RESEARCH ARTICLE

Physiology of phototrophic iron(II)-oxidizing bacteria: implications for modern and ancient environments

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Abstract

Phototrophic iron(II) [Fe(II)]-oxidizing bacteria are present in modern environments and evidence suggests that this metabolism was present already on early earth. We determined Fe(II) oxidation rates depending on pH, temperature, light intensity, and Fe(II) concentration for three phylogenetically different phototrophic Fe(II)-oxidizing strains (purple nonsulfur bacterium *Rhodobacter ferrooxidans* sp. strain SW2, purple sulfur bacterium *Thiodictyon* sp. strain F4, and green sulfur bacterium *Chlorobium ferrooxidans* strain KoFox). While we found the overall highest Fe(II) oxidation rates with strain F4 (4.5 mmol L^{-1} day^{-1}, 800 lux, 20 °C), the lowest light saturation values [at which maximum Fe(II) oxidation occurred] were determined for strain KoFox with light saturation already below 50 lux. The oxidation rate per cell was determined for *R. ferrooxidans* strain SW2 to be 32 pmol Fe(II) h^{-1} per cell. No significant toxic effect of Fe(II) was observed at Fe(II) concentrations of up to 30 mM. All three strains are mesophiles with upper temperature limits of *c.* 30 °C. The main pigments were identified to be spheroidene, spheroidenone, OH-spheroidenone (SW2), rhodopinal (F4), and chlorobactene (KoFox). This study will improve our ecophysiological understanding of iron cycling in modern environments and will help to evaluate whether phototrophic iron oxidizers may have contributed to the formation of Fe(III) on early earth.

Introduction

Iron(III) [Fe(III)] oxy(hydr)oxides are abundant both in terrestrial and in aquatic environments (Canfield, 1989). While Fe(III) can serve as an electron acceptor for microorganisms mineralizing organic matter (Lovley, 1991), the product of their metabolism, i.e. iron(II) [Fe(II)], can be reoxidized by Fe(II)-oxidizing microorganisms either under oxic or under anoxic conditions (Kappler & Straub, 2005). At acidic pH, the ferrous iron is relatively stable even in the presence of O_2 due to kinetic limitations (Stumm & Morgan, 1995) so that mainly microorganisms, and not chemical oxidation processes, are responsible for the oxidation of Fe(II), for example in acid mine drainage processes (Baker & Banfield, 2003). At circumneutral pH, however, microorganisms have to compete with the rapid chemical oxidation of Fe(II) by molecular oxygen and thrive best under microoxic conditions (Emerson & Moyer, 1997). In anoxic environments, iron-oxidizing bacteria can oxidize iron either using nitrate as an electron acceptor (Straub *et al.*, 1996) or phototrophically using light as an energy source (Widdel *et al.*, 1993). These tightly coupled microbial iron reduction and oxidation processes allow efficient iron cycling on very small spatial scales as demonstrated recently in several studies (Weber *et al.*, 2006 and references therein). The iron cycle is tightly coupled to carbon, nutrient, and trace metal biogeochemical cycles, making its full understanding vital to the interpretation of natural systems (Thamdrup, 2000).

In both freshwater and marine sediments, Fe(III) reduction is controlled by microorganisms and not by chemical reduction of Fe(III) minerals (Thamdrup, 2000). Thus, steady-state Fe(II) concentrations, balanced by microbial Fe(III) reduction and various chemical and microbiological Fe(II) oxidation processes, lead to Fe(II) concentrations of 10^{-3} mol L^{-1} in sediments (Canfield, 1989; Stumm & Sulzberger, 1992; Thamdrup, 2000; Gerhardt *et al.*, 2005). It was suggested that each Fe atom undergoes about 100–300 redox

cycles until it gets buried in a biounavailable form in the sediment (Canfield *et al.*, 1993).

Furthermore, anaerobic and aerobic iron oxidation, as well as iron reduction, have not only drawn interest because of their presence in modern environments but also because it is likely that these processes were important on early earth about 3.5–1.8 Ga ago, possibly even being responsible for the deposition of banded iron formations (BIFs) (Johnson *et al.*, 2003; Kappler *et al.*, 2005). BIFs represent the largest iron deposits worldwide (Beukes *et al.*, 1992), but even to date the mechanism of Fe(II) oxidation of these formations at different time points of earth history is not yet fully understood. Microorganisms were suggested to have played a major role in the deposition of the oldest BIFs (Cloud, 1968) when the atmosphere of the early earth was probably still anoxic (Farquhar *et al.*, 2000). The traditional model proposes that molecular oxygen produced by *Cyanobacteria* was responsible for the oxidation of the iron (Cloud, 1968; Beukes *et al.*, 1992). Phototrophic Fe(II)-oxidizing bacteria were suggested to have played a major role in BIF deposition (Garrels *et al.*, 1973; Hartmann, 1984). However, only recent analysis of the genome of anoxygenic-phototrophic bacteria showing that anoxygenic photosynthesis likely evolved earlier than oxygenic photosynthesis (Xiong, 2006) in combination with evidence from eco-physiological, biomarker, and isotope studies (Johnson *et al.*, 2003; Brocks *et al.*, 2005; Kappler *et al.*, 2005; Rashby *et al.*, 2007) made a strong case for early BIF deposition via phototrophic iron-oxidizing bacteria.

While much is known about the biochemical and ecological role of acidophilic aerobic iron oxidation in acid mine drainage processes (e.g. Blake *et al.*, 1993; Baker & Banfield, 2003), much less is known about the biochemical mechanism of iron oxidation at circumneutral pH. In the last few years, the ecological significance of aerobic Fe(II) oxidation both in freshwater and in deep-sea environments has been investigated intensively and their activity in the environment has been localized and quantified by microsensor studies (Edwards *et al.*, 2003; Emerson & Weiss, 2004). In contrast, anaerobic iron oxidation has been largely unexplored although it closes a gap in the iron cycle under O_2-free conditions. This group of anaerobic Fe(II)-oxidizing bacteria include nitrate-dependent and phototrophic Fe(II)-oxidizing cultures. As of now, seven cultures of phototrophic Fe(II) oxidizers are known: the freshwater strains *Rhodobacter ferrooxidans* strain SW2 (Ehrenreich & Widdel, 1994), *Rhodopseudomonas palustris* strain TIE-1 (Jiao *et al.*, 2005), *Chlorobium ferrooxidans* strain KoFox (Heising *et al.*, 1999), *Thiodictyon* sp. strain F4 (Croal *et al.*, 2004), and *Rhodomicrobium vannielii* strain BS-1 (Heising & Schink, 1998) as well as the marine strains *Rhodovulum iodosum* and *Rhodovulum robiginosum* (Straub *et al.*, 1999). These bacteria oxidize Fe(II) according to the following stoichiometry (Ehrenreich & Widdel, 1994):

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

Phototrophic iron-oxidizing bacteria metabolize dissolved Fe(II), i.e. Fe^{2+}, and can also oxidize the soluble minerals FeS and $FeCO_3$ but not the poorly soluble Fe_3O_4 and FeS_2 (Kappler & Newman, 2004). So far, these strains have been isolated from both fresh and saltwater settings, notably freshwater ditches (Ehrenreich & Widdel, 1994; Heising *et al.*, 1999), a freshwater marsh (Croal *et al.*, 2004), and North Sea coastal sediments (Straub *et al.*, 1999). Only recently has it been demonstrated that the biochemical pathway(s) of Fe(II) oxidation in the two phototrophic Fe(II)-oxidizing bacteria *R. palustris* strain TIE-1 and *R. ferrooxidans* strain SW2 involve *c*-type cytochromes from where the electrons are transported to the photosynthetic reaction center (Croal *et al.*, 2007; Jiao & Newman, 2007). Interestingly, one of these studies even suggested that the Fe(II) is oxidized in the periplasm (Jiao & Newman, 2007) and not at the outer membrane as suggested previously (Kappler & Newman, 2004).

In order to understand the potential ecological role of phototrophic Fe(II)-oxidizing bacteria in various modern and ancient environments, this study aimed to quantify the Fe(II) oxidation rates for this type of microbial metabolism under different conditions in terms of pH, light intensity, temperature, and Fe(II) concentration. The overall goal of this study was to help evaluate a potential role of phototrophic Fe(II)-oxidizing bacteria in deposition of Precambrian banded iron formation (e.g. future modeling studies) by providing relevant Fe(II) oxidation rates at circumneutral pH and different light and temperature conditions. For these experiments, we chose three strains of distinct phylogenetic groups: the *Alphaproteobacteria* (*R. ferrooxidans* strain SW2), the *Betaproteobacteria* (*C. ferrooxidans* strain KoFox), and the *Gammaproteobacteria* (*Thiodictyon* sp. strain F4). Additionally, we identified the main carotenoids these strains produce in order to assess whether any specific carotenoids are present and can potentially be used as a biomarker for this type of metabolism, especially with respect to the proposed role of these organisms in the deposition of BIFs.

Materials and methods

Sources of microorganisms

The strains *R. ferrooxidans* strain SW2, *Thiodictyon* sp. strain F4, and *C. ferrooxidans* strain KoFox were kindly originally provided by F. Widdel (Max-Planck Institute, Bremen,

Germany) and B. Schink (University of Konstanz, Germany) to D.K. Newman (MIT), from where A. Kappler transferred them into his lab strain collection at the University of Tuebingen, Germany.

Media and growth conditions

All strains were cultivated in serum bottles (58 mL volume, 25 mL medium) in freshwater medium modified from Ehrenreich & Widdel (1994) containing 0.6 g L^{-1} potassium phosphate (KH_2PO_4), 0.3 g L^{-1} ammonium chloride (NH_4Cl), 0.5 g L^{-1} magnesium sulfate ($MgSO_4 \cdot 7H_2O$), and 0.1 g L^{-1} calcium chloride ($CaCl_2 \cdot 2H_2O$). The medium was buffered at pH 6.8–6.9 with 22 mmol L^{-1} bicarbonate that was autoclaved separately under a N_2/CO_2 atmosphere (90 : 10). The medium was cooled to room temperature under a N_2/CO_2 atmosphere (90 : 10) and 1 mL L^{-1} trace element solution (Tschech & Pfennig, 1984), 1 mL L^{-1} selenate–tungstate solution (Widdel, 1980), and 1 mL L^{-1} sterile filtered vitamin solution (Widdel & Pfennig, 1981) were added. If necessary, the pH was adjusted to pH 6.8 with 0.5 mol L^{-1} Na_2CO_3 or 1 mol L^{-1} HCl. If mineral-free cultures were required, either acetate was added from a 1 mol L^{-1} stock solution to a concentration of 10 mmol L^{-1} or the headspace was flushed with a mixture of dihydrogen and CO_2 (90 : 10 v/v).

Two different kinds of Fe(II)-containing growth media were used in this study: filtered and unfiltered Fe(II)-containing medium. For unfiltered medium, $FeCl_2$ was added [1 mol L^{-1} stock solution of 99.9% ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) dissolved in anoxic water (boiled and then flushed with nitrogen during cooling)] to freshwater medium. A greenish-whitish precipitate formed immediately [likely consisting of Fe(II) phosphate, possibly vivianite containing small amounts of Ca and S; J. Miot, pers. commun.]. The unfiltered medium [containing the precipitated Fe(II) minerals] was used for some of the microbial Fe(II) oxidation experiments without further treatment. In contrast, the preparation of filtered medium provided a medium in which only dissolved Fe(II) was present and neither dissolution processes of Fe(II) minerals nor sorption of Fe(II), Fe(III), or cells to Fe(II) minerals played a role. This medium was filtered in an anoxic chamber with a 0.22-μm filter (polyethersulfone, Millipore) 2 h after the $FeCl_2$ solution was added and Fe(II) minerals precipitated. The pH was checked before filtering and adjusted to pH 6.8 if necessary. Approximately 40% of the Fe(II) of a 10 mM Fe(II) solution remained in solution (supersaturated with regard to siderite); this medium was stable for about 4 weeks without any further visible Fe(II) mineral precipitation. Within the typical time frame of our experiments, loss of iron by photochemical oxidation, precipitation, or adsorption to the glass was not observed in noninoculated controls. Standard incubation conditions were at 20 °C and 650 lux, the light source was a tungsten light bulb, and the light intensity was varied by changing the distance to the light bulb.

To adjust the pH, 1 M HCl or 0.5 M Na_2CO_3 was added with a syringe to bottles sealed with a rubber stopper already containing Fe(II) in order to avoid a loss of CO_2 in the case of a decreasing pH and thus a loss of buffer. pH was checked at the beginning and the end of the growth experiments and did not change by more than 0.1 pH units.

Analytical techniques

Fe(II) and Fe(III) were quantified using a spectrophotometric assay with ferrozine modified from Stookey (1970). Samples of Fe(II)-grown cultures were taken under sterile conditions using a syringe through the butyl-rubber stopper. About 200-μL culture suspension was withdrawn after vigorous shaking and transferred into an Eppendorf tube. One hundred microliters of the sample was taken quickly with a transfer pipette and added to 700 μL of 1 mol L^{-1} HCl to stabilize Fe(II) and to dissolve the Fe(III) minerals. Twenty microliters from this solution was transferred into a well of a microtiter plate containing either 80 μL of 1 mol L^{-1} HCl to quantify Fe(II) or 80 μL of a solution of the reducing agent hydroxylamine hydrochloride (50% w/v in 1 M HCl) to quantify the total iron content [i.e. Fe(II) plus Fe(III)]. Three subsamples were analyzed per sample at each time point for Fe(II) and three for total iron. One hundred microliters of ferrozine solution (10% w/v ammonium acetate, 0.1% w/v ferrozine) was added after 30 min. The ferrozine was allowed to react for 5 min with the Fe(II) and the $A_{562\,nm}$ was measured using a FlashScan 550 plate reader (Jena Analytics, Jena, Germany).

Ferrous iron concentration was plotted vs. time. The curve obtained was inverse sigmoidal while the turning point of this curve denotes the maximum oxidation rate under a given condition, such as pH or temperature. We approximated this by a regression through the visibly steepest part of the curve as shown in Fig. 1 yielding the oxidation rate per time. Each experiment consists of three parallel cultures. The oxidation rates were determined for each of these parallels, averaged, and the SD was calculated. The uninoculated control was used to quantify loss of Fe(II) either by sorption to the glass wall of the serum bottles or by a possible abiotic oxidation by light.

Cell numbers were determined by microscopic cell counts. Cells in aliquots of cell-mineral suspensions were fixed with 3.7% formaldehyde (30 min), centrifuged, washed with 0.9% NaCl, and resuspended in 1.1 mL of 0.9% NaCl. The Fe(III) minerals were dissolved by addition of 1 mL anoxic ferrous ethylene diammonium sulfate solution [$C_2H_4(NH_3)_2SO_4$] from a 100 mM anoxic stock

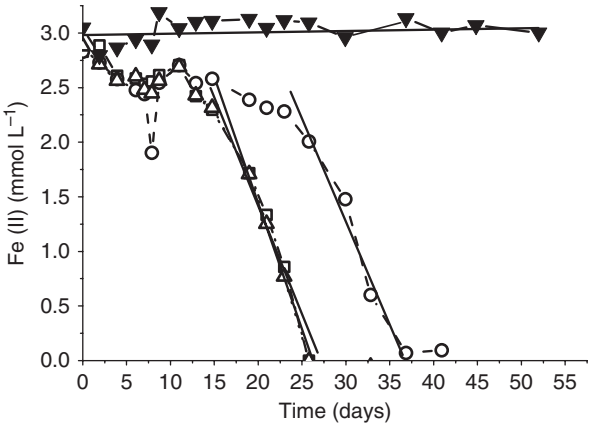

Fig. 1. Fe(II) concentration plotted over time for three parallel cultures of *Rhodobacter ferrooxidans* strain SW2 (inoculated with the same inoculum) growing in filtered 3 mM Fe(II)-containing medium. Maximum oxidation rates were determined at the steepest part of the Fe(II) curve (indicated by the solid lines). Although the lag-time can differ (probably due to small differences in the inoculation procedure) in the three parallel cultures (□, ○, △), the maximum rate and the total extent of oxidation are similar for the three parallel cultures while in the noninoculated control (▼) no oxidation was observed at all.

solution and 8.9 mL oxalate solution (pH 3, autoclaved). 10 μL of 4′,6-diamidino-2-phenylindole (5 mg L^{-1} stock) was added as a fluorescent dye and 0.5–1 mL of the cell suspension was sucked onto a filter (Millipore, polycarbonate filter, 0.22 μm pore size). Cells were counted microscopically.

Microscopy

Light microscopy images were taken using an AxioVison microscope (Zeiss) and an oil immersion objective lens. Scanning electron micrographs (SEMs) were taken on either a Zeiss Leo Gemini 1550 VP or a Zeiss Crossbeam Leo 1540 XB FE-SEM at the Natural and Medical Sciences Institute at the University of Tuebingen. Samples were prepared for SEM as described elsewhere (Schaedler *et al.*, 2008).

Data analysis

The data for pH and temperature dependence of Fe(II) oxidation were fitted according to a model published by Rosso *et al.* (1995) that can be used to determine the minimal, optimal, and maximal pH and the temperature of microbial metabolism based on experimental data. The data obtained for the light-dependent Fe(II) oxidation rates were modeled with equation (2) with v, oxidation rate; v_{max}, maximum oxidation rate; I, light intensity; and x_{HLS}, light intensity at which the oxidation rate equals 50% of v_{max}. For all data for light-dependent Fe(II) oxidation, half-light saturation values x_{HLS} at which the oxidation rate is at 50% of the maximum rate are reported.

$$v = \frac{v_{max} I}{I + x_{HLS}} \quad (2)$$

Identification of pigments

Cells from hydrogen- or acetate-grown stationary growth phase cultures were harvested by centrifugation at 7800 ***g***. The pigments were extracted in the dark with 5 mL of acetone and ethanol (1 : 1). After sonification for 2 min, the mixture was incubated at 20 °C for 1 h. The pigments were then transferred to hexane by adding 3 mL of hexane and 0.5 mL of water. The upper hexane phase was exchanged until it remained clear. The hexane extracts were collected, concentrated 10-fold under a stream of nitrogen, and stored at − 20 °C. Pigments were separated using a normal phase thin-layer chromatography system with silica adsorbed (Kieselgel 60, Merck, Darmstadt, Germany) with petrolether and acetone (4 : 1) as the mobile phase. After scraping the colored bands from the thin layers, the pigments were extracted with dichloromethane and filtered through glass wool. The pigments were transferred to hexane and absorbance spectra of the individual pigments were recorded in a spectrophotometer. The pigments were identified by comparison with reference absorption spectra and published TLC-R_f-values (Züllig & Rheineck, 1985; Britton, 1995a, b). No differences in pigment content between Fe(II)-grown cells and hydrogen-/acetate-grown cells were observed in TLC plates and due to higher cell mass; therefore, acetate- and H_2-grown cells were used for pigment identification. All steps of the pigment separation and identification were carried out in the dark or at least with dim light.

Results

Fe(II) oxidation by phototrophic Fe(II)-oxidizing bacteria

Phototrophic Fe(II)-oxidizing bacteria oxidize Fe(II) and produce Fe(III) that precipitates as Fe(III) mineral (Fig. 2). In the cultures investigated in this study, a significant fraction, but never all cells were associated with the Fe(III) minerals produced during Fe(II) oxidation (Fig. 2). Furthermore, the cells were not encrusted in iron minerals, but rather loosely attached to the Fe(III) minerals.

The maximum rates of Fe(II) oxidation by the strains were determined by analyzing the decrease of Fe(II) over time. In some cases, for the same strain the lag phase of independent or even parallel cultures varied by up to 14 days either due to small differences in culture age, and therefore physiological status of the inoculated cells, or due to slightly different amounts of inoculum, and thus a variation of the number of cells added. Nevertheless, the maximum rates

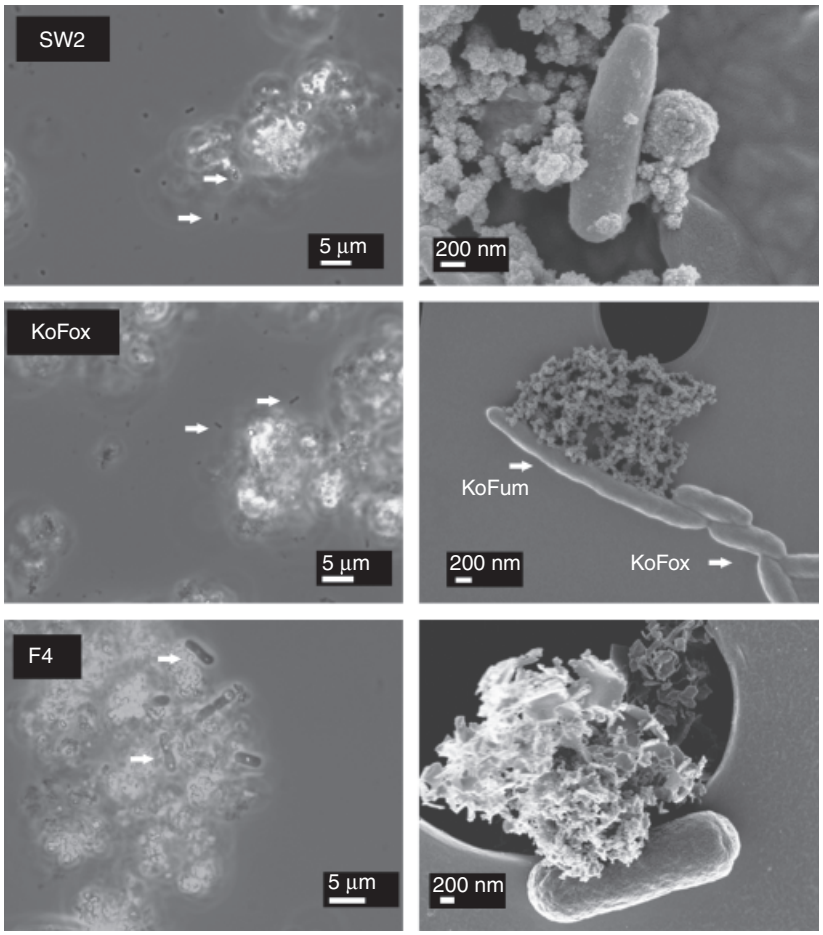

Fig. 2. Light microscopy (left) and SEM images (right) of the phototrophic Fe(II)-oxidizing bacteria *Rhodobacter ferrooxidans* strain SW2, *Chlorobium ferrooxidans* strain KoFox (with the coculture KoFum in the SEM picture) (Heising *et al.*, 1999), and *Thiodictyon* sp. strain F4. Images were taken of samples from cultures growing with Fe(II) as the electron donor. Cells are indicated with arrows.

and the extent of Fe(II) oxidation were similar in all cultures (Fig. 1). Therefore, the maximum oxidation rates are dependent on the concentration and activity of cells at the point of maximum Fe(II) oxidation and not on the number and physiological status of the inoculated cells. Oxidation rates can thus be compared between independent cultures of the same strain.

Oxidation of dissolved Fe(II) and Fe(II) minerals by anoxygenic phototrophs

The medium used for cultivation of anoxygenic phototrophic Fe(II)-oxidizing bacteria typically contains 20–30 mM bicarbonate as buffer and 4 mM of phosphate as mineral salt (Ehrenreich & Widdel, 1994; Kappler & Newman, 2004). Upon Fe(II) addition, Fe(II) phosphate precipitates form (J. Miot, pers. commun.). We compared oxidation rates for cultures of *R. ferrooxidans* strain SW2 with only dissolved Fe(II) with cultures that contained a mix of dissolved Fe(II) and Fe(II) minerals and observed similar rates of *c.* 0.5 mmol L^{-1} h^{-1} at 2 and 4 mM Fe(II) for both filtered and nonfiltered medium (Fig. 3a). Only at 8 mM Fe(II) was a significant difference found, with higher rates in the precipitate-free medium compared with the Fe(II)-mineral-containing medium. All further experiments investigating light, temperature, and pH dependence were performed at Fe(II) concentrations of 2–4 mM. As no difference in oxidation rate was found dependent on the presence of Fe(II) minerals at these concentrations, we carried out these experiments in either filtered or nonfiltered medium and consider the results as comparable to each other.

Growth yields and oxidation rates per cell

Initial cell numbers and cell numbers after Fe(II) oxidation as well as oxidation rates per cell were determined for *R. ferrooxidans* strain SW2. The initial cell number after inoculation was $1.6 \pm 0.1 \times 10^8$ cells mL^{-1}. During oxidation of 4 mM Fe(II) at 20 °C at circumneutral pH and light saturation, the cell number increased to $9.8 \pm 0.1 \times 10^8$ cells mL^{-1}. The oxidation rate for these conditions was determined to be 0.75 mmol mL^{-1} day^{-1}, which yielded a per-cell oxidation rate of 32 pmol Fe(II) h^{-1} per cell. According to the equation for microbially catalyzed

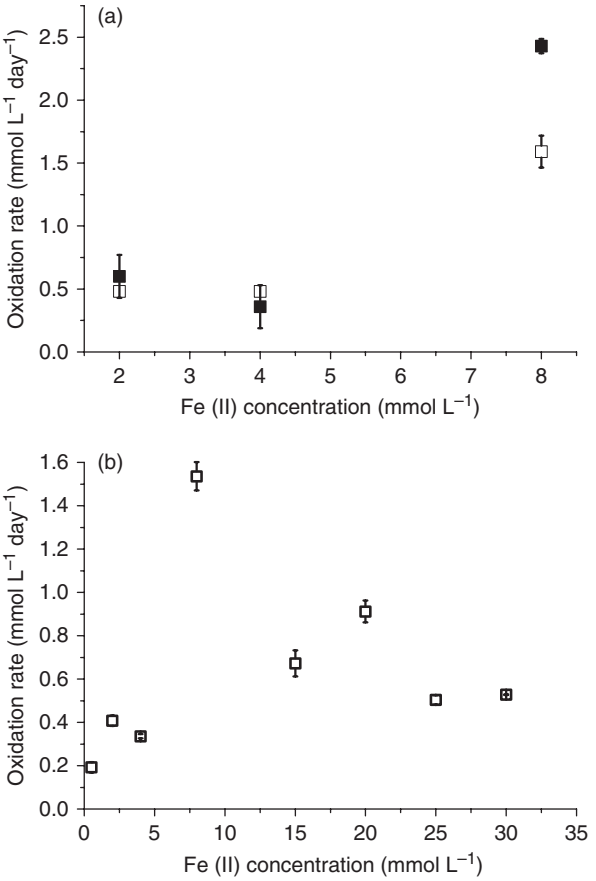

Fig. 3. Rates of Fe(II) oxidation by *Rhodobacter ferrooxidans* strain SW2 at various Fe(II) concentrations. (a) Comparison of Fe(II) oxidation rates in filtered (■) and nonfiltered (□) medium. (b) Fe(II) oxidation rates in nonfiltered medium. In both setups, each data point represents two experiments with at least four cultures each from which the SD was calculated.

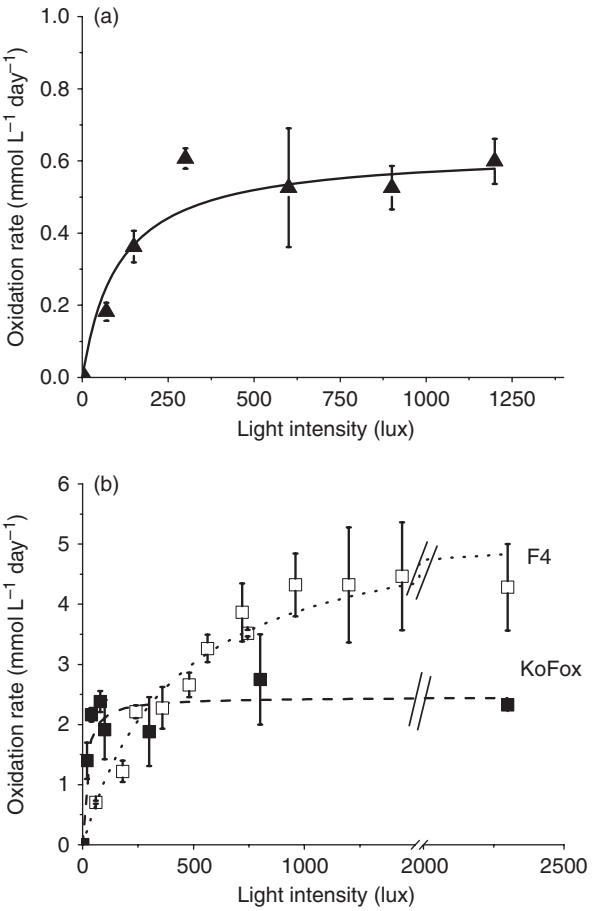

Fig. 4. Light dependence of Fe(II) oxidation by the phototrophic Fe(II)-oxidizing bacteria *Rhodobacter ferrooxidans* strain SW2 (a) and *Chlorobium ferrooxidans* strain KoFox (■) and *Thiodictyon* sp. strain F4 (□) (b). The dashed, punctuated, and solid lines represent the light dependence of Fe(II) oxidation modeled with equation (2) using the experimentally determined data. Each data point represents two independent experiments with at least two cultures each. The error bars represent SDs calculated from all experiments. The rates were determined for strains KoFox and F4 with a 10 mM Fe(II)-containing unfiltered medium whereas the rates for strain SW2 were determined in filtered medium containing 4 mM dissolved Fe(II). Because we showed that at these Fe(II) concentrations the oxidation rates in filtered and nonfiltered Fe(II)-containing medium are similar (Fig. 3a), we consider these experiments to be comparable.

photoautotrophic Fe(II) oxidation [equation (1)], by oxidation of 4 mM Fe(II) the cells can produce 1 mM $< CH_2O >$ (used as proxy for biomass) meaning about 30 mg biomass. This growth yield in photoautotropic Fe(II)-oxidizing cultures of strain SW2 was confirmed already by others (Ehrenreich & Widdel, 1994).

Concentration dependence of Fe(II) oxidation rates

The dependence of Fe(II) oxidation rates on the amount of Fe(II) initially present was tested with *R. ferrooxidans* strain SW2 at concentrations of Fe(II) in the range of 0.2–30 mM. We observed an increase in oxidation rates from 0.2 to 8 mM Fe(II), with the highest oxidation rate at 8 mM (Fig. 3b). At higher concentrations of 15 mM and above, the oxidation rate decreased to about 40–50% of the maximum rate; however, up to 30 mM no substantial toxic effect of Fe(II) was observed.

Light dependence of Fe(II) oxidation rates

In order to determine the light dependence of *C. ferrooxidans* strain KoFox, *Thiodictyon* sp. strain F4, and *R. ferrooxidans* strain SW2, we incubated cultures at various distances from tungsten light bulbs, measured the light intensity, and determined the Fe(II) oxidation rates by quantifying Fe(II) over time. For *R. ferrooxidans* strain SW2, we observed a light saturation (light intensity at which maximum oxidation rates are observed) of *c.* 400 lux with a half-light saturation (HLS) (light intensity at which we observed 50% of the maximum oxidation rate) of 113 ± 50 lux (Fig. 4a). *Thiodictyon* sp. strain

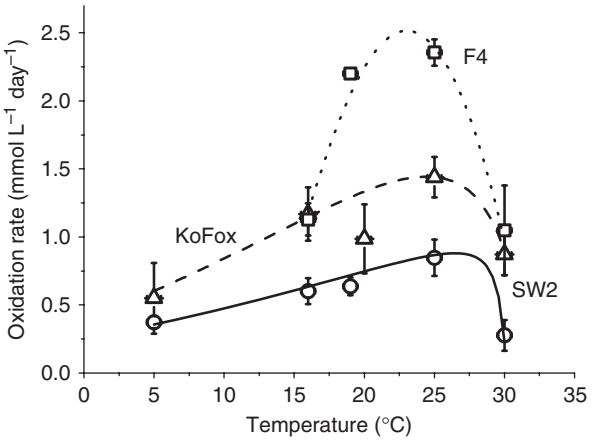

Fig. 5. Temperature dependence of Fe(II) oxidation rates for *Chlorobium ferrooxidans* strain KoFox (△), *Thiodictyon* sp. strain F4 (□), and *Rhodobacter ferrooxidans* strain SW2 (○). All rates were determined with filtered medium containing 4 mM dissolved Fe(II). The dashed and solid lines are calculated from the data with the model described by Rosso *et al*. (1995). Each data point represents two independent experiments with at least four cultures each. The error bars represent SDs calculated from all experiments.

F4 has a light saturation of 800 lux with a HLS of 350 ± 68 lux (Fig. 4b). In contrast to the purple sulfur and purple nonsulfur strains F4 and SW2, the Fe(II) oxidation rate of the green-sulfur bacterium *C. ferrooxidans* sp. strain KoFox reaches light saturation already below 50 lux with an HLS of 11 ± 5 lux (Fig. 4b).

Temperature dependence of Fe(II) oxidation rates

The optimal and maximal temperatures for phototrophic Fe(II) oxidation were determined for the three strains *C. ferrooxidans* strain KoFox, *Thiodictyon* sp. strain F4, and *R. ferrooxidans* strain SW2 (Fig. 5). We found that all three strains investigated are mesophilic, with maximum Fe(II) oxidation rates at 23 °C for strain F4, 26 °C for SW2, and 25 °C for strain KoFox and an upper temperature limit for Fe(II) oxidation of 32 °C for F4, 30 °C for SW2, and 32 °C for KoFox. We also incubated the strains at lower temperatures and observed that strain F4 did not oxidize Fe(II) at 10 °C, but the strains SW2 and KoFox oxidized Fe(II) at appreciable rates even at 5 °C.

pH dependence of Fe(II) oxidation rates

In order to determine the pH dependence of *C. ferrooxidans* strain KoFox, *Thiodictyon* sp. strain F4, and *R. ferrooxidans* strain SW2, we incubated the strains in nonfiltered medium containing 4 mM Fe(II) at pH 5–8. We determined the optimal pH for Fe(II) oxidation for strain F4 and SW2 to be at 6.5, while for strain KoFox we observed maximum oxidation rates at pH 6.9 (Table 1). The maximum pH at which we observed Fe(II) oxidation was 7.2 for strain F4 and 7.5 for strains SW2 and KoFox. The lowest pH at which the strains oxidized Fe(II) was 5.4 for strains F4 and KoFox and 5.2 for strain SW2.

Table 1. pH dependence of Fe(II) oxidation by the phototrophic Fe(II)-oxidizing strains *Thiodictyon* sp. strain F4, *Chlorobium ferrooxidans* strain KoFox and *Rhodobacter ferrooxidans* strain SW2

Strain	pH_{opt}	pH_{min}	pH_{max}	R^2
F4	6.5 ± 0.1	5.4 ± 0.1	7.2 ± 0.1	0.98
KoFox	6.9 ± 0.1	5.4 ± 0.1	7.5 ± 0.1	0.97
SW2	6.5 ± 0.0	5.2 ± 0.1	7.5 ± 0.1	0.92

Shown are the optimal pH (pH_{opt}) and the lowest and highest pH at which significant Fe(II) oxidation occurred, pH_{min} and pH_{max}, respectively. R^2 indicates the goodness of the fit.

Table 2. Main carotenoids (and their color) identified in the phototrophic Fe(II)-oxidizing bacteria *Rhodobacter ferrooxidans* sp. SW2, *Chlorobium ferrooxidans* strain KoFox, and *Thiodictyon* sp. strain F4

Strain	Compound	Absorption maxima (nm)
SW2	Spheroidene (yellow)	430, 455, 486
	Spheroidenone (pinkish-red)	465, 480, 517
	OH-spheroidene (yellow)	430, 454, 486
KoFox	Chlorobactene (yellow)	435, 462, 491
F4	Rhodopinal (purple-red)	362, 490–512 (broad)

Carotenoids in phototrophic Fe(II)-oxidizing bacteria

We identified the main carotenoids in the phototrophic Fe(II)-oxidizing bacteria *C. ferrooxidans* strain KoFox, *Thiodictyon* sp. strain F4, and *R. ferrooxidans* SW2 (Table 2). Spheroidene, spheroidenone, and OH-spheroidene were identified as the main pigments in the purple nonsulfur strain SW2, whereas rhodopinal was the main pigment in the purple-sulfur strain F4 and chlorobactene in the green sulfur strain KoFox. All pigments identified represented the main pigments in these strains when grown either photoautotrophically with Fe(II) as an electron source or photoautotrophically, with either H_2 (strains SW2 and KoFox) or acetate (strain F4).

Discussion

Ecophysiology of anoxygenic phototrophic Fe(II) oxidizers

Understanding the physiological constraints of microbially catalyzed phototrophic Fe(II) oxidation enhances our understanding of the iron cycle over the last 3.5 Ga until today. In modern environments, these organisms close the iron cycle in near-surface environments where Fe(II) produced in deeper layers by Fe(III)-reducing microorganisms reaches the light-exposed surface. With regard to ancient

environments, physiology experiments with modern phototrophic Fe(II)-oxidizing bacteria constrain the likely ecological limits for this metabolism in the past. The fact that modern phototrophic Fe(II)-oxidizing bacteria were isolated from environments as varied as a marsh (*Thiodictyon* sp. F4) (Croal *et al*., 2004), iron-rich ditches (*C. ferrooxidans* strain KoFox, *R. ferrooxidans* strain SW2, and *R. palustris* strain TIE-1) (Ehrenreich & Widdel, 1994; Heising *et al*., 1999; Jiao *et al*., 2005), and ocean sediments (*R. iodosum* and *R. robiginosum)* (Straub *et al*., 1999) highlights the ubiquity and likely environmental significance of this metabolism.

In natural environments, bacteria must cope with temperature, pH, and light conditions that change in different time intervals (minutes to months). Especially in anoxic near-surface environments required by phototrophic Fe(II) oxidizers, temperature and light intensity, but also pH, can change rapidly. In this regard, the physiology of the isolated strains reflects the habitats from which they were isolated: moderate temperatures and a circumneutral pH. Two of the strains even grew at temperatures of 4 °C (KoFox and SW2). As of now, no phototrophic Fe(II)-oxidizing strain is known that is able to oxidize Fe(II) at high temperatures, for example in iron-rich hot springs. Iron-rich, neutrophilic hot springs reflect habitats thought to have been omnipresent on ancient earth. Likewise, attempts made to isolate thermophilic aerobic Fe(II)-oxidizing bacteria to date have also been without success (e.g. Emerson & Weiss, 2004).

Adaptation to pH changes in the environment

When Fe(III) precipitates from solution, protons are formed

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_{3,\,ppt} + 3\,H^+ \quad (3)$$

and thus the vicinity of a cell can acidify (depending on the buffer capacity of the environment). As a consequence, it is essential for these strains to be able to cope with lower pH values not only because of pH changes caused by environmental processes but also due to metabolism-driven pH changes. It has been suggested previously that the pH for Fe(II) oxidation by *R. ferrooxidans* strain SW2 ranges from 5.5 to 7 (Ehrenreich & Widdel, 1994). We found the range to be slightly broader (pH 5.2–7.5). The slightly wider range we report here for strain SW2 might be due to adaptation and mutation of the strain or to a slightly different culturing technique (such as the use of other salts that may contain concentrations of a trace element not supplemented with the trace element solution). Yet, the differences between the values reported previously and the values we determined are minor. Also, the other phototrophic Fe(II)-oxidizing bacteria that we have tested (strains F4 and KoFox) showed pH ranges similar to strain SW2 for phototrophic Fe(II) oxidation. Such a relatively wide pH range is essential for such strains considering their environmental habitats. Over the course of the day, the pH in sediments changes due to the generation of protons by the microbial formation of Fe(III) hydroxides (during the day when light is present), but also due to uptake of bicarbonate/CO_2 by the cells. Indeed, a range of pH fitting the limits for the phototrophic Fe(II)-oxidizers described here occur in naturally forming microbial mats (e.g. Pierson *et al*., 1999).

Consequences of light dependence for habitat choice of phototrophic Fe(II) oxidizers

Phototrophic Fe(II)-oxidizing bacteria require very restricted and specialized habitats as they need to be close to the surface to get light but also require a reduced environment devoid of oxygen. In the presence of O_2, the Fe(II) substrate would be oxidized chemically by molecular oxygen rather than by the phototrophic Fe(II)-oxidizing cells. These organisms circumvent this problem either by tolerating low concentrations of oxygen, which allows them to live closer to the surface [requiring that the cells oxidize the Fe(II) faster than the molecular oxygen], or by lowering their requirement for light and being able to live deeper in soils and sediments where the concentration of Fe(II), replenished from below, is higher. In this regard, *C. ferrooxidans* strain KoFox, which needs the lowest light intensities for the highest Fe(II) oxidation rates, has adapted best to living deeper in a sediment or soil. *Rhodobacter ferrooxidans* strain SW2 and *Thiodictyon* strain F4 require higher light intensities for fast Fe(II) oxidation and thus cannot live as deep in sediments as strain KoFox.

In modern microbial iron-rich mats, light penetrates on average about 2–3 mm and thus phototrophic bacteria will live in the upper anoxic millimeter of such an environment (e.g. Pierson *et al*., 1999). The amount of light the phototrophic Fe(II)-oxidizing strains need for maximum Fe(II) oxidation follows the general observation that green-sulfur bacteria (such as strain KoFox) have a very low light saturation, while purple sulfur and purple nonsulfur bacteria need more light (in our case represented by strains F4 and SW2, respectively) (Overmann & Garcia-Pichel, 2000).

Oxidation of dissolved Fe(II) and relatively soluble Fe(II) minerals

We found that the oxidation rate is the same at low Fe(II) concentrations [2 and 4 mM Fe(II)] for filtered [no Fe(II) minerals present] and unfiltered medium [including Fe(II) minerals]. This suggests that at these concentrations, the dissolution rate of the Fe(II) minerals supports iron oxidation rates as fast as the ones obtained with only dissolved Fe(II). In contrast, at higher concentrations [e.g. 8 mM Fe(II)], the oxidation rates were much faster in the presence of only dissolved Fe(II) compared with a setup containing

dissolved Fe(II) as well as Fe(II) minerals. This suggests that at such high Fe(II) concentrations, in particular in the presence of high amounts of Fe(II) minerals, the dissolution rate of the Fe(II) minerals limits the overall oxidation rate. This may be due to Fe(III) mineral formation at the surface of the Fe(II) minerals, thus reducing the Fe(II) mineral dissolution rate. Additionally, the organisms might directly oxidize Fe(II) minerals by transferring electrons from the mineral surface into the cells and not only dissolved Fe(II) as suggested previously (Croal *et al.*, 2007; Jiao & Newman, 2007). These authors provide evidence that Fe(II) oxidation takes place in the periplasm of phototrophic Fe(II)-oxidizing bacteria that would require a dissolved Fe(II) species to be transported at some step. In contrast, also oxidation of Fe(II) minerals is possible. This not necessarily requires the presence of dissolved Fe(II) which could explain why in the presence of higher amounts of Fe(II) minerals the oxidation rates are lower than in the presence of only dissolved Fe(II). The bulk of the Fe(II) minerals are not accessible to the microorganisms via direct contact. In order to distinguish the contribution of Fe(II) arising from the poorly crystalline minerals vs. the Fe(II) from solution, further experiments are needed.

Pigments in anoxygenic phototrophic Fe(II) oxidizers

Carotenoids function both as radical detoxifiers and as light-harvesting pigments (Britton, 1995a, b), harvesting photons used for energy generation in cyclic photophosphorylation, but also protecting cells from oxidative damage by radicals. Because radical formation is stimulated by Fe-catalyzed Fenton reactions, in particular, photosynthetic Fe(II)-oxidizing bacteria are potentially faced with high radical concentrations. As a consequence, it can be speculated that these organisms might contain unique and specialized carotenoids that help the cells to cope with these radicals. We therefore determined the identity of the main carotenoids in the photosynthetic Fe(II)-oxidizing strains *R. ferrooxidans* strain SW2, *C. ferrooxidans* strain KoFox, and *Thiodictyon* sp. F4. However, the pigments identified (spheroidene, spheroidenone, OH-spheroidene, rhodopinal, and chlorobactene) suggest that these organisms use standard carotenoids that are also present in non-Fe(II)-oxidizing relatives of these phototrophic organisms (Britton, 1995a, b). Despite the presence of high concentrations of Fe and exposure to light, radical formation (e.g. due to light-catalyzed radical formation and/or Fenton reactions) obviously does not represent a major problem for these organisms.

Potential role of anoxygenic phototrophic Fe(II) oxidizers on ancient earth

In order to evaluate a potential role of phototrophic Fe(II)-oxidizing bacteria in the formation of Precambrian banded iron formations, eco-physiological studies with modern organisms help constrain the conditions required for such organisms and whether it is plausible that they thrived at all in Precambrian oceans. While it is true that these modern analogue organisms could have acquired new functions via horizontal gene transfer or have evolved over time including adaptation of their physiology to modern environmental conditions, evolutionary phylogenetic studies and eco-physiological studies with modern strains are one way to constrain possible scenarios in the past (Posth *et al.*, 2008).

To date, modeling of ocean pH on early earth yielded a pH value of *c.* 7 (Grotzinger & Kasting, 1993) and a wide range of temperatures of 25–70 °C (Knauth & Lowe, 2003; Robert & Chaussidon, 2006; Jaffrés *et al.*, 2007; Shields & Kasting, 2007) with the most recent suggestion of 10–33 °C (Kasting *et al.*, 2006). While certainly not offering direct proof, the physiological data presented in this study for three phylogenetically distant phototrophic Fe(II)-oxidizers support the notion that these organisms may have lived in a moderate-temperature Precambrian ocean actively contributing to BIF deposition. The Fe(II) oxidation rates provided in the present study even support a recent report in which we used first preliminary physiological data to suggest that the rates of oxidation by phototrophic Fe(II)-oxidizing bacteria can indeed sustain the high Fe(III) precipitation rates required for the deposition of the BIFs (Kappler *et al.*, 2005). Additionally, the oxidation rates provided here for several strains of phototrophic Fe(II) oxidizers under various environmental conditions allow in future studies not only evaluation of Fe(III) precipitation rates under different conditions. They also allow to better understand modern and ancient Fe cycling as attempted previously by (Konhauser *et al.*, 2005), who used Fe(II) oxidation rates to model the ancient Fe-cycle involved in the deposition of BIFs.

Because the heavily debated upper temperature limit suggested for Precambrian oceans (see e.g. Jaffrés *et al.*, 2007) is *c.* 50–70 °C, the isolation of a thermophilic phototrophic iron-oxidizing strain e.g. from iron-rich hot springs would offer a modern analogue strain that could offer more information about the role of these organisms in BIF deposition at higher temperatures. In addition, the model of BIF deposition by anoxygenic phototrophs assumes that these strains thrived in the water column of the archean ocean (Kappler *et al.*, 2005). As all modern organisms isolated thus far stem from ditches or sediments, two of them from marine sediments, it would be beneficial to isolate such a strain from the water column. Unfortunately modern oceans are either oxic or sulfidic, no modern analogue of an Fe(II)-rich ocean exists. However, a number of lakes with seasonal stratification and significant concentrations of dissolved Fe(II) in the water column are good candidates for isolation of Fe(II)-oxidizing organisms from the water column. Interestingly, however, it has been

demonstrated recently that even the strains isolated from sediments can maintain a significant concentration of planktonic cells in the water column when grown in batch cultures (Konhauser *et al.*, 2005).

Conclusions

Knowledge about the physiological limitations of phototrophic Fe(II) oxidizers better constrains metabolic activities that close the iron cycle under anoxic conditions and in particular microbially catalyzed processes that lead to the formation of Fe(III) oxides in the upper centimeters of the subsurface. The Fe(II) oxidation rates determined for phototrophic iron-oxidizing bacteria under varying conditions of pH, temperature, and light and will help to understand the interactions of iron oxidizers and iron reducers. Fe(II)-oxidizing bacteria provide the Fe(III) for the iron reducers that mineralize organic matter and in turn the iron reducers provide the Fe(II) that is used by the anoxygenic phototrophs.

The activity of anoxygenic phototrophic Fe(II) oxidizers is limited to Fe(II)-rich and light-penetrated environments such as the surface of sediments, ditches, or soils [although so far no phototrophic Fe(II) oxidizers have been isolated from soils]. However, the fact that these organisms can tolerate a wide range of pH values and temperature, can cope with low light intensities, and can live with low and high concentrations of Fe(II) suggests that they find niches in many environments. However, further research is needed to determine the full ecological role of this metabolism. Additionally, more biochemical research is needed in order to understand the mechanism of anaerobic Fe(II) oxidation and the strategies that these organisms developed to deal with a poorly soluble metabolic product, i.e. Fe(III) (hydr)oxides.

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