RESEARCH ARTICLE

Ecophysiology and the energetic benefit of mixotrophic Fe(II) oxidation by various strains of nitrate-reducing bacteria

Eva Marie Muehe[1], Simone Gerhardt[2], Bernhard Schink[2] & Andreas Kappler[1]

[1]Geomicrobiology, Center for Applied Geosciences, University of Tübingen, Tübingen, Germany; and [2]Microbial Ecology, Department of Biology, University of Konstanz, Konstanz, Germany

Correspondence: Bernhard Schink, Microbial Ecology, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany. Tel.: +49 7531 88 2140; fax: +44 7531 88 4047; e-mail: bernhard.schink@uni-konstanz.de

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Abstract

In order to assess the importance of nitrate-dependent Fe(II) oxidation and its impact on the growth physiology of dominant Fe oxidizers, we counted these bacteria in freshwater lake sediments and studied their growth physiology. Most probable number counts of nitrate-reducing Fe(II)-oxidizing bacteria in the sediment of Lake Constance, a freshwater lake in Southern Germany, yielded about 10^5 cells mL^{-1} of the total heterotrophic nitrate-reducing bacteria, with about 1% (10^3 cells mL^{-1}) of nitrate-reducing Fe(II) oxidizers. We investigated the growth physiology of *Acidovorax* sp. strain BoFeN1, a dominant nitrate-reducing mixotrophic Fe(II) oxidizer isolated from this sediment. Strain BoFeN1 uses several organic compounds (but no sugars) as substrates for nitrate reduction. It also reduces nitrite, dinitrogen monoxide, and O_2, but cannot reduce Fe(III). Growth experiments with cultures amended either with acetate plus Fe(II) or with acetate alone demonstrated that the simultaneous oxidation of Fe(II) and acetate enhanced growth yields with acetate alone (12.5 g dry mass mol^{-1} acetate) by about 1.4 g dry mass mol^{-1} Fe(II). Also, pure cultures of *Pseudomonas stutzeri* and *Paracoccus denitrificans* strains can oxidize Fe(II) with nitrate, whereas *Pseudomonas fluorescens* and *Thiobacillus denitrificans* strains did not. Our study demonstrates that nitrate-dependent Fe(II) oxidation contributes to the energy metabolism of these bacteria, and that nitrate-dependent Fe(II) oxidation can essentially contribute to anaerobic iron cycling.

Introduction

Iron is abundant in terrestrial and aquatic environments and exists in di- and trivalent redox states. These two redox species can be converted into each other either abiotically or by microbial catalysis, leading to a complex system of Fe-cycling processes (Kappler & Straub, 2005; Weber *et al.*, 2006a, b). For a long time, only aerobic Fe(II)-oxidizing bacteria were described, which grew either at neutral (Emerson & Revsbech, 1994; Emerson & Moyer, 1997; Emerson, 2000) or at acidic pH (Wood, 1988; Baker & Banfield, 2003). In the mid 1990s, the discovery of anaerobic Fe(II)-oxidizing bacteria (Widdel *et al.*, 1993; Hafenbradl *et al.*, 1996; Straub *et al.*, 1996) introduced the concept of microbially controlled iron cycling within anoxic environments (see, e.g. Kappler & Straub, 2005). The feasibility of such a redox cycle was also shown recently in lab experiments with defined cultures of Fe(III)-reducing and Fe(II)-oxidizing bacteria (Straub *et al.*, 2004; Roden *et al.*, 2004).

Most described nitrate-dependent Fe(II)-oxidizing strains depend on an organic cosubstrate such as acetate (Straub *et al.*, 1996; Benz *et al.*, 1998; Kappler *et al.*, 2005). Truly lithoautotrophic nitrate-reducing strains have not been isolated in a pure culture (Straub & Buchholz-Cleven, 1998). Weber *et al.* (2006c) isolated an Fe(II)-oxidizing bacterium that was suggested to be able to oxidize Fe(II) autotrophically; however, this strain could not be transferred continuously in a lithoautotrophic culture. Only one enrichment culture was described that oxidizes Fe(II) lithoautotrophically with nitrate (Straub *et al.*, 1996; Weber *et al.*, 2001). Recently, some strains of nitrate-dependent bacteria oxidizing Fe(II) in the absence of an organic cosubstrate were isolated from the deep sea (Edwards *et al.*, 2003), but it is unclear whether these strains can be

FEMS MICROBIOLOGY ECOLOGY

cultivated for successive generations with Fe(II) as the sole electron donor.

One of the key questions to answer is whether Fe(II) oxidation is beneficial and may even be induced specifically by Fe(II)-oxidizing bacteria (Chan *et al.*, 2004) or harmful to nitrate-reducing bacteria that simultaneously oxidize organic compounds (acetate). Microbial Fe(II) oxidation at a neutral pH may result in cell encrustation (Emerson &Moyer, 1997; Kappler *et al.*, 2005; Miot *et al.*, 2009; Schaedler *et al.*, 2009), potentially leading to a limitation of nutrient and substrate uptake into the cell (Hallberg & Ferris, 2004) or to constrained cell mobility (Kappler *et al.*, 2005). On the other hand, Fe(III) mineral coatings may act as a conductive mineral phase, allowing an electron transfer from mineral-bound Fe(II) via the iron mineral crust to the cell surface. Such an electron transfer via iron minerals was shown recently for hematite in abiotic systems (Williams & Scherer, 2004; Yanina & Rosso, 2008). It is also unknown as to how other neutrophilic Fe(II)-oxidizing strains successfully avoid encrustation by iron minerals (Edwards *et al.*, 2003; Kappler & Newman, 2004; Hegler *et al.*, 2008).

In the present study, we quantified Fe(II)-oxidizing nitrate-reducing bacteria in the sediment of a freshwater lake. We characterized the dominant nitrate-reducing Fe(II) oxidizer *Acidovorax* sp. strain BoFeN1 with respect to its growth physiology and a possible benefit of Fe(II) oxidation in the presence of acetate. Iron oxide encrustation was followed over time in parallel with Fe(II) oxidation and growth. Finally, we also analyzed the capacity for nitrate-dependent Fe(II) oxidation in known species of nitrate-reducing bacteria.

Materials and methods

Bacterial strains and cultivation conditions

Acidovorax sp. strain BoFeN1 was isolated from the most probable number (MPN) dilution series as described earlier (Kappler *et al.*, 2005). The following pure cultures were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany: *Thiobacillus denitrificans*: DSM 807, ATCC 29685; *Pseudomonas stutzeri*: DSM 5190, ATCC 17588; *Pseudomonas fluorescens* IV: DSM 50415, ATCC 12983; and *Paracoccus denitrificans*: DSM 65, ATCC 17741.

An oxygen-free, nonreduced bicarbonate/CO_2-buffered freshwater mineral medium (pH 7.0) (Ehrenreich & Widdel, 1994; Kappler *et al.*, 2005) under a headspace of N_2/CO_2 (90/10 v/v) was used in all cultivation studies. Ferrous iron (8 or 10 mM) was added from an anoxic 1 M $FeCl_2$ or 1 M $FeSO_4$ stock solution. Growth experiments were performed either in serum bottles closed with butyl rubber stoppers or in test tubes closed with rubber septa. Gaseous substrates [H_2, CO_2, dinitrogen monoxide (N_2O), and O_2] were supplied with syringes into the headspace.

For experiments quantifying the growth of the strain BoFeN1 in the presence or absence of Fe(II) by cell counts, 100 -mL glass serum bottles containing 50 mL of mineral medium were prepared. Bottles were amended either with $NaNO_3$ (10 mM), Na-acetate (5 mM) and $FeSO_4$ (10 mM), or with 10 mM $NaNO_3$ plus 5 mM acetate without $FeSO_4$.

Reduction of Fe(III) was tested in 100-mL glass serum bottles containing 50-mL mineral medium. Cultures were amended with acetate (5 mM) or H_2 (5% in the gas phase) as the electron donor and either ferrihydrite [$Fe(OH)_3$] (5 mM) or ferric citrate ($FeC_6H_5O_7$) (5 mM) as the electron acceptor.

Counts of nitrate-reducing bacteria in lake sediments

Sediment samples were taken at 1–2 m water depth at the Southern shore of the north-western arm of Lake Constance (Überlinger See), close to the island Mainau, where the sediment is slightly sandy and covered with thin limestone precipitates. Plastic core tubes (8-cm diameter) were immersed into the sediment. Inoculum material for direct dilution in agar shake tubes was taken at 1-cm sediment depth, at the transition zone between the brownish surface sediment and gray deeper sediment. Oxygen penetrated into this sediment down to about 4-mm depth (Gerhardt *et al.*, 2005). Agar shake dilutions (10 × dilutions, three replicates per dilution) (Widdel & Bak, 1992) were run in the medium described above with 2 mM acetate, 5 mM $FeSO_4$, and 5 mM $NaNO_3$ as substrates. Parallel dilution series were run with acetate plus nitrate or with ferrous sulfate plus nitrate as substrates. Colonies appearing after 4–5 weeks of incubation at 28 °C were suspended and diluted again in agar shake cultures and transferred into a liquid medium after microscopic checking for purity. The nitrate-reducing Fe(II)-oxidizing *Acidovorax* sp. strain BoFeN1 had been isolated in this way previously from one of the highest positive dilution tubes (Kappler *et al.*, 2005).

Analytical methods

For analysis of total Fe(II) and Fe(III), 100-μL samples were taken at different time points under sterile and anoxic conditions with a syringe (Kappler & Newman, 2004) and analyzed using the ferrozine assay (Stookey, 1970). Fe(II) was determined directly after dissolution of a sample (100 μL) taken from the culture fluid in 1 M HCl (900 μL). Total Fe was determined by reducing an aliquot of the sample with hydroxylamine hydrochloride before addition of the ferrozine reagent. Fe(III) was calculated by subtracting the amount of Fe(II) from the amount of total Fe. The purple ferrozine–Fe(II) complex was quantified at 562 nm

using a microtiter plate reader (FlashScan 550, Analytik Jena, Germany). Three parallel measurements were performed per sample. A calibration line with 0, 10, 25, 50, 100, 250, and 500 μM ferrous iron ($FeCl_2 \times 4H_2O$) was used for calculation.

N_2O was quantified by GC using a thermal conductivity detector (Carlo Erba VEGA 6000 with HWD 430). Samples (0.3 mL) were separated on a 45/60 Carboxen 1000 column with helium as the carrier gas (25 mL min^{-1}). Oven temperature was 120 °C, detector block 180 °C, and filament temperature was 270 °C.

For microscopic quantification of cell growth, 1-mL samples were taken sterilely from acetate- and iron-acetate-grown cultures and fixed for 30 min at room temperature with 0.1 mL of 37% formaldehyde. The fixative and the supernatant were removed after centrifugation (10 min at 8000 ***g***), and the cell–mineral aggregates were resuspended in 1 mL of sterile 0.9% NaCl. This sample and 1-mL anoxic ferrous ethylene diammonium sulfate [$FeC_2H_4(NH_3)_2SO_4$] from a 100 mM anoxic stock solution were added to 8.9 mL oxalate solution (0.23 M ammonium oxalate, 0.17 M oxalic acid, pH 3, filter sterilized) to dissolve Fe(III) minerals. Ten microliters of 4′,6-diamidino-2-phenylindole (5 mg mL^{-1} stock) was added and 0.5–1 mL of the cell suspension was sucked onto a filter (polycarbonate, Millipore, 0.22-μm pore size). Cells were counted under a microscope (Axioscop 2 plus, Zeiss, objective ×40) and documented using the SPOT advanced software program (Diagnostic Instruments Inc.). For each sample, two to four filters with eight images each were counted.

Light microscopy and scanning electron microscopy (SEM)

To quantify dead and alive cells, a 10-μL sample was taken from growing cultures at different time points and mixed with 3 μL live/dead stain (BacLight, bacterial viability kit, Molecular Probes Inc.). Cells were visualized under a light microscope (Axioscop 2 plus, Zeiss, objective ×40) and documented using the SPOT advanced program (Diagnostic Instruments Inc.).

For SEM, samples were prepared and investigated as described previously (Schaedler *et al.*, 2008). Briefly, 1-mL samples were taken anoxically and sterilely from the cultures inside the glove box. Samples were fixed for 20 min at room temperature with a half-strength Karnovsky solution (2% glutaraldehyde plus 4% formaldehyde in 20 mM KH_2PO_4, 80 mM $Na_2HPO_4 \times 2H_2O$ phosphate buffer). After fixation, the cells and minerals were washed three times in phosphate-buffered saline (PBS). Twenty microliters of PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4)-suspended cells were pipetted onto a carbon-coated electron microscope grid and dried overnight. The grid was washed for 10 min in PBS and immersed into a dehydration dilution series of distilled water mixed with isopropanol (0%, 15%, 30%, 50%, 70%, 80%, 90%, and > 95% isopropanol). Images were taken using the Scanning Electron Microscope LEO Model 1450 VP (Everhart-Thornley SE-Detector, 4-Quadrant BSE-Detector) of the Institute for Geoscience at the University of Tübingen.

Results

Quantification of total denitrifying bacteria and nitrate-dependent Fe(II) oxidizers in a freshwater lake sediment

Nitrate-dependent Fe(II)-oxidizing bacteria were quantified in two different sampling periods during fall and in winter 2002 at two different depths (5 and 30 mm) in the littoral sediment of Lake Constance, and were compared with the number of acetate-oxidizing heterotrophic nitrate-reducing bacteria (Table 1). At both depths, we found similar numbers of acetate-oxidizing denitrifiers, with slightly higher numbers in the surface layer (8×10^5 cells mL^{-1}) than in the deeper layer (2×10^5 cells mL^{-1}). Also, the numbers of mixotrophic nitrate-dependent Fe(II) oxidizers were similar at both depths, with slightly higher numbers in the surface layer (8×10^3 cells mL^{-1}) than in the deeper layer (1.5×10^3 cells mL^{-1}). In the dilution series with only ferrous iron plus nitrate, similar numbers of colonies developed as in the mixotrophic cultivation series, but these colonies only developed very late and remained much smaller than in the mixotrophic cultivation series. This indicates that mixotrophic Fe(II)-oxidizing nitrate reducers represent about 1% of the heterotrophic denitrifying community in these freshwater sediments. One representative nitrate-dependent Fe(II) oxidizer, *Acidovorax* sp. strain BoFeN1, has been isolated from the highest positive dilution step of the mixotrophic dilution series (Kappler *et al.*, 2005). On the basis of 16S rRNA gene sequence analysis, this strain had been assigned to the genus *Acidovorax* (Kappler *et al.*, 2005).

Utilization of alternative substrates by *Acidovorax* sp. strain BoFeN1

Strain BoFeN1 grew with the following substrates in anoxic incubations with 5 mM $NaNO_3$ as the electron acceptor: acetate, propionate, butyrate, pyruvate, malate, fumarate, succinate, ethanol, 1-propanol, 1-butanol (each 2 mM), and H_2/CO_2 (80/20). No growth was observed with $FeCl_2$ (5 mM), glucose, fructose, xylose, arabinose, ribose, glycerol, ethylene glycol, glycolate, citrate, acetone, isopropanol, isopropylamine (each 2 mM), formate (10 mM), and thiosulfate (10 mM). Acetate (2 mM) served as a growth substrate in the presence of $NaNO_3$, $NaNO_2$ (2 mM),

Table 1. Counts of nitrate-reducing bacteria in two different horizons of the littoral sediment of Lake Constance, Germany

	Colonies (mL^{-1})		
Depth (mm)	Colonies in acetate/nitrate medium	Fe-oxidizing colonies in acetate/Fe(II)/nitrate medium	Fe-oxidizing colonies in Fe(II)/nitrate medium
5	$8 \pm 6 \times 10^5$	$8 \pm 5 \times 10^3$	$6.5 \pm 4.5 \times 10^3$
30	$2.0 \pm 1.2 \times 10^5$	$1.5 \pm 0.3 \times 10^3$	$0.8 \pm 0.3 \times 10^3$

Sediment slurries were diluted in a bicarbonate-buffered anoxic mineral medium containing 2 mM acetate, 5 mM nitrate, and 8 mM $FeSO_4$. Colonies were counted after 9 weeks of incubation at 28 °C; those in the Fe(II)/nitrate medium became visible only after 8 weeks of incubation. Numbers give means ± standard variations of colony counts obtained in two independent counting experiments in fall and winter 2002.

N_2O (5%), or O_2 (20%). Thiosulfate or sulfate was not reduced. Neither $Fe(OH)_3$ nor dissolved $FeC_6H_5O_7$ was reduced with acetate as the electron donor.

Growth physiology and the energetic benefit from mixotrophic Fe(II) oxidation

Acidovorax sp. strain BoFeN1 grew between 4 °C (very slowly) and 37 °C, with an optimum temperature at 30 °C; no growth was observed at 45 °C. Growth was observed between pH 6.0 and 8.5, with an optimum at pH 7.0–7.5; weak growth was still observed at pH 9.0.

Strain BoFeN1 grew heterotrophically with acetate and also mixotrophically with acetate plus Fe(II) (Kappler *et al.*, 2005). In the present study, Fe(II) oxidation started 24 h after inoculation and was usually completed after 48 h (Fig. 1a), whereas Kappler *et al.* (2005) observed that Fe(II) oxidation started after 2 days and was completed after 4 or 5 days. In the presence of 10 mM Fe(II) and 5 mM acetate, cell numbers increased from 5×10^7 to 8.2×10^8 cells mL^{-1} within the first 2 days when Fe(II) was oxidized completely. The cell number increased further to 1.1×10^9 cells mL^{-1} on day 4.

On comparing cell numbers in acetate- vs. acetate-plus-Fe(II)-grown cultures, we observed a similar development of cell numbers within the first 2 days (Fig. 1b): the exponential growth phase was entered shortly after inoculation and both cultures reached a cell number of about 8×10^8 cells mL^{-1} after 2 days. While the cell number in acetate-grown cultures did not increase after day 2, the cell number in acetate-plus-Fe(II)-grown cultures increased further to 1.1×10^9 cells mL^{-1} within 4 days, although the Fe(II) was already completely oxidized after 2 days. These results show that acetate-plus-Fe(II)-grown cultures yielded about 2.1×10^8 cells mL^{-1} more than acetate-grown cultures. From the calibration of cell number counts via ODs of acetate-grown cultures to cell dry masses, the molar growth yield with acetate was calculated to be 12.5 g mol^{-1} acetate. The

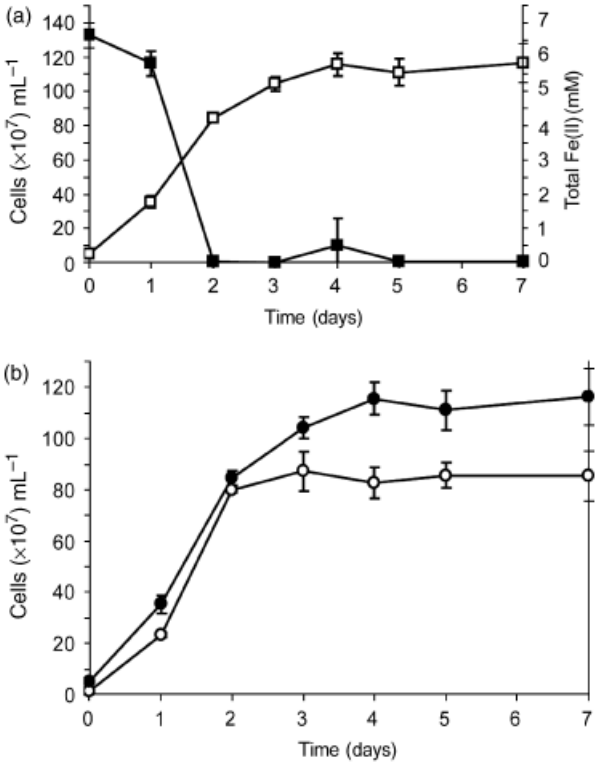

Fig. 1. Fe(II) oxidation and cell growth over time in Fe(II)-plus-acetate and acetate-grown cultures of strain BoFeN1. (a) Fe(II) (■) and concordant cell numbers (□) in Fe(II)-plus-acetate-grown cultures of strain BoFeN1 [10 mM nitrate, 5 mM acetate, 10 mM Fe(II)]. (b) Cell numbers of cultures grown either with iron plus acetate (●) or with acetate only (○) [10 mM nitrate, 5 mM acetate ± 10 mM Fe(II)]. All cultures were inoculated from a culture pregrown on acetate for two transfers (5 mM acetate, 10 mM nitrate).

yield increase by Fe(II) oxidation was calculated to be 1.42 g mol^{-1} Fe(II). Similar results were obtained in acetate-limited continuous cultures of strain BoFeN1 at the half-maximal growth rate in the presence or absence of 8 mM Fe(II) nitrilotriacetate (results not shown).

Utilization of ferrous iron depended on the presence of acetate; in the absence of acetate or other cosubstrates, Fe(II) was oxidized only extremely slowly and without measurable growth. Acetate already supported Fe(II) oxidation at a very low concentration: for stoichiometric oxidation of 4 mM Fe(II), only 0.2 mM acetate was required (Fig. 2).

Cell–mineral interactions and cell encrustation

SEM showed that acetate-grown cells did not change their shape during the different growth phases (Fig. 3a), whereas cells grown with Fe(II) plus acetate as the electron donor accumulated mineral crusts at their cell surface in the exponential growth phase (Fig. 3b) and were fully encrusted in the stationary phase (Fig. 3c and d). Despite the encrustation of cells with minerals and the formation of tight

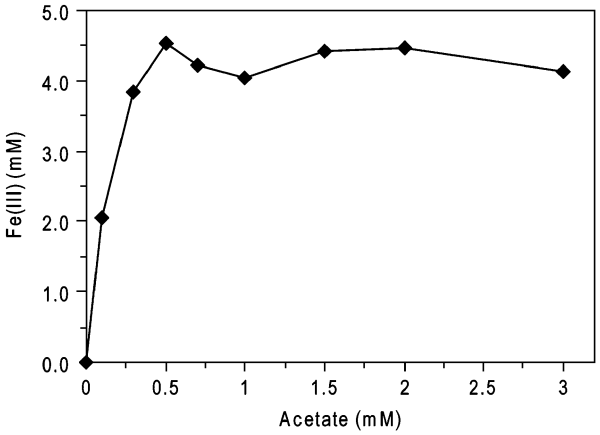

Fig. 2. Dependence of Fe(II) oxidation by strain BoFeN1 on the supply of acetate as a cosubstrate. One series of representative data is shown from five experimental series with similar results.

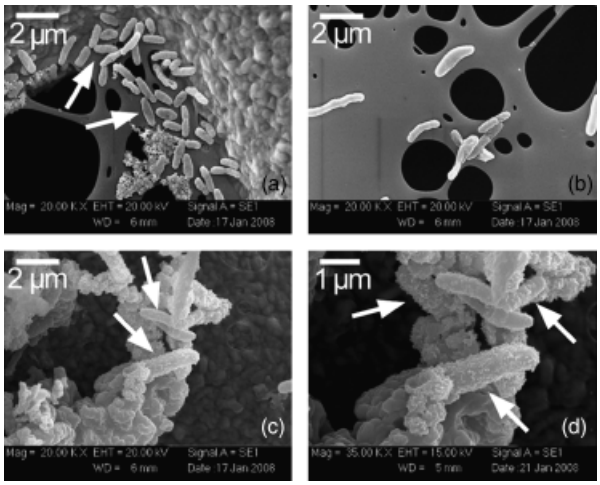

Fig. 3. Scanning electron micrographs of cells of strain BoFeN1. (a) Culture grown with 5 mM acetate plus 10 mM nitrate after 8 days, in comparison with cultures grown with acetate, NO_3^- plus 10 mM Fe(II): (b) after 36 h, (c) 8 days, (d) higher resolution image of the 8-day image. White arrows indicate cells. All setups were inoculated with a culture pregrown with acetate for two transfers.

cell–mineral aggregates, most of the cells were alive after one month according to the dead–live stain (Fig. 4).

Ferrous iron oxidation by other nitrate-reducing bacteria

In order to check whether the capacity for ferrous iron oxidation is widespread among nitrate-reducing bacteria, we examined some well-studied nitrate reducers for this capacity (Table 2). We found that the extent of Fe(II) oxidation by the tested strains of *P. fluorescens* and *T. denitrificans* remained below that of the abiotic control assays, whereas the *P. stutzeri* and *P. denitrificans* strains

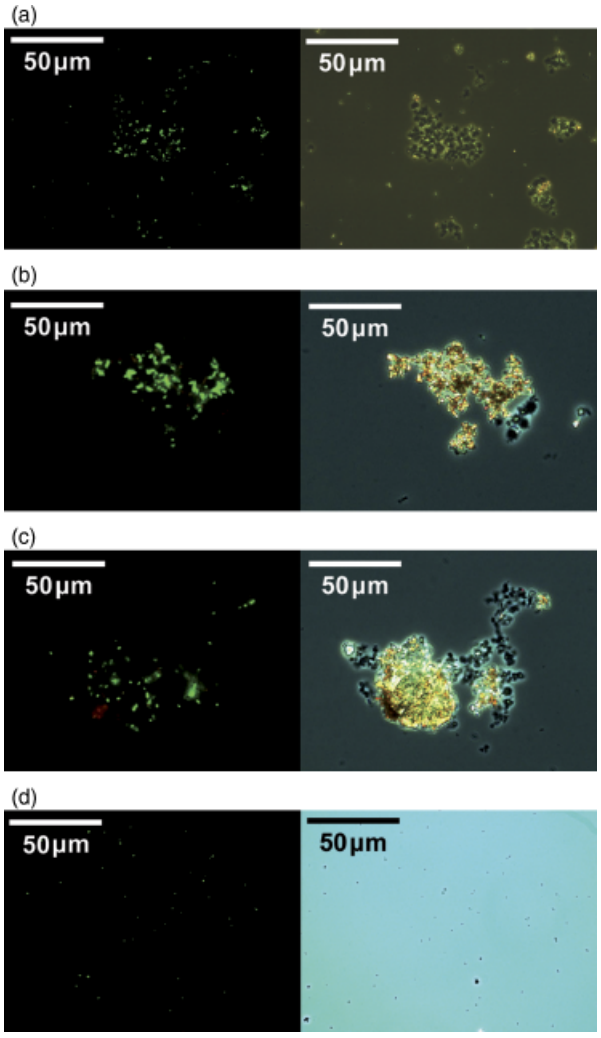

Fig. 4. Light microscopic images of dead–live stained cells of the strain BoFeN1 (green, alive; red, dead). Fluorescence images are shown on the left, and bright field images on the right. Images show samples from cultures amended with 10 mM Fe(II), 5 mM acetate plus 10 mM nitrate: (a) $t = 36$ h (exponential growth phase), (b) $t = 8$ days (stationary phase), (c) $t = 1$ month (death phase). In (d), for comparison, a light microscopy image of a culture amended with only acetate plus NO_3^- [no Fe(II)] is shown (sample taken after 8 days, stationary phase). All setups were inoculated with a culture pregrown with acetate for two transfers.

exhibited significant Fe(II) oxidation after 4 weeks of incubation, but at much lower rates than *Acidovorax* sp. strain BoFeN1.

Discussion

Ecological importance of mixotrophic nitrate-dependent Fe(II) oxidation

MPN counts with freshwater lake sediments revealed that the numbers of mixotrophic nitrate-reducing iron oxidizers

Table 2. Oxidation of Fe(II) by nitrate-reducing pure cultures

	Pure cultures tested				
	Pseudomonas stutzeri	*Pseudomonas fluorescens*	*Thiobacillus denitrificans*	*Paracoccus denitrificans*	Control
Fe(II) sulfate supplied* (mM)	6.4	6.9	7.3	4.5	7.0
Fe(III) formed† (mM)	3.2	1.2	0.6	3.8	1.2
Electrons from Fe(II) oxidation (mM)	3.2	1.2	0.6	3.8	1.2
Nitrate supplied (mM)	2.9	3.6	3.7	3.3	3.1
Nitrate consumed (mM)	0.5	0‡	0.03	0.7	0.02
Nitrite formed (mM)	0	0	0	0	0
N_2O formed (mM)	0.02	0.02	0.01	0.003	0.01
Electrons consumed in nitrate reduction (mM)	2.5	0.1	0.2	3.5	0.1

Cultures and control tubes were incubated in triplicate for 4 weeks until product formation was assayed.

*Initial Fe(II) content including Fe(II) in the fresh medium plus remaining Fe(II) transferred from inoculum.

†Fe(III) content from the inoculum was subtracted.

‡'0' means below the detection limit (< 0.005 mM).

were about two orders of magnitude lower than the numbers of total acetate-oxidizing denitrifiers. As shown here and in former field studies (Straub & Buchholz-Cleven, 1998; Hauck *et al.*, 2001), indeed, in most environments, the number of Fe(II)-oxidizing nitrate reducers is significantly lower than that of heterotrophic denitrifiers. It also appears that mixotrophic Fe(II) oxidation with organic cosubstrates is the preferred process for nitrate-dependent Fe(II) oxidation. Counts of Fe(II) oxidizers in the agar shake series in the presence or absence of acetate as a cosubstrate yielded similar numbers, but colonies were significantly larger in the presence of acetate. Because traces of organic substrates, for example acetate, are always available in sediments, iron oxidation in these environments is likely to proceed mixotrophically rather than lithoautotrophically. Thus, although mixotrophic Fe(II)-oxidizing nitrate reducers make up only a minor part of the total nitrate-reducing community, they may contribute significantly to Fe(II) oxidation within the anoxic part of the lake sediment. Beyond our new isolate, two out of four pure cultures of well-known nitrate-reducing bacteria were also found to be able to oxidize Fe(II) with nitrate, although slowly, thus indicating that this capacity may be widespread among nitrate reducers.

Physiology of mixotrophic Fe(II) oxidation

Strain BoFeN1 oxidized ferrous iron only in the presence of a cosubstrate such as acetate, similar to other isolates described earlier (Straub *et al.*, 1996, 2004; Benz *et al.*, 1998; Lack *et al.*, 2002). The only pure culture reported to grow lithoautotrophically with ferrous iron and nitrate exhibited only very weak growth, with hardly two doublings in one cultivation period (Weber *et al.*, 2006c). The reason for this dependence on a cosubstrate may be twofold: lithoautotrophic growth requires a means of autotrophic CO_2 fixation, for example, the Calvin cycle, as this is being used by nearly all aerobic or nitrate-reducing lithoautotrophic bacteria (McFadden & Shively, 1991). Beyond this, autotrophic CO_2 fixation requires reducing equivalents carried by NAD(P)H ($E' = -320$ mV). Most lithoautotrophs, especially those oxidizing sulfur and nitrogen compounds, use reversed electron transport systems to supply the necessary low-potential electrons (Ferguson, 1988), and such a reversed electron transport system may be missing in strain BoFeN1. Oxidation of ferrous iron at pH 7.0 releases electrons at a redox potential of around 0 ± 100 mV, depending on the type of ferric oxide formed (Straub *et al.*, 2005). Obviously, these electrons are only used in energy metabolism. If they enter the electron transport chain at the level of ubiquinone ($E' = +113$ mV), the ATP gain is rather limited. As shown in an earlier study on the bioenergetics of nitrate reduction (Strohm *et al.*, 2007), ubiquinol oxidation with nitrate can lead to a translocation of a maximum of 16 protons per five ubiquinol oxidized, which is equivalent to the oxidation of 10 ferrous iron ions. Thus, only a maximum of 0.4 ATP can be synthesized per ferrous iron oxidized. In our present study, we showed that mixotrophic oxidation of ferrous iron plus acetate increased the acetate-dependent growth yield by 1.4 g dry mass mol^{-1} Fe(II). In a former study on the energetics of denitrification, we calculated a Y_{ATP} value of 3.3 g mol^{-1} ATP for *Pseudomonas* and *Paracoccus* strains (Strohm *et al.*, 2007). Using this Y_{ATP} value, we would expect a cell mass increase of 1.3 g mol^{-1} Fe(II) oxidized, which is nearly identical to our experimental result. However, some Fe(II) electrons may also bypass the quinone cycle and be transferred directly to cytochrome *c*, with correspondingly lower ATP yields.

The ratio of acetate requirement vs. Fe(II) oxidized does not appear to be a constant value. In an earlier study, we

found that a brackish water isolate, strain HidR2, oxidized Fe(II) at a ratio of four Fe(II) per acetate (Benz *et al.*, 1998). In the present study, strain BoFeN1 requires only 0.2 acetate for oxidation of four Fe(II), i.e., the ratio of acetate to Fe(II) is lower by a factor of five. Obviously, this ratio differs between different bacterial isolates, perhaps depending on the cell-internal organization of electron flow from the two electron donors to nitrate.

Consequences of cell encrustation and Fe(III) mineral formation

The isolation of metabolically diverse Fe(II)-oxidizing bacteria either being encrusted (Emerson & Moyer, 1997; Kappler *et al.*, 2005; Schaedler *et al.*, 2009) or nonencrusted by iron minerals (Benz *et al.*, 1998; Edwards *et al.*, 2003; Kappler & Newman, 2004; Schaedler *et al.*, 2009) has raised many questions in the past. As a consequence of encrustation, cells might be limited in nutrient and substrate uptake (Hallberg & Ferris, 2004) or limited in their mobility and hence have a lower life expectancy (Kappler *et al.*, 2005). Microscopic observations indicate that encrusted cells are still metabolically active, and therefore, substrate transport may be possible (Miot *et al.*, 2009). Even division of encrusted cells has been observed (Schaedler *et al.*, 2009). On the other hand, these iron crusts may be beneficial by protecting cells from UV radiation, as Fe(III) was shown to absorb this radiation in sediments and microbial crusts (Pierson *et al.*, 1993), or from predation or dehydration (Phoenix & Konhauser, 2008). Fe(III) mineral coatings may even act as a conductive layer allowing the transfer of electrons from sorbed Fe(II) via the conductive iron mineral crust to the cells, as it was shown for abiotic mineral systems (Williams & Scherer, 2004). This tight association with conductive iron minerals might artificially increase the surface of cells to accept electrons from sorbed Fe(II) and thus allow the cells to gain more energy. In a natural environment, this process might allow this strain to compete effectively with other Fe(II) oxidizers in its habitat.

Scanning electron micrographs and light microscopy images of strain BoFeN1 showed that the cells already begin to encrust in the logarithmic growth phase after 36 h. Kappler *et al.* (2005) previously observed that many BoFeN1 cells lose their iron crust when they are transferred to a fresh medium. Obviously, the cells are able to cope with the mineral formation, and substrate and nutrient uptake are still possible despite the encrustation. Several ways of how bacterial cells could avoid encrustation have been suggested in the literature; however, no explicit answer has been found yet (for an overview, see Schaedler *et al.*, 2009). A slightly acidic microenvironment around cells or Fe(III)-complexing organic ligands excreted by the Fe(II)-oxidizing bacteria could retain Fe(III) in solution (Sobolev & Roden, 2001, 2004; Kappler & Newman, 2004). Extracellular organic compounds were suggested to serve to scavenge the Fe(III) and thus avoid Fe(III) mineral precipitation at the cell surface (Hanert, 1981; Hallberg & Ferris, 2004; Miot *et al.*, 2009). In contrast to these Fe(II) oxidizers, which can avoid cell encrustation by one or a combination of these mechanisms, cells of strain BoFeN1 are obviously not able to prevent sorption of Fe(III) and precipitation of Fe(III) minerals at the cell surface. However, this strain obviously is able to grow and break out of its crust when the growth conditions change.

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