fe quantification were taken every second to third day over a period of 14 days. Additionally, samples were taken after 24 days to ensure that in samples where in the first 14 days no Fe(II) oxidation was detected, there was still no Fe(II) oxidation. Sampling for Fe quantification was done as described above for the standard growth curve.

Temperature dependence of Fe(II) oxidation rates

To quantify the temperature dependence of Fe(II) oxidation rates of the isolate from Norsminde Fjord, cultures were incubated at different temperatures, ranging from 7°C to 45°C at a light intensity of 380 lux. The tested temperatures were as follows: 7°C, 10°C, 20°C, 25°C, 30°C, 37°C and 45°C. At each temperature, triplicate cultures were incubated. Samples for Fe quantification were taken every second to third day over a period of up to 16 days. At the lowest temperature, an additional sample was taken after 22 days.

Salinity dependence of Fe(II) oxidation rates

For quantifying the salinity dependence of Fe(II) oxidation rates, the isolate from Norsminde Fjord was grown on medium with different mixing ratios of an ASW medium (adjusted to a salinity of 50) and a freshwater medium (Modified Wolfe's Mineral Medium, MWMM). Per liter, the ASW medium with a salinity of 50 contained the following salts: 37.6 g NaCl, 18.7 g $MgCl_2 \times 6 H_2O$, 0.082 g $MgSO_4 \times 7 H_2O$, 2.1 g $CaCl_2 \times 2H_2O$ 0.86 g KCl, 0.13 g KBr, 0.54 g NH_4Cl and 0.87 g KH_2PO_4. The MWMM contained the following salts per liter: 0.1 g NH_4Cl, 0.2 g $MgSO_4 \times 7H_2O$, 0.1 g $CaCl_2 \times 2H_2O$, 0.05 g K_2HPO_4. The buffer, the pH and the addition of vitamin and trace element solutions were the same as for the ASW medium with a salinity of 23. The MWMM had a salinity of 1.6 (measured with a multimeter (WTW; Multi 3430) equipped with a conductivity electrode (WTW; TetraCon92)). The salinity range tested was consequently 1.6 (only MWMM) to 50 (only ASW) and within that range eight different salinities were tested in triplicates. The tested salinities were 1.6, 2.6, 6.4, 11.3, 16.1, 25.8, 35.5 and 50. For quantification of Fe(II) oxidation rates at different salinities, initially every second day samples were taken. After 10 days, the sampling interval was changed to 5–6 days.

Fe(II) oxidation by *Chlorobium luteolum* DSM 273

To determine whether *C. luteolum* DSM 273, which is the closest relative of our isolate (98% sequence identity), is able to oxidize Fe(II), a culture of *C. luteolum* DSM 273 was purchased from the DSMZ, Germany. To test the strains' ability to phototrophically oxidize Fe(II), we incubated this strain at different Fe(II) concentrations (100 μM to 10 mM) and tested two different media, i.e. ASW medium with a salinity of 23 and the medium recommended by the DSMZ. The viability of the strain was confirmed by growing it under the standard growth conditions suggested by the DSMZ (with H_2S as electron donor). To test for the toxicity of Fe(II) and H_2S, we also cultivated *C. luteolum* DSM 273 in medium containing varying concentrations (100 μM to 1 mM) of either only Fe(II), only H_2S or a combination of Fe(II) plus H_2S.

Microscopic analysis of photoferrotrophs

Cell counts

Relative cell counts of abundances of autofluorescent *Chlorobium* sp. cells and total cells were done in order to confirm the purity of the culture. These counts were done for cultures of the isolate that were grown on different substrates under phototrophic conditions. For the counts, the cells were immobilized on glass slides. Therefore, a sample of the culture (that was fixed with PFA) was diluted 1:10 with 1×PBS and mixed 1:1 with 0.2% ca. 40°C warm agarose. A drop of this mixture was applied on glass slide and dried at 60°C. Cells were stained with SYTO 9 (Molecular Probes) and CitiFlour AF1 was added onto the glass slide. Cells were counted using a Leica DM 6000 epifluorescence microscope. In random view fields 500 green fluorescent, SYTO nine stained cells were counted (filter set L5; excitation filter: BP480/40 nm; dichromatic mirror: 505 nm; suppressor filter: BP 527/39). In the same view fields, the number of red autofluorescent cells was counted (filter set Y3 (excitation filter: BP 543/30; dichromatic mirror: 565 nm; suppressor filter: BP 610/75)). The red autofluorescence of *Chlorobium* sp. was reported before (e.g. Bird and Karl 1991; Tuschak, Glaeser and Overmann 1999).

For quantitative cell counts of a culture that was grown with Fe(II), the Fe(III) minerals formed during Fe(II) oxidation needed to be dissolved first. To this end, the PFA-fixed cell suspension was mixed 1:10 with an oxalate solution (per liter: 28 g $(NH_4)_2C_2O_4{}^*H_2O$ and 15 g $C_2H_2O_4$) and incubated for 1 h to dissolve Fe(III) minerals. After Fe(III) mineral dissolution, the sample was centrifuged (7 min; 13 000 g), the supernatant was discarded and the pellet was resuspended in 200 μl of 1×PBS. To immobilize the cells, they were filtered on a black polycarbonate filter (GTTB, 0.2 μm pore size, Millipore). 1/8 of a filter was used for cell counts. To fix the cells on the filter, the filter piece was dipped into a drop of ca. 40°C warm 0.2% agarose and dried at 60°C. Afterwards, the filter was mounted on a glass slide with one drop of CitiFlour and the cells were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes). Cells were counted using a Leica DM 6000 epifluorescence microscope with the L5 filter. At least 70 counting grids or 500 cells were counted per sample. The dilution of the sample was optimized to reach cell numbers of 30–100 cells per counting grid.

Helium-ion microscopy

A sample of a culture that was grown under standard conditions (10 mM $FeCl_2$, 20°C, pH 6.8–7.0) was fixed overnight with glutaraldehyde (2.5% final concentration) at 4°C. Afterwards, cells were washed twice with $NaHCO_3$ buffer (30 mM). Dehydration was performed by an ethanol dilution series with increasing ethanol concentrations (30%, 70%, 95% and 2 × 100%). Samples were then immersed in hexamethyldisilazane (HMDS; Sigma-Aldrich St. Louis, USA) twice for 30 s and dried at room temperature. No coating was applied to the samples as it is one of the advantages of using HIM. Imaging was performed with a Zeiss Orion NanoFab HIM (Carl Zeiss, Peabody, MA, USA) (Joens *et al.* 2013).

Identification of Fe minerals produced during photoferrotrophic growth

The Fe-mineral precipitates of the Norsminde Fjord isolate, grown under standard conditions (10 mM $FeCl_2$, 20°C, pH 6.8–7.0), were analyzed by Mössbauer spectroscopy. Due to the potential presence of oxygen-sensitive Fe(II)-containing minerals, samples were prepared inside an anoxic glovebox (100% N_2). Ten

milliliters of culture were filtered with a 0.45-μm syringe filter which was then fixed between two stripes of Kapton tape and stored frozen in a Schott bottle filled with N_2 until measurements. For measurements, the sample was inserted into a closed-cycle exchange gas cryostat (Janis cryogenics). Spectra were recorded at 77 K and 5 K in transmission geometry using a constant acceleration drive system (WissEL). A ^{57}Co source embedded in a Rhodium matrix was used as gamma radiation source. The sample spectra were calibrated against a 7-μm thick α-^{57}Fe foil at room temperature. The RECOIL software suite (University of Ottawa, Canada) was used for the calibration and the modeling of the spectra. The spectra were modeled using Voigt-based line shapes. The Lorentz half-with-half-maximum value was kept constant at 0.141 mm s^{-1} (determined from the minimum line width of the 3 and 4 peaks of the calibration foil) in the models, and the Gauss' sigma (σ) parameter was used to account for line broadening until the fitting was reasonable. The sample spectra were analyzed in respect to the center shift values (CS), the quadrupole shift or splitting (ε, QS) and the hyperfine field (H).

Quantification of Fe(II) and Fe(III) concentrations and calculation of Fe(II) oxidation rates

The concentrations Fe(II) and total Fe in the samples that were stabilized with HCl were determined with the spectrophotometric Ferrozine assay (Stookey 1970). For quantification of Fe(II), 20 μl of the sample was added to 80 μl 1 M HCl in a 96-well microtiter plate. To determine Fe total concentrations, 20 μl of the sample was added to 80 μl of the reducing agent hydroxylamine hydrochloride (HAHCl; 10% (w/v) hydroxylamine in 1 M HCl) and incubated for 30 min in the dark. The concentration of Fe(II) was then determined by adding 100 μl ferrozine solution (50% (w/v) $C_2H_3O_2NH_4$ and 0.1% (w/v) ferrozine in MQ water) and, after 5 min of incubation in the dark, measuring the absorption at 562 nm with a spectrophotometric plate reader (FlashScan 550; Analytic, Jena, Germany or Multiskan GO, Thermo Fisher Scientific, USA). Standards with concentrations ranging from 0.005 to 1 mM Fe(II) ($Fe(NH_4)_2SO_4$ in 1 M HCl) were treated in the same way and measured to determine a calibration curve. For calculation of Fe(III) concentrations, the concentration of Fe(II) was subtracted from the total Fe concentration.

Fe(II) oxidation rates were quantified by applying linear regression to the measured Fe(II) concentrations over time. Linear regression was applied over the visually steepest part of the Fe(II) curve and at least four points were used for the calculation. For the light dependence of Fe(II) oxidation rates, the maximum Fe(II) oxidation rate (V_{max}) and the half-light saturation (K_m) were calculated by modeling a Michaelis–Menten-like behavior. The values for V_{max} and K_m were determined by minimizing the value of χ^2 values using the Solver tool in Microsoft Excel, where χ^2 values were determined by comparing the fit of the resulting model to the measured Fe(II) oxidation rates.

RESULTS

Isolation and phylogenetic analysis

Hungate tubes containing ASW medium amended with 10 mM Fe(II) and an inoculum of Norsminde Fjord sediment showed the first orange rusty patches after 4 to 5 weeks of incubation in the light (as described previously, Laufer *et al.* 2016), suggesting the activity of phototrophic Fe(II) oxidizers. Cell growth was confirmed by fluorescence microscopy (data not shown). The highest dilution tube showing Fe(II) oxidation and presence of cells was used for the preparation of a further dilution series. After transfers over more than 10 dilution series, the cell morphology in the Norsminde Fjord culture was homogeneous. In fluorescence microscopy, the cells appeared to be small rods that are ca. 1.5–1.8 μm long and 0.5–0.6 μm wide. During the isolation procedure, sulfate was the only S source indicating that our isolate, like its close relatives *Chlorobium luteolum* and *C. ferrooxidans* (Heising *et al.* 1999; Frigaard and Bryant 2008), is not dependent on sulfide as a S source.

The phylogenetic relationship of the isolate with its closest relatives and other known photoferrotrophs from the purple-sulfur and purple non-sulfur bacteria based on the 16S rRNA gene is shown in Fig. 1. Phylogenetic analysis revealed that the closest relative of our photoferrotrophic culture from Norsminde Fjord is *C. luteolum* DSM 273 (Schmidle 1901; Pfennig and Trueper 1971) with 98% sequence identity. The closest relative photoferrotrophic strain is *C. ferrooxidans* KoFox (Heising *et al.* 1999) with 94% sequence identity. We designated the name *Chlorobium* sp. strain N1 to our isolate.

Physiological characterization

Fe(II) oxidation and growth

Under standard growth conditions (10 mM $FeCl_2$, 20°C, pH 6.8–7.0), we found maximum Fe(II) oxidation rates of 0.77 ± 0.02 mM d^{-1}. Within 11 days 94% and after 16 days, 97% of the Fe(II) was oxidized (Fig. 2). Concurrent with Fe(II) oxidation, cell numbers in the culture were increasing from initially 3.1 × 10^7 cells ml^{-1} to 6.7 × 10^8 cells ml^{-1}after 11 days, yielding a doubling time of ca. 0.4 days (9.6 h). With the amount of oxidized Fe(II) and the experimentally determined increase in cell numbers, we can estimate a stoichiometry of $Fe(II)_{oxidized}$ to C_{fixed}. Within 11 days, per ml 6.4 × 10^8 cells were formed and per ml 0.008 mmoles Fe(II) were oxidized. Assuming an average dry weight of a microbial cell of 0.2 × 10^{-12} g and a carbon content of 50% per cell weight, as commonly assumed for soil and subsurface bacteria (Bratbak and Dundas 1984; Whitman, Coleman and Wiebe 1998), we can estimate that the ratio of $Fe(II)_{oxidized}$ to C_{fixed} was 1.5:1. This value is lower than the 4:1 stoichiometry that is expected when following Equation 1. The difference between the theoretical value and the estimated value based on the experimental data is likely explained by the estimations for weight and C content per cell used for this calculation. To reach the 4:1 stoichiometry, the cells would need to be about 62% smaller than the theoretically assumed value, yielding an average C content per cell of 3.8 × 10^{-14} g (dw) and a cellular size of about 1.8 × 0.5 μm, what fits well to the above described cell size. To determine a more accurate stoichiometry of $Fe(II)_{oxidized}$ to C_{fixed} it would be necessary to actually quantify the increase in organic carbon in the culture during growth on Fe(II).

Alternative growth substrates

Tests for phototrophic growth on alternative substrates revealed that the isolate from Norsminde Fjord can grow phototrophically with several other organic and inorganic electron donors (Table 1). On most substrates on which growth was observed, growth was fast and a greenish color, indicative for pigments of green-sulfur bacteria, was observed within <1 week. The only exceptions were H_2 and elemental sulfur, where a greenish color was detected only after >2 weeks and >1 month, respectively. Cultures that showed growth were at least transferred one more time on the same substrate to confirm growth-related consumption of this substrate. Furthermore, the comparison of total cell

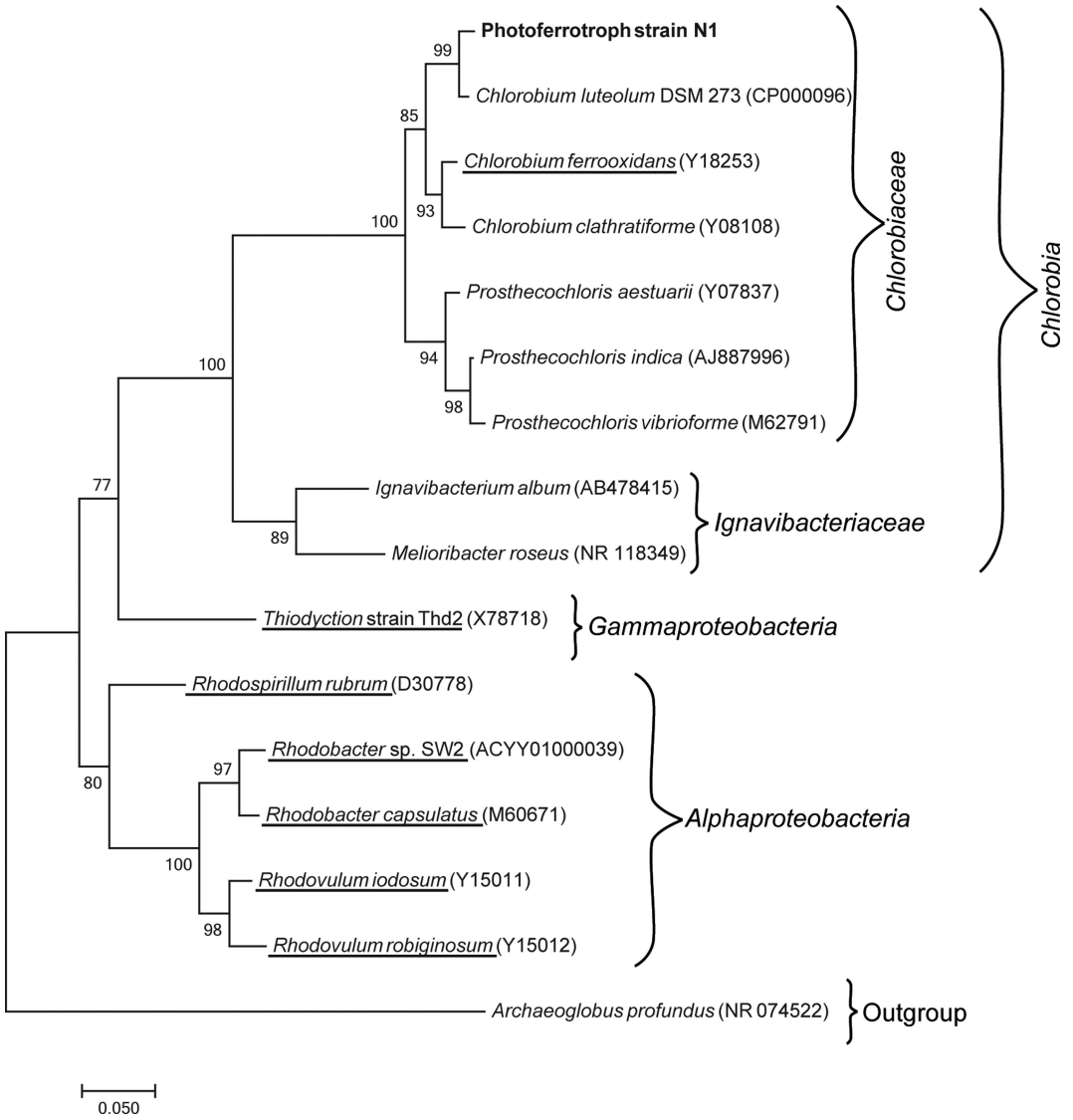

Figure 1. Phylogenetic tree constructed with the maximum-likelihood method based on the 16S rRNA gene showing the relation of the photoferrotrophic isolate from Norsminde Fjord (in bold) and its closest relative, *C. luteolum*, to other *Chlorobia* and other anoxygenic phototrophic bacteria that were reported to be capable of Fe(II) oxidation (underlined). Archaeoglobus profundus was included as outgroup. The scale bar corresponds to 0.05 nucleotide substitutions per site. At the branches, bootstrap values (from 1000 replications) are indicated.

numbers and the number of autofluorescent cells for cultures grown under phototrophic conditions showed that 97.8%–100% of the total cells were also autofluorescent (Table S1; Fig. S1, Supporting Information). We did not observe growth with synthetic FeS (Table S1) what was surprising because the isolate grew both with either Fe(II) or H_2S in individual setups. Additionally, no growth was found with any of the tested complex media under oxic conditions. We did also not detect anaerobic growth by reduction of Fe(III), SO_4^{2-} or NO_3^-. Based on the results of the cell counts and the lack of growth in the dark (with organic carbon and sulfate, nitrate or Fe(III) as electron acceptor), we are confident to claim that we have a pure culture of GSB and we can rule out the possibility of having a secret sulfur cycle (e.g. by a sulfate reducer that is able to use organics as e-donor in the dark).

Light dependence of Fe(II) oxidation rates

We found a sharp increase of Fe(II) oxidation rates between 0 and 470 lux and a less steep increase between 470 and 1270 lux (Fig. 3A). At light intensities of 1270 and 470 lux, only a short lag phase of 3 days was observed whereas at 47 lux a longer lag phase of 7 days occurred (Fig. S2, Supporting Information). Fitting a Michaelis–Menten kinetic model to the calculated rates revealed that half-light saturation (K_m) was reached at a light intensity of 220 lux and the maximum light dependent Fe(II) oxidation rates were around 0.59 mM d^{-1}. The fit of the model was good and yielded a R^2 of 0.99 (Fig. 3A).

pH dependence of Fe(II) oxidation rates

Fe(II) was oxidized between pH 6.3 and 8.4 with an optimum at pH 7.0–7.3, where Fe(II) oxidation rates of 0.53–0.54 mM d^{-1} were reached (Fig. 3B, Fig. S3, Supporting Information). Between pH 6.7 and 7.9, no lag phase was observed while at pH 6.3, 6.5 and 8.4 a lag phase of 7 days was observed (Fig. S3). Within 24 days of incubation, no Fe(II) oxidation was detected at the lowest and highest tested pH values of 6 and 9.1 (Fig. 3B; Figs S3 and S4, Supporting Information).

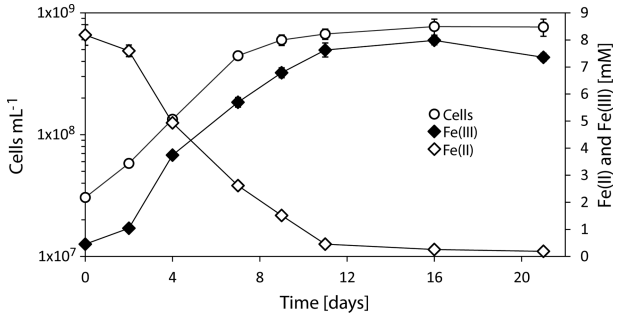

Figure 2. Fe(II) oxidation and growth curve of the photoferrotrophic isolate from Norsminde Fjord in ASW medium with a salinity of 23, 10 mM $FeCl_2$, 20°C and 380 lux. Error bars indicate standard deviation of triplicate cultures (in some cases smaller than symbol size).

Temperature dependence of Fe(II) oxidation rates

Fe(II) was oxidized between 10°C and 37°C (Fig. 3C; Fig. S5, Supporting Information). No Fe(II) oxidation was detected at 7°C or 45°C within 22 days. Highest rates of Fe(II) oxidation were measured at 25°C (0.72 mM d^{-1}) (Fig. 3C). No lag phase was observed at all temperatures except for the 10°C incubation, where a lag phase of 10 days was found (Fig. S5).

Salinity dependence of Fe(II) oxidation rates

The Fe(II) oxidation rates determined in this experiment were generally rather low compared to the rates found in the other experiments. Fe(II) was oxidized at all tested salinities at comparable rates, with the exception of the highest tested salinity, where the rates were significantly lower (Fig. 3D; Fig. S6, Supporting Information). No lag phases were observed at salinities from 1.6 to 25.8. At salinities of 35.6 and 50, a lag phase of 6 days was observed (Fig. S6).

Fe(II) oxidation tests with the closest relative, *Chlorobium luteolum* DSM 273

We did not observe any Fe(II) oxidation by *C. luteolum* DSM 273 under all conditions tested. However, in the simultaneous presence of Fe(II) and H_2S, that formed FeS as indicated by a black precipitate, growth was observed. Growth occurred without oxidation of Fe(II), suggesting that the culture was growing by oxidation of sulfide released from the FeS. These results suggested that the Fe(II) is not toxic to this strain, but that this microorganism is not able to oxidize Fe(II).

Microscopic investigation of cell–mineral aggregates formed by the photoferrotrophic isolate

Fluorescence microscopy analysis of the isolate from Norsminde Fjord that was grown on Fe(II) revealed a close association of cells and Fe(II) minerals (Fig. 4). Almost all cells were attached to minerals. HIM imaging of the culture also revealed a close association of cells and minerals, the absence of encrusted cells and clearly demonstrated that the cell surfaces are smooth and free of Fe(III) minerals (Fig. 4).

Identification of Fe minerals present in cultures of the photoferrotrophic isolate

After 7 days of incubation under standard conditions (10 mM $FeCl_2$, 20°C, pH 6.8–7.0), when ca. 70% of the Fe(II) was oxidized (Fig. 2), a sample for identification of Fe mineralogy by Mössbauer spectroscopy was taken. At 77 K, the Mössbauer spectrum showed two clearly separated paramagnetic doublets (Fig. 5). The narrow one with a center shift (CS) = 0.46 mm/s and a quadrupole splitting (QS) = 0.78 mm/s (Table S2, Supporting Information) consists of two components (not shown here) and corresponds to Fe(III) oxyhydroxide (Murad and Schwertmann 1980). The wider one has a CS = 1.28 mm/s and a QS =

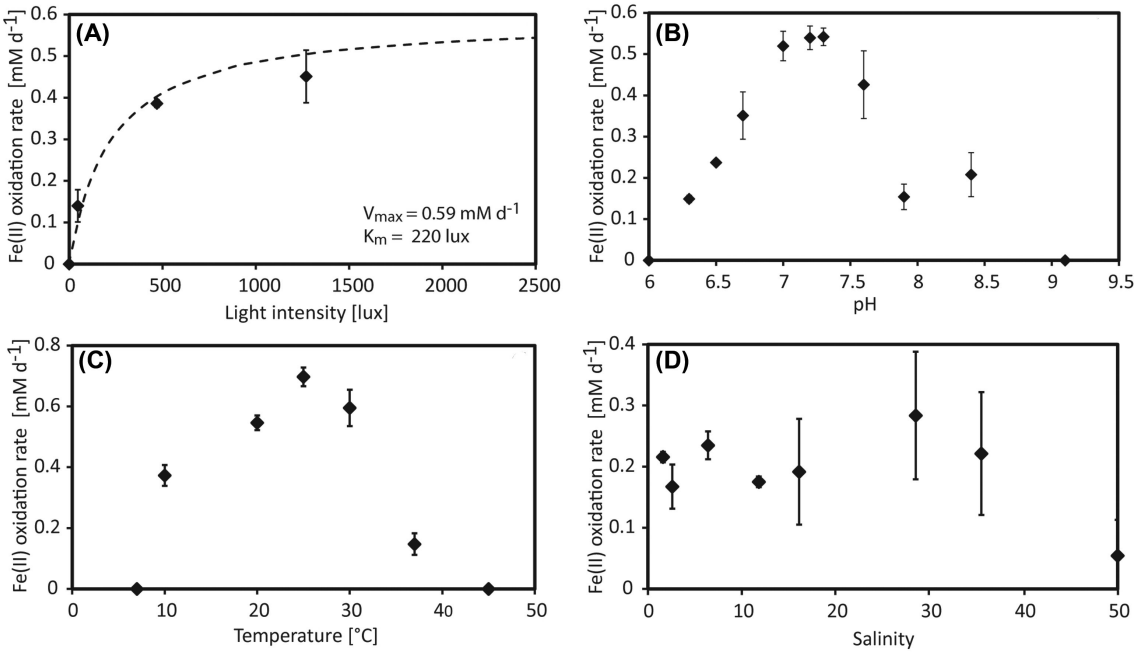

Figure 3. Dependence of Fe(II) oxidation rates of the photoferrotrophic isolate from Norsminde Fjord on (**A**) light intensity, (**B**) pH, (**C**) temperature and (**D**) salinity. Means of rate calculations from triplicate cultures are shown. Error bars indicate standard deviations of triplicates (sometimes smaller than symbol size). In panel **A**, the dashed line indicates the light dependence of Fe(II) oxidation that was modeled by applying Michaelis–Menten kinetics.

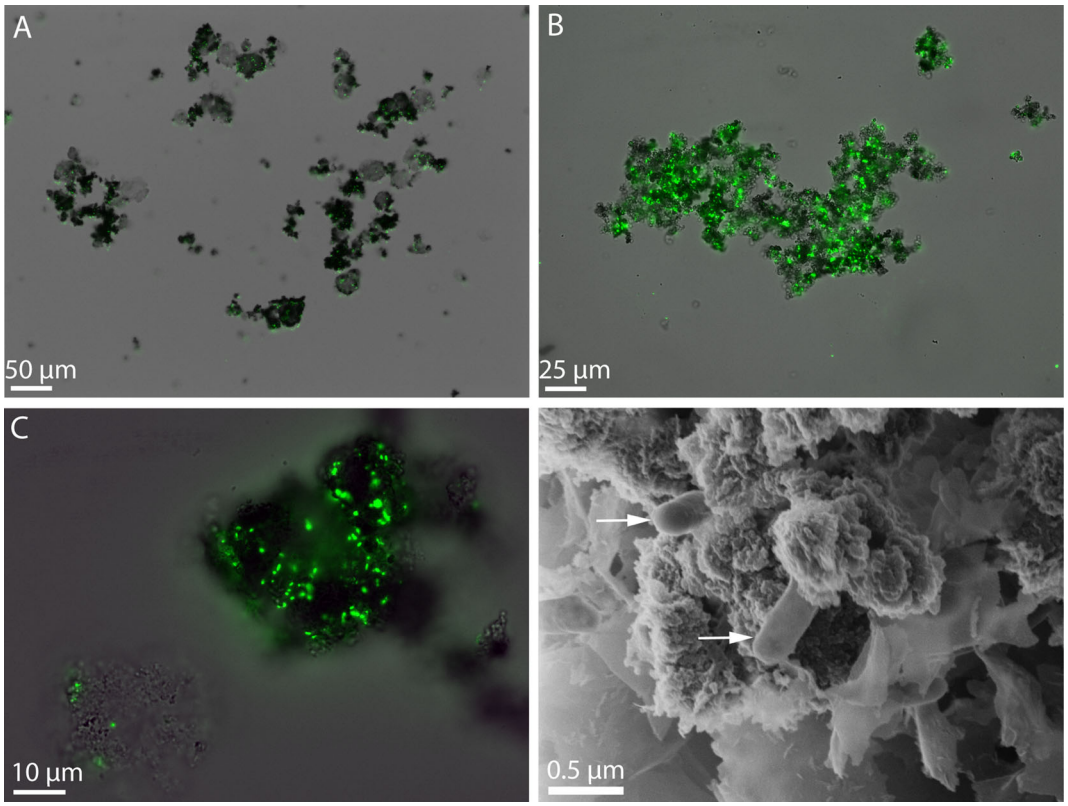

Figure 4. Associations of Fe(III) minerals with cells of the isolate from Norsminde Fjord when grown under standard conditions (20°C, pH 6.8–7.0) with 10 mM of Fe(II). Panels A–C are overlays of fluorescence and bright field micrographs. Cells are green fluorescent (stained with LIFE/DEAD stain). Panel D shows a helium-ion micrograph of the sample acquired by Matthias Schmidt (ProVIS/UFZ). White arrows point on microbial cells.

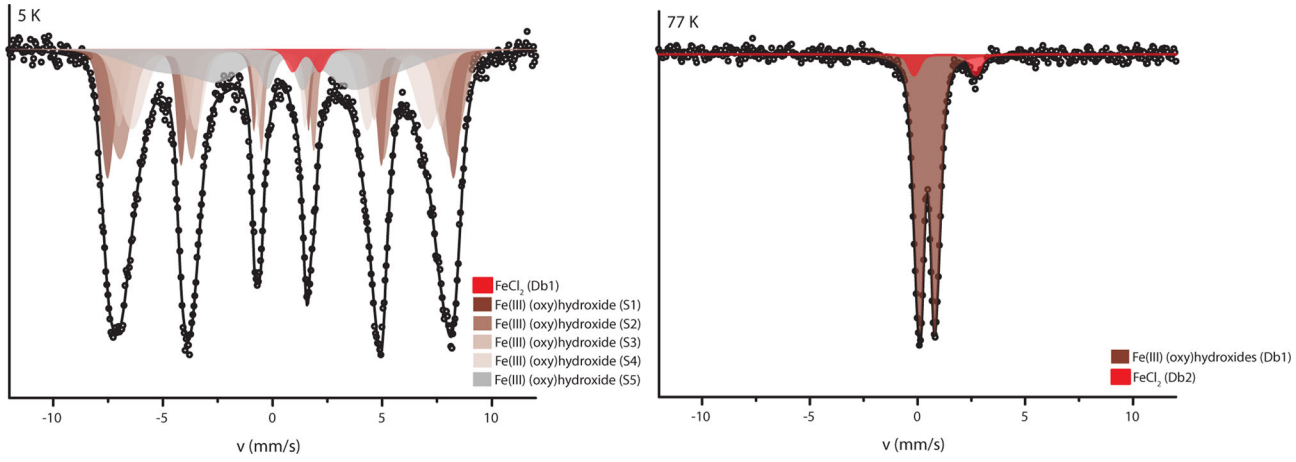

Figure 5. Mössbauer spectra of the Fe minerals produced by the isolate from Norsminde Fjord when oxidizing 5.5 mM of Fe(II) (ca. 70% of total Fe(II)). Left: Mössbauer spectrum collected at 5 K showing a sextet with broad absorption lines and inner line broadening. The spectrum was fitted with five sextets with different H values ranging from 30 to 49 T and a narrow doublet. Right: Mössbauer spectrum collected at 77 K fitted with Fe(III) and Fe(II) doublet.

2.86 mm/s indicating a Fe(II) phase (Murad 2010). At 5 K, a sextet has been established that is characterized by very broad absorption lines with inner line broadening (Fig. 5). In total five different sextets with hyperfine field (H) values ranging in total from 30 T to 49 T were needed to fit this absorption pattern (Table S2). The sextet with the lowest H value (S5) is collapsed (Fig. 5). When lowering the temperature to 5 K, the Fe(II) phase is still present, but with a lowered QS of 1.25 mm/s (Table S2). On the one hand, the lack of magnetic splitting at 77 K which would be indicative of more crystalline Fe(III) phases suggests that mainly an amorphous or poorly crystalline Fe(III) phase-like ferrihydrite exists. On the other hand, the occurrence of several sextets including a collapsed one at 5 K with partly negative quadrupole shift (ε)values does not support the presence of ferrihydrite only. Instead, the fit suggests a more complex mixture of Fe(III) minerals potentially consisting of ferrihydrite, akaganeite and/or lepidocrocite. Alternatively, the sextets could represent different degrees of structural distortion of the Fe(III) mineral due to association of organics or incorporation of other cations. The presence of a collapsed sextet at 5 K is very unusual because

it indicates the transition from paramagnetic to magnetic behavior of a Fe phase, but even amorphous ferrihydrite is already magnetically ordered at temperatures above 5 K. That means that a highly disturbed amorphous Fe(III) phase must be present in the mineral sample produced by our phototrophic Fe(II) oxidizer.

DISCUSSION

Physiology of the isolated marine anoxygenic phototrophic Fe(II)-oxidizing green-sulfur bacterium—implications for the ecological niche occupation of these Fe(II) oxidizers

The photoferrotrophic isolate from Norsminde Fjord presented here is only the third isolate of a photoferrotrophic green-sulfur bacterium, and the first isolate from a marine sediment. The other two isolates are the freshwater photoferrotrophic green-sulfur bacteria *Chlorobium ferrooxidans* KoFox, and *C. phaeoferrooxidans* that were isolated from a ditch close to the University of Konstanz (Germany) and ferruginous waters of Lake Kivu (East Africa), respectively (Heising *et al.* 1999; Llirós *et al.* 2015; Crowe *et al.* 2017). Additionally, there are further recent reports about natural populations of photoferrotrophic green-sulfur bacteria in stratified lakes (Crowe *et al.* 2008; Walter *et al.* 2014; Llirós *et al.* 2015). Our isolate is the first photoferrotrophic green-sulfur bacterium that was isolated from a marine habitat, and it has therefore potential to be used as a model strain for reconstruction of photoferrotrophy in ancient oceans.

Besides our novel green-sulfur photoferrotrophic isolate, there are two further isolates of phototrophic Fe(II) oxidizers from marine sediments, the purple non-sulfur bacteria *Rhodovulum iodosum* and *R. robiginosum,* which were both isolated from a mud flat in the German Wadden Sea (Straub, Rainey and Widdel 1999). Compared to the isolate from Norsminde Fjord, these strains have a much more narrow pH range and only oxidize Fe(II) between pH 6.3 and 6.8 (Straub, Rainey and Widdel 1999). The pH range in which Fe(II) oxidation was possible for our isolate (6.3–8.5) was much broader than for the two *Rhodovulum* strains and was overlapping with the range of the freshwater isolates (5.5–7.5; Hegler *et al.* 2008) but was shifted more towards higher pH ranges (6.3–8.5). This is beneficial considering that the pH of seawater is around 8 and that the pH in the pore water of the anoxic layers of sunlit sediments usually varies between ca. 8.0 and 6.9 (Ben-Yaakov 1973; Marion *et al.* 2011) and implies that our isolate is much better adapted to perform Fe(II) oxidation in marine habitats than the two *Rhodovulum* strains.

A specific characteristic of green-sulfur bacteria is that they are well adapted to low-light conditions (Overmann 2006). Compared to *C. ferrooxidans* strain KoFox, which reaches light saturation already below 50 lux (Hegler *et al.* 2008), our isolate has a relatively high half-light saturation of 220 lux and reaches saturation only above 400 lux, above which Fe(II) oxidation rate is not further increasing. However, the Fe(II) oxidation rate of our isolate under standard conditions (0.77 ± 0.02 mM d^{-1}, at 20°C and 380 lux) was comparable to the rate measured for strain KoFox (0.82 ± 0.7 mM d^{-1} at 20°C and 450 lux) (Gauger *et al.* 2016). More precise rate measurements with more frequent sampling time points and under lower light intensities should be made to more precisely determine the Fe(II) oxidation rates of the isolate at low-light intensities.

In addition to their adaptation to relatively low-light intensities, a further characteristic of the green-sulfur bacteria of the *Chlorobiaceae* family is that they are generally very restricted in their metabolic capacities. They are obligately phototrophic and strictly anaerobic (Overmann 2006; Imhoff 2014). With only one exception, which is the Fe(II)-oxidizing *C. ferrooxidans* KoFox (Heising *et al.* 1999), all *Chlorobiaceae* can oxidize sulfide, elemental sulfur and some can also use thiosulfate and sulfite (Trüper 1981; Imhoff 2014). As a green-sulfur bacterium, our new isolate from Norsminde Fjord is remarkable since it uses, besides different inorganic substrates like H_2S or H_2, also a broad range of organic compounds, including fatty acids, alcohols, sugar and even yeast extract for growth. In general, green-sulfur bacteria can only use a limited range of organic compounds (Trüper 1981) in their carbon metabolism. For most *Chlorobiaceae* only acetate and pyruvate are known to be used and only in the presence an inorganic electron donor-like hydrogen (Imhoff 2014). Due to these limited metabolic capacities, the ecological niche of green-sulfur bacteria is rather restricted and usually they are thought to only occur in the narrow zone where the gradients of oxygen and sulfide overlap in sunlit environments. The finding of broad range of growth substrates, which can be used by our isolate from Norsminde Fjord, widens the ecological niche of this bacterial group.

Absence of oxidation of FeS minerals by the isolated green-sulfur photoferrotroph—implications for Fe(II) oxidation mechanism

Despite the ability of the photoferrotrophic isolate to grow on Fe(II) and H_2S (when these substrates are provided individually), it could not grow on synthetic FeS. Usage of FeS as an electron donor would be an important capability for this marine photoferrotroph, as this Fe(II) mineral is present in significant quantities in marine sediments (Canfield 1989; Moeslund, Thamdrup and Jørgensen 1994). Kappler and Newman (2004) showed that the freshwater photoferrotrophs *Rhodobacter ferrooxidans* strain SW2, *C. ferrooxidans* strain KoFox and *Thiodyction* sp. strain F4 were able to grow on FeS, however, with significantly lower Fe(II) oxidation rates compared to growth on ca. 5 mM dissolved Fe(II). Their measurements suggest that the FeS used in their study was relatively soluble and the strains were probably oxidizing dissolved Fe(II) rather than the solid substrate. Fe(II) oxidation rates would then be controlled by the dissolution rates of the used FeS mineral. Since such a behavior was not observed in our experiments, it is likely that in our incubations the FeS was less soluble, probably due to the age and crystallinity which might have been higher for our used FeS (Berner 1967; Rickard 2006). Therefore, the Fe(II) and the sulfide concentrations were obviously too low to support growth of our isolate. This also means that our isolate is probably not able to use solid-phase electron donors and implies that, as suggested before, Fe(II) oxidation is happening in the periplasm and requires a dissolved Fe(II) species (Croal, Jiao and Newman 2007).

To understand the mechanism of Fe(II) oxidation, it is essential to identify the molecular components involved in Fe(II) oxidation. A study that searched for homologs of a gene that was recently proposed to encode for an outer membrane cytochrome that is involved in microaerophilic Fe(II) oxidation, $Cyc2_{PV\text{-}1}$, found this gene to be present in the genome of the Fe(II)-oxidizing green-sulfur bacterium *C. ferrooxidans* strain KoFox and other sulfur-oxidizing species of the *Chlorobiaceae,* including *C. luteolum* DSM 273, the closest relative of our photoferrotrophic isolate from Norsminde Fjord (Kato *et al.* 2015). $Cyc2_{PV\text{-}1}$ shares homology with the outer membrane Fe oxidase of acidophilic Fe(II) oxidizers (Castelle *et al.* 2008; Barco *et al.* 2015). This indicates that the oxidation of solid-phase Fe(II) should

be possible when the cells can have direct contact with the solid substrate. However, although the closest relative of our isolate, *C. luteolum* DSM 273, also contains a homolog of this gene, it was unable to oxidize Fe(II). This could either be just a result of the incubation conditions, although we tested different Fe(II) concentrations and different media, or it could also be that genes encoding other proteins involved in Fe(II) oxidation, which are yet not known, are missing in this strain. Indeed, there is one photoferrotrophic strain that is known to be able to oxidize solid-phase Fe(II), i.e. the purple non-sulfur bacterium *Rhodopseudomonas palustris* TIE-1, which was even shown to be able to grow on electrodes or on magnetite, which is a mixed Fe(II)/Fe(III) mineral (Bose *et al.* 2014; Byrne *et al.* 2015). However, for the majority of the remaining isolates, including our isolate, evidence for the ability to use solid-phase Fe(II) as electron source is missing.

Minerals produced by the photoferrotrophic isolate from Norsminde Fjord

Mössbauer spectroscopy revealed that the product of microbial Fe(II) oxidation by the isolate from Norsminde Fjord, the Fe(III) minerals, consists of a mixture of poorly crystalline and/or short range ordered Fe(III) minerals such as ferrihydrite, lepidocrocite and/or akaganeite, similar to previous studies with photoferrotrophic bacteria (Straub, Rainey and Widdel 1999; Kappler and Newman 2004; Wu *et al.* 2014; Gauger *et al.* 2016).

Microscopic analyses showed a close association of cells and minerals, but mostly non-encrusted cells, as previously observed for photoferrotrophic bacteria (Jiao *et al.* 2005; Schädler *et al.* 2009; Gauger, Konhauser and Kappler 2015; Gauger *et al.* 2016). Wu *et al.* (2014) also found a close association of cells and Fe(III) minerals in marine photoferrotrophs but no encrustation of cells with Fe(III) minerals. By confocal laser scanning microscopy using different specific dyes they could determine that the Fe(III) instead was closely associated with extracellular polymeric substances (EPS), suggesting that EPS can help to prevent cell encrustation with Fe(III) minerals (Schädler *et al.* 2009; Wu *et al.* 2014). A tight association of organics and minerals is also possible in strain N1 because of the presence of a poorly ordered sextet phase in the 5 K spectrum which is not expected of pure short range ordered phases (e.g. ferrihydrite, lepidocrocite or akaganeite) and perhaps indicates the presence of organics which constrained the mineral growth (Chen, Kukkadapu and Sparks 2015; Mehlhorn *et al.* 2016).

Potential for photoferrotrophy in situ in the sediment of Norsminde Fjord

In the sediment from Norsminde Fjord, bacteria are exposed to dynamic conditions including fluctuations of salinity, temperature, pH or light intensity. These changes can occur on a short-term (hours-days) and long-term (weeks-months) timescale. Temperatures in the upper few mm of the sediment can vary from close to or even below zero in winter to 30°C –40°C in summer when solar irradiation is heating the shallow-water sediment. The isolate is well adapted for the higher range of temperatures, but less good for the lower temperature range. However, it was found that at low temperatures (around 2°C –5°C), when bacterial activities in the sediment are generally lower, oxygen is penetrating deeper into the sediment (5–6 mm) than light (ca. 2 mm) (Laufer *et al.* 2016). Therefore, in winter the activity of green-sulfur bacteria or any other anoxygenic phototrophic bacteria that are strict anaerobes should be inhibited by oxygen. Our isolate from Norsminde Fjord can also cope remarkably well with changes in salinities. This is a significant advantage in an estuarine sediment, such as in Norsminde Fjord sediment, where we measured salinities ranging from 14 to 23 (Laufer *et al.* 2016). However, based on wind and precipitation probably also much lower salinities can be encountered. Also the pH range of the isolate fits well to the conditions that are found in the sediment from Norsminde Fjord (Laufer *et al.* 2016). In summary, based on the physiological characteristics of the photoferrotrophic isolate and the geochemical characteristics of the sediment of Norsminde Fjord, the isolate should be able to perform Fe(II) oxidation *in situ* in the sediment as long as temperature is not too low.

Based on the metabolic capabilities and the resulting potential for metabolic flexibility of the isolate from Norsminde Fjord, one major question remaining is, what is the metabolism on which strain N1 actually lives *in situ* in the sediment? In the sediment from Norsminde Fjord, the isolate probably has simultaneously access to several possible electron donors including Fe(II), different organic compounds or H_2S. It is known that whether a photoferrotroph prefers Fe(II) or organic compounds, when both are available simultaneously, depends on the identity of the carbon source (Melton *et al.* 2014a) and that the preferences are different for different strains (Ehrenreich and Widdel 1994). Some strains are even known to oxidize organic carbon and Fe(II) simultaneously (Ehrenreich and Widdel 1994; Melton *et al.* 2014a). It has also been shown that organic carbon can stimulate phototrophic Fe(II) oxidation (Kopf and Newman 2012). Also for *C. ferrooxidans* strain KoFox, it was shown that Fe(II) oxidation is stimulated in the presence of hydrogen, acetate, pyruvate, fumarate, cysteine or thiosulfate, despite it could not grow on thiosulfate alone (Heising *et al.* 1999). Thus, the presence of organic carbon or other inorganic electron donors, which are thermodynamically even more favorable than Fe(II), does not necessarily lead to a preference of these substrates over Fe(II). To the best of our knowledge, there is currently no study involving Fe(II)-oxidizing bacteria that has investigated the co-occurrence of several potential electron donors, including Fe(II), at environmentally relevant concentrations, which would be necessary to draw reliable conclusions about the metabolism on which these bacteria live in their natural habitats. It has to be elucidated in future experiments which substrate the photoferrotrophic isolate from Norsminde Fjord would prefer when it has access to several electron donors at environmentally relevant concentrations and if it can use concurrently Fe(II) and other organic or inorganic substrates with a growth benefit.

Suitability of the isolate from Norsminde Fjord as a model organism for photoferrotrophy on early Earth

Conditions suggested for the Archean when photoferrotrophs could have thrived and contributed to the production of Fe(III) minerals that formed the BIFs (Konhauser *et al.* 2002; Kappler *et al.* 2005; Posth, Konhauser and Kappler 2013) fit well to the physiological range of our photoferrotrophic isolate from Norsminde Fjord. In addition to high Fe(II) concentrations in the Archean of 50 μM to 1 mM (Holland 1973; Morris 1993), it is known that the Archean atmosphere was anoxic (Saito, Sigman and Morel 2003; Canfield 2005). The salinity of the Archean ocean was probably up to two times the present salinity (Knauth 2005), while sulfate concentrations were low (Crowe *et al.* 2014), and dissolved silica concentrations were high (>1 mM) (Treguer *et al.* 1995; Jones *et al.* 2015). The pH is assumed to have been >6.5 (Saito, Sigman and Morel 2003). The estimates for temperatures

in the Archean ocean range from 10°C to 85°C (Knauth 2005; Kasting *et al.* 2006; Robert and Chaussidon 2006; Jaffrés, Shields and Wallmann 2007; Shields and Kasting 2007), with temperatures around 10°C–33°C being most likely (Kasting *et al.* 2006; Posth *et al.* 2008). In summary, the photoferrotrophic isolate from Norsminde Fjord would be able to thrive under conditions that prevailed in the Archean, however, only in the lower range of the estimates for salinity and temperature. It furthermore remains to be tested how the strain can cope with high silica concentrations. Only recently a study by Gauger *et al.* (2016) demonstrated that the presence of dissolved silica even stimulated Fe(II) oxidation rates of the freshwater photoferrotroph *C. ferrooxidans* KoFox probably because silica decreased the toxicity of Fe(II) due to changes in Fe complexation and Fe speciation (Gauger *et al.* 2016).

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGEMENTS

We thank Karin Stögerer (deceased) for help with the molecular biological analyses and Wiebke Ruschmeier for cultivation of *C. luteolum*. Furthermore, we thank Dr Cindy Lockwood for useful advice on how to build the phylogenetic tree. The authors are grateful for using the helium ion microscope at ProVIS - Centre for Chemical Microscopy at the Helmholtz Centre for Environmental Research.

FUNDING

This project was supported by the European Research Council under the European Union's Seventh Framework Program (FP/2007–2013)/ERC Grant, agreement no. 307320-MICROFOX and no. 294200. The research underlying this article has been co-funded by the Danish National Research Foundation/grant Agreement nr. DNRF104. The ProVis-Centre for Chemical Microscopy at the Helmholz Centre for Environmental Research is supported by Europäischer Fonds für regionale Entwicklung (EFRE), Freistaat Sachsen and the Helmholtz Association.

Conflict of interest. None declared.

REFERENCES

Barco RA, Emerson D, Sylvan JB *et al.* New insight into microbial iron oxidation as revealed by the proteomic profile of an obligate iron-oxidizing chemolithoautotroph. *Appl Environ Microb* 2015;**81**:5927–37.

Bekker A, Slack JF, Planavsky N *et al.* Iron formation: the sedimentary product of a complex interplay among mantle, tectonic, oceanic, and biospheric processes. *Econ Geol* 2010;**105**:467–508.

Ben-Yaakov S. pH buffering of pore water of recent anoxic marine sediments. *Limnol Oceanogr* 1973;**18**:86–94.

Berner RA. Thermodynamic stability of sedimentary iron sulfides. *Am J Sci* 1967;**265**:773–85.

Beukes NJ, Klein C, Holland HD *et al. Proterozoic Atmosphere and Ocean*. New York: Cambridge University Press, 1992.

Bird DF, Karl DM. Microbial biomass and population diversity in the upper water column of the Black Sea. *Deep Sea Res* 1991;**38**:S1069–82.

Bose A, Gardel EJ, Vidoudez C *et al.* Electron uptake by iron-oxidizing phototrophic bacteria. *Nat Commun* 2014;**5**, DOI: 10.1038/ncomms4391.

Bratbak G, Dundas I. Bacterial dry matter content and biomass estimates. *Appl Environ Microb* 1984;**48**:755–7.

Byrne JM, Klueglein N, Pearce C *et al.* Redox cycling of Fe(II) and Fe(III) in magnetite by Fe-metabolizing bacteria. *Science (80-)* 2015;**347**:1473–6.

Canfield DE. Reactive iron in marine sediments. *Geochim Cosmochim Acta* 1989;**53**:619–32.

Canfield DE. The early history of atmospheric oxygen: homage to Robert M. Garrels. *Annu Rev Earth Pl Sc* 2005;**33**:1–36.

Canfield DE, Rosing MT, Bjerrum C. Early anaerobic metabolisms. *Philos T Roy Soc B* 2006;**361**:1819–36.

Castelle C, Guiral M, Malarte G *et al.* A new iron-oxidizing/O_2-reducing supercomplex spanning both inner and outer membranes, isolated from the extreme acidophile *Acidithiobacillus ferrooxidans*. *J Biol Chem* 2008;**283**:25803–11.

Chen C, Kukkadapu R, Sparks DL. Influence of coprecipitated organic matter on $Fe^{2+}_{(aq)}$-catalyzed transformation of ferrihydrite: implications for carbon dynamics. *Environ Sci Technol* 2015;**49**:10927–36.

Cornell RM, Schwertmann U. *The Iron Oxides: Structure, Properties, Reactions, Occurrences and Uses*. Weinheim: John Wiley & Sons, 2003.

Croal LR, Jiao Y, Newman DK. The fox operon from *Rhodobacter* strain SW2 promotes phototrophic Fe(II) oxidation in *Rhodobacter capsulatus* SB1003. *J Bacteriol* 2007;**189**:1774–82.

Croal LR, Johnson CM, Beard BL *et al.* Iron isotope fractionation by Fe(II)-oxidizing photoautotrophic bacteria. *Geochim Cosmochim Acta* 2004;**68**:1227–42.

Crowe SA, Hahn AS, Morgan-Lang C *et al.* Draft genome sequence of the pelagic photoferrotroph *Chlorobium phaeoferrooxidans*. *Genome Announc* 2017;**5**, DOI: 10.1128/genomeA.01584-16.

Crowe SA, Jones C, Katsev S *et al.* Photoferrotrophs thrive in an Archean Ocean analogue. *P Natl Acad Sci USA* 2008;**105**:15938–43.

Crowe SA, Paris G, Katsev S *et al.* Sulfate was a trace constituent of Archean seawater. *Science (80-)* 2014;**346**:735–9.

Druschel GK, Emerson D, Sutka R *et al.* Low-oxygen and chemical kinetic constraints on the geochemical niche of neutrophilic iron(II) oxidizing microorganisms. *Geochim Cosmochim Acta* 2008;**72**:3358–70.

Ehrenreich A, Widdel F. Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Appl Environ Microb* 1994;**60**:4517–26.

Eickhoff M, Birgel D, Talbot HM *et al.* Oxidation of Fe(II) leads to increased C-2 methylation of pentacyclic triterpenoids in the anoxygenic phototrophic bacterium *Rhodopseudomonas palustris* strain TIE-1. *Geobiology* 2013;**11**:268–78.

Emerson D, Moyer C. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl Environ Microb* 1997;**63**:4784–92.

Frigaard N-U, Bryant DA. Genomic insights into the sulfur metabolism of phototrophic green sulfur bacteria. In: Hell R, Dahl C, Knoff D *et al.* (eds). *Sulfur Metabolism in Phototrophic Organisms*, Netherlands: Springer, 2008, 337–55.

Gauger T, Byrne JM, Konhauser KO *et al.* Influence of organics and silica on Fe(II) oxidation rates and cell–mineral aggregate formation by the green-sulfur Fe(II)-oxidizing bacterium *Chlorobium ferrooxidans* KoFox – Implications for Fe(II) oxidation in ancient oceans. *Earth Planet Sci Lett* 2016;**443**:81–9.

Gauger T, Konhauser K, Kappler A. Protection of phototrophic iron (II)-oxidizing bacteria from UV irradiation by biogenic iron (III) minerals: implications for early Archean banded iron formation. *Geology* 2015;**43**:1067–70.

Hegler F, Posth NR, Jiang J *et al.* Physiology of phototrophic iron(II)-oxidizing bacteria: implications for modern and ancient environments. *FEMS Microbiol Ecol* 2008;**66**:250–60.

Heising S, Richter L, Ludwig W *et al.* *Chlorobium ferrooxidans* sp. nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "*Geospirillum*" sp. strain. *Arch Microbiol* 1999;**172**:116–24.

Holland HD. The oceans; a possible source of iron in iron-formations. *Econ Geol* 1973;**68**:1169–72.

Imhoff JF. The family *Chlorobiaceae*. In: Rosenberg E, DeLong EF, Lory S *et al.* (eds). *The Prokaryotes*. Berlin-Heidelberg: Springer, 2014, 501–14.

Jaffrés JBD, Shields GA, Wallmann K. The oxygen isotope evolution of seawater: a critical review of a long-standing controversy and an improved geological water cycle model for the past 3.4 billion years. *Earth-Science Rev* 2007;**83**: 83–122.

Jiao Y, Kappler A, Croal LR *et al.* Isolation and characterization of a genetically tractable photoautotrophic Fe(II)-oxidizing bacterium, *Rhodopseudomonas palustris* strain TIE-1. *Appl Environ Microb* 2005;**71**:4487–96.

Joens MS, Huynh C, Kasuboski JM *et al.* Helium Ion Microscopy (HIM) for the imaging of biological samples at sub-nanometer resolution. *Sci Rep* 2013;**3**:3514.

Jones C, Nomosatryo S, Crowe SA *et al.* Iron oxides, divalent cations, silica, and the early earth phosphorus crisis. *Geology* 2015;**43**:135–8.

Kappler A, Newman DK. Formation of Fe(III)-minerals by Fe(II)-oxidizing photoautotrophic bacteria. *Geochim Cosmochim Acta* 2004;**68**:1217–26.

Kappler A, Pasquero C, Konhauser KO *et al.* Deposition of banded iron formations by anoxygenic phototrophic Fe(II)-oxidizing bacteria. *Geology* 2005;**33**:865–8.

Kasting JF, Howard MT, Wallmann K *et al.* Paleoclimates, ocean depth, and the oxygen isotopic composition of seawater. *Earth Planet Sci Lett* 2006;**252**:82–93.

Kato S, Ohkuma M, Powell DH *et al.* Comparative genomic insights into ecophysiology of neutrophilic, microaerophilic iron oxidizing bacteria. *Front Microbiol* 2015;**6**, DOI: 10.3389/fmicb.2015.01265.

Knauth LP. Temperature and salinity history of the Precambrian ocean: implications for the course of microbial evolution. *Palaeogeogr Palaeocl* 2005;**219**:53–69.

Konhauser KO, Hamade T, Raiswell R *et al.* Could bacteria have formed the Precambrian banded iron formations? *Geology* 2002;**30**:1079–82.

Kopf SH, Newman DK. Photomixotrophic growth of *Rhodobacter capsulatus* SB1003 on ferrous iron. *Geobiology* 2012;**10**:216–22.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;**33**, msw054.

Lane DJ, Pace B, Olsen GJ *et al.* Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *P Natl Acad Sci USA* 1985;**82**:6955–9.

Laufer K, Nordhoff M, Røy H *et al.* Co-existence of microaerophilic, nitrate-reducing, and phototrophic Fe (II)-oxidizers and Fe (III)-reducers in coastal marine sediment. *Appl Environ Microb* 2016;**82**:1433–47.

Llirós M, García–Armisen T, Darchambeau F *et al.* Pelagic photoferrotrophy and iron cycling in a modern ferruginous basin. *Sci Rep* 2015;**5**, DOI: 10.1038/srep13803.

Majzlan J, Navrotsky A, Schwertmann U. Thermodynamics of iron oxides: part III. Enthalpies of formation and stability of ferrihydrite (~$Fe(OH)_3$), schwertmannite (~$FeO(OH)_{3/4}(SO_4)_{1/8}$), and ε-Fe_2O_3. *Geochim Cosmochim Acta* 2004; **68**:1049–59.

Marion GM, Millero FJ, Camoes MF *et al.* pH of seawater. *Mar Chem* 2011;**126**:89–96.

Mehlhorn J, Byrne JM, Kappler A *et al.* Time and temperature dependency of carbon dioxide triggered metal(loid) mobilization in soil. *Appl Geochem* 2016;**74**:122–37.

Melton ED, Schmidt C, Behrens S *et al.* Metabolic flexibility and substrate preference by the Fe (II)-oxidizing purple non-sulphur bacterium *Rhodopseudomonas palustris* strain TIE-1. *Geomicrobiol J* 2014a:**31**:835–43.

Melton ED, Swanner ED, Behrens S *et al.* The interplay of microbially mediated and abiotic reactions in the biogeochemical Fe cycle. *Nat Rev Microbiol* 2014b;**12**:797–808.

Mloszewska AM, Pecoits E, Cates NL *et al.* The composition of Earth's oldest iron formations: the Nuvvuagittuq Supracrustal Belt (Québec, Canada). *Earth Planet Sci Lett* 2012;**317**:331–42.

Moeslund L, Thamdrup B, Jørgensen BB. Sulfur and iron cycling in a coastal sediment: Radiotracer studies and seasonal dynamics. *Biogeochemistry* 1994;**27**:129–52.

Morris RC. Genetic modelling for banded iron-formation of the Hamersley Group, Pilbara Craton, Western Australia. *Precambrian Res* 1993;**60**:243–86.

Murad E, Schwertmann U. The Mössbauer spectrum of ferrihydrite and its relations to those of other iron oxides. *Am Mineral* 1980;**65**:1044–9.

Murad E. Mössbauer spectroscopy of clays, soils and their mineral constituents. *Clay Miner* 2010;**45**:413–30.

Muyzer G, Teske A, Wirsen CO *et al.* Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* 1995;**164**:165–72.

Overmann J. The Family Chlorobiaceae. In: Dworkin M, Falkow S, Rosenberg E *et al.* (eds.) The Prokaryotes: Proteobacteria: Delta, Epsilon Subclass vol. **7**. New York, NY: Springer, 2006, 359–78.

Pfennig N, Trueper HG. New nomenclatural combinations in the phototrophic sulfur bacteria. *Int J Syst Bacteriol* 1971;**21**:11–14.

Posth NR, Hegler F, Konhauser KO *et al.* Alternating Si and Fe deposition caused by temperature fluctuations in Precambrian oceans. *Nat Geosci* 2008;**1**:703–8.

Posth NR, Konhauser KO, Kappler A. Microbiological processes in banded iron formation deposition. *Sedimentology* 2013;**60**:1733–54.

Raiswell R, Canfield DE. The iron biogeochemical cycle past and present. *Geochem Perspect* 2012;**1**:1–2.

Rickard D. The solubility of FeS. *Geochim Cosmochim Acta* 2006;**70**:5779–89.

Robert F, Chaussidon M. A palaeotemperature curve for the Precambrian oceans based on silicon isotopes in cherts. *Nature* 2006;**443**:969–72.

Saito MA, Sigman DM, Morel FMM. The bioinorganic chemistry of the ancient ocean: the co-evolution of cyanobacterial metal requirements and biogeochemical cycles at the Archean–Proterozoic boundary? *Inorg Chim Acta* 2003;**356**:308–18.

Schädler S, Burkhardt C, Hegler F *et al.* Formation of cell-iron-mineral aggregates by phototrophic and nitrate-reducing anaerobic Fe(II)-oxidizing bacteria. *Geomicrobiol J* 2009;**26**:93–103.

Schmidle W. Neue Algen Aus Dem Gebiete Des Oberrheins. Beih Bot Zentralbl 1901;**11**:544–55.

Shields GA, Kasting JF. Palaeoclimatology: evidence for hot early oceans? *Nature* 2007;**447**:E1.

Schwertmann U, Cornell RM. *Iron Oxides in the Laboratory*. Weinheim: John Wiley & Sons, 2008.

Stookey LL. Ferrozine—a new spectrophotometric reagent for iron. *Anal Chem* 1970;**42**:779–81.

Straub KL, Benz M, Schink B *et al*. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl Environ Microb* 1996;**62**:1458–60.

Straub KL, Rainey FA, Widdel F. *Rhodovulum iodosum* sp. nov. and *Rhodovulum robiginosum* sp. nov., two new marine phototrophic ferrous-iron-oxidizing purple bacteria. *Int J Syst Bacteriol* 1999;**49**:729–35.

Swanner ED, Wu W, Schoenberg R *et al*. Fractionation of Fe isotopes during Fe (II) oxidation by a marine photoferrotroph is controlled by the formation of organic Fe-complexes and colloidal Fe fractions. *Geochim Cosmochim Acta* 2015;**165**:44–61.

Taylor SR, McLennan SM. *The Continental Crust: Its Composition and Evolution*. CA: Blackwell Science Ltd, 1985.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;**22**:4673–80.

Treguer P, Nelson DM, Van Bennekom AJ *et al*. The silica balance in the world ocean: a reestimate. *Science (80-)* 1995;**268**:375.

Trüper HG. Photolithotrophic sulfur oxidation. In: Bothe H, Trebst A (eds). *Biology of Inorganic Nitrogen and Sulfur*. Berlin-Heidelberg: Springer, 1981, 199–211.

Tschech A, Pfennig N. Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch Microbiol* 1984;**137**:163–7.

Tuschak C, Glaeser J, Overmann J. Specific detection of green sulfur bacteria by in situ hybridization with a fluorescently labeled oligonucleotide probe. *Arch Microbiol* 1999;**171**:265–72.

Walter XA, Picazo A, Miracle MR *et al*. Phototrophic Fe(II)-oxidation in the chemocline of a ferruginous meromictic lake. *Front Microbiol* 2014;**5**:713.

Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: the unseen majority. *P Natl Acad Sci USA* 1998;**95**:657865–83.

Widdel F. Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten Sufat reduzierender Bakterien. *Dissertation* 1980.

Widdel F, Pfennig N. Studies on dissimilatory sulfate-reducing bacteria that compose fatty acids- I. Isolation of a new sulfate-reducer enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. *Arch Microbiol* 1981;**129**:395–400.

Widdel F, Schnell S, Heising S *et al*. Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature* 1993;**362**:834–6.

Wu W, Swanner ED, Hao L *et al*. Characterization of the physiology and cell-mineral interactions of the marine anoxygenic phototrophic Fe(II)-oxidizer *Rhodovulum iodosum* - implications for Precambrian Fe(II) oxidation. *FEMS Microbiol Ecol* 2014;**88**:503–15.

Xiong J. Photosynthesis: what color was its origin. *Genome Biol* 2006;**7**:245.