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Abiotic oxidation of Fe(II) by reactive nitrogen species in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax* sp. BoFeN1 – questioning the existence of enzymatic Fe(II) oxidation

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ABSTRACT

Nitrate-reducing, Fe(II)-oxidizing bacteria were suggested to couple with enzymatic Fe(II) oxidation to nitrate reduction. Denitrification proceeds via intermediates (NO_2^-, NO) that can oxidize Fe(II) abiotically at neutral and particularly at acidic pH. Here, we present a revised Fe(II) quantification protocol preventing artifacts during acidic Fe extraction and evaluate the contribution of abiotic vs. enzymatic Fe(II) oxidation in cultures of the nitrate-reducing, Fe(II) oxidizer *Acidovorax* sp. BoFeN1. Sulfamic acid used instead of HCl reacts with nitrite and prevents abiotic Fe(II) oxidation during Fe extraction. Abiotic experiments without sulfamic acid showed that acidification of oxic Fe(II) nitrite samples leads to 5.6-fold more Fe(II) oxidation than in anoxic samples because the formed NO becomes rapidly reoxidized by O_2, therefore leading to abiotic oxidation and underestimation of Fe(II). With our revised protocol using sulfamic acid, we quantified oxidation of approximately 7 mM of Fe(II) by BoFeN1 within 4 days. Without addition of sulfamic acid, the same oxidation was detected within only 2 days. Additionally, abiotic incubation of Fe(II) with nitrite in the presence of goethite as surface catalyst led to similar abiotic Fe(II) oxidation rates as observed in growing BoFeN1 cultures. BoFeN1 growth was observed on acetate with N_2O as electron acceptor. When adding Fe(II), no Fe(II) oxidation was observed, suggesting that the absence of reactive N intermediates (NO_2^-, NO) precludes Fe(II) oxidation. The addition of ferrihydrite [$Fe(OH)_3$] to acetate/nitrate BoFeN1 cultures led to growth stimulation equivalent to previously described effects on growth by adding Fe(II). This suggests that elevated iron concentrations might provide a nutritional effect rather than energy-yielding Fe(II) oxidation. Our findings therefore suggest that although enzymatic Fe(II) oxidation by denitrifiers cannot be fully ruled out, its contribution to the observed Fe(II) oxidation in microbial cultures is probably lower than previously suggested and has to be questioned in general until the enzymatic machinery-mediating Fe(II) oxidation is identified.

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INTRODUCTION

Ferrous iron (Fe(II)) is important not only as a nutrient for virtually all organisms (Andrews *et al.*, 2003) but also as an electron and even energy source for many microorganisms (Weber *et al.*, 2006a; Bird *et al.*, 2011; Konhauser *et al.*, 2011). Aerobic acidophilic (Bonnefoy & Holmes, 2011), micro-aerobic neutrophilic (Emerson & Moyer, 1997; Krepski *et al.*, 2011), as well as anaerobic bacteria using light (Widdel *et al.*, 1993; Hegler *et al.*, 2008) or nitrate (Straub *et al.*, 1996) have been discovered and studied for their ability to oxidize ferrous iron. Several strains and enrichments of neutrophilic nitrate-reducing, Fe(II)-oxidizing bacteria have previously been isolated (Kappler *et al.*, 2005; Weber *et al.*, 2009; Chakraborty *et al.*, 2011), almost all of which have been shown to be

able to use nitrate (NO_3^-) and the intermediates of the denitrification process nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) as electron acceptors (Philippot, 2002; Picardal, 2012). Autotrophic growth has been claimed only for *Pseudogulbenkiania* sp. strain 2002 (Weber *et al.*, 2006b, 2009) and for the coculture KS (Straub *et al.*, 1996). All other strains are mixotrophic, that is, they need an organic cosubstrate in addition to Fe (II) for continuous growth (Benz *et al.*, 1998; Kappler *et al.*, 2005).

The first step of denitrification, the reduction of nitrate, leads to the formation of nitrite, and depending upon the geochemical conditions, such as temperature, organic matter concentration, pH and carbon sources, nitrite can accumulate to mM concentrations in the environment (Betlach & Tiedje, 1981; Constantin & Fick, 1997; Glass & Silverstein, 1998). In cultures of nitrate-reducing, Fe (II)-oxidizing bacteria with several mM concentrations of electron donor (acetate, Fe(II)) and acceptor (nitrate), nitrite was shown to accumulate at mM concentrations as well (Kappler *et al.*, 2005; Weber *et al.*, 2006b). At acidic pH, nitrite is not very stable both in soils and in aqueous solution. Under acidic pH conditions, there is a shift in the equilibrium ($pK_a = 3.3$) from nitrite (NO_2^-) toward nitrous acid (HNO_2) (equation 1), and at a pH below 5, nitrous acid shows spontaneous self-decomposition into NO_2 and NO (Nelson & Bremner, 1970a; Park & Lee, 1988) (equation 2).

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \quad (1)$$

$$2HNO_2 \rightarrow NO_2 + NO + H_2O \quad (2)$$

The N species that are produced are fairly strong oxidants in aqueous solution (Van Cleemput & Baert, 1984; Van Cleemput & Samater, 1996) and can potentially react with the Fe(II) present in cultures of Fe(II)-oxidizing bacteria. The abiotic oxidation of ferrous iron by nitrite has been investigated intensively (Wullstein & Gilmour, 1966; Buresh & Moraghan, 1976; Ibrahim *et al.*, 2001). Van Cleemput & Baert (1983) showed an enhanced decomposition of nitrite in the presence of 800 mg L^{-1} Fe^{2+} at pH values below 4. At neutral pH, the reaction of nitrite with ferrous iron is slower but still significant (Buresh & Moraghan, 1976; Van Cleemput & Baert, 1983). Additional factors can stimulate the decomposition of nitrous acid, for example, the presence of other metallic cations including Cu^{2+} (Nelson & Bremner, 1970b; Ottley *et al.*, 1997), which is normally also present at low µM concentration in microbial culture media. Furthermore, catalytic reactions can occur at the surface of Fe minerals, such as lepidocrocite, green rusts, siderite,or hydrous ferric oxide (Sorensen & Thorling, 1991; Hansen *et al.*, 1994; Rakshit, 2007; Tai & Dempsey, 2009), which are minerals that were also shown to be present in cultures of anaerobic nitrate-reducing, Fe(II)-oxidizing bacteria (Miot *et al.*, 2009; Larese-Casanova *et al.*, 2010; Pantke *et al.*, 2012). For quantification of Fe(II) and Fe(III) in cultures of Fe-metabolizing bacteria by the spectrophotometric ferrozine assay (Stookey, 1970), samples are commonly acidified with HCl for dissolution of Fe(II)/Fe(III) minerals and stabilization of Fe(II). However, decreasing the pH is expected to lead to rapid decomposition of the HNO_2 formed from nitrite present (equations 1 & 2) followed by rapid abiotic oxidation of Fe(II) by the reactive NO_2 and NO (Bonner & Pearsall, 1982) (equations 3 & 4) with N_2O as final N product (equation 5).

$$NO_2 + 2Fe^{2+} + 2H^+ \rightarrow 2Fe^{3+} + NO + H_2O \quad (3)$$

$$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + HNO \quad (4)$$

$$2HNO \rightarrow N_2O + H_2O \quad (5)$$

These processes can lead to systematic errors in the Fe (II) and Fe(III) values measured by photometric quantification and consequently to an overestimation of Fe(II) oxidation rates by iron(II)-oxidizing bacteria. Although this problem has been described already by Weber *et al.* (2001) and recently by Picardal (2012), little attention has been paid to iron quantification in the presence of nitrite in some publications describing nitrate-dependent iron oxidizers (Senko *et al.*, 2005; Bosch *et al.*, 2012).

Recent studies have actually even suggested that abiotic reactions by nitrite probably have a much more pronounced influence on the Fe(II) oxidation observed in these cultures than previously thought and thereby raise the question to which extent these abiotic side effects contribute to the quantified rates and extent of nitrate-dependent Fe(II) oxidation (Carlson *et al.*, 2012; Picardal, 2012). The goals of the present study therefore were (i) to use a revised Fe(II) quantification protocol to determine *in situ* Fe(II) oxidation rates preventing artifacts during acidic Fe extraction and (ii) to evaluate the contribution of abiotic vs. enzymatic Fe(II) oxidation in cultures of the nitrate-reducing, Fe(II)-oxidizing strain *Acidovorax* sp. BoFeN1.

MATERIALS AND METHODS

Source of micro-organism

Strain BoFeN1 is a chemoorganotrophic, nitrate-reducing ß-proteobacterium closely related to *Acidovorax* sp. isolated from Lake Constance sediments (Kappler *et al.*, 2005). The strain was kept in the authors' laboratory since its original isolation. BoFeN1 grows under denitrifying conditions mixotrophically oxidizing ferrous iron with acetate as an organic cosubstrate (Muehe *et al.*, 2009).

Microbial growth media and growth conditions

For routine cultivation of strain BoFeN1, 10 mM Na nitrate and 5 mM Na acetate were added to 22 mM bicarbonate-buffered low-phosphate mineral medium (pH 7.1), which was prepared anoxically as described in detail by Hegler *et al.* (2008) and Hohmann *et al.* (2010). For mineral precipitation experiments, 10 mM Fe(II) was added from an anoxic, sterile 1 M $FeCl_2$ stock solution upon which precipitation of whitish, poorly crystalline Fe(II) carbonates and Fe(II) phosphates followed (Kappler & Newman, 2004). Precipitate-free medium was prepared by sterile filtration (0.22 μm, mixed cellulose esters, Millipore) in an anoxic chamber (Braun, Germany, 100% N_2 atmosphere) according to Kappler & Newman (2004), which contained a final Fe(II) concentration of 6–8 mM and a remaining concentration of 10–20 μM phosphate.

Experimental setup

Serum bottles (58 mL) were washed with 1 M HCl and distilled water prior to sterilization by autoclaving. Twenty-five mL of filtered medium was filled anoxically into each bottle [headspace N_2/CO_2 (90/10, v/v)], and bottles were sealed with butyl stoppers and crimped. Bottles were amended with anoxic Na nitrate (10 mM) and Na acetate (5 mM) and inoculated with 5% of a fresh BoFeN1 culture grown on acetate/nitrate (approximately 5×10^6 cells mL^{-1}). All cultures were incubated at 28 °C in the dark. For abiotic experiments, bottles containing medium were amended with different nitrite concentrations (0.5–8 mM). Additionally, 2 mM abiogenic goethite (Bayferrox, Lanxess, Leverkusen, Germany) was added to abiotic experiments testing the effects of mineral presence on abiotic Fe(II) oxidation. For growth with nitrous oxide, bottles contained initially 100 μM nitrate to induce the N_2O gene cluster, and every day, 5%, that is, 1.25 mL, of the headspace was exchanged with N_2O accumulating to approximately 500 μmol total N_2O (20 mM N_2O in our 25-mL medium bottles) within 10 days. Ferrihydrite was synthesized according to Raven *et al.* (1998), washed four times with Millipore water, deoxygenated by vacuum, and autoclaved. Approximately 7 mM ferrihydrite was added to a nitrate/acetate-containing BoFeN1 culture. Samples for cell counts were taken every day as described later. For Fe(III) reduction experiments, ferrihydrite was added in exchange for nitrate.

Analytical methods

For quantification of Fe(II) and Fe(III), 100 μL of culture suspension was withdrawn anoxically with a syringe and dissolved in 900 μL of 0.5 M HCl for 1 h at room temperature. After Fe mineral dissolution, dissolved Fe(II) and Fe (III) were quantified in the liquid phase by the ferrozine assay (Stookey, 1970). Total Fe was determined by reducing an aliquot of the sample with hydroxylamine hydrochloride (10% w/v in 1 M HCl) 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, Jena, Germany). All iron measurements with the ferrozine assay were carried out in triplicates. For the revised ferrozine assay, the samples were not dissolved in HCl but rather in 40 mM sulfamic acid (pH approximately 1.8) that reacts rapidly with nitrite, thus preventing oxidation of Fe(II) by the nitrite at acidic pH (Granger & Sigman, 2009). Sulfamic acid does not function as reducing agent, and no reduction of Fe(III) to Fe(II) by sulfamic acid was observed (data not shown). Control experiments with the poorly crystalline Fe(III) oxyhydroxide, ferrihydrite, showed that both 0.5 M HCl and 40 mM sulfamic acid were able to dissolve ferrihydrite to a similar extent (data not shown). A separate calibration curve was prepared for the revised ferrozine assay that uses sulfamic acid. For analysis under anoxic conditions, samples were withdrawn, incubated, and reacted with the ferrozine reagent inside of an anoxic chamber (100% N_2) and were exposed only briefly to air/oxygen for approximately 2 min during absorbance measurements. For analysis under oxic conditions, all samples (in HCl or sulfamic acid) were incubated, and the ferrozine assay was performed in the presence of air. Maximum rates of microbial iron oxidation for the individual setups were calculated from the steepest slope between two subsequent data points of Fe(II) concentrations. Samples for NO_3^- and NO_2^- quantification were stored anoxically at 5 °C for a maximum of 7 days before analysis by a flow injection analysis (FIA) system containing a special membrane for iron removal to prevent reactions between nitrite and iron during analysis (3-Quattro; Bran & Lübbe, Norderstedt, Germany). Acetate was quantified by HPLC (Class vp with RID 10 A & DAD SPM 10 A vp dectors, Shimadzu, Japan; pre-column: Micro guard cation H cartridge; main column: Aminex HPX-87H Ion exclusion column 300 mm × 7.8 mm, Bio-Rad, Vienna, Austria; eluent: 5 mM H_2SO_4 in MQ water). For quantification of cell growth, optical density (OD) was quantified at 660 nm (SPEKOL 1300; Analytik Jena). For microscopic quantification of cell growth, samples were taken aseptically from nitrate/acetate- or iron(III)/nitrate/acetate-grown cultures and fixed for 30 min at room temperature with formaldehyde (final concentration 9%). The fixative and the supernatant were removed after centrifugation (10 min at 8000 *g*), and the cells were resuspended in 1 mL of sterile 0.9% NaCl solution. Cells without iron were further diluted in PBS buffer. Samples containing iron were mixed with 1 mL anoxic ferrous ethylene diammonium sulfate [$FeC_2H_4(NH_3)_2(SO_4)_2$] from a 100 mM anoxic stock solution and with 8 mL oxalate solution (0.23 M ammonium

oxalate, 0.17 M oxalic acid, pH 3, filter sterilized) to dissolve the Fe(III) minerals. Cells were immobilized on a filter (polycarbonate, Millipore, 0.22 μm pore size), stained with 4,6-diamidino-2-phenylindole (DAPI) (final concentration of 5 mg mL^{-1}) and counted using a fluorescence microscope (CTR 5500; Leica, Wetzlar, Germany). Siderophores in the supernatants of iron-free cultures were detected with the Chrome Azurol S (CAS) assay performed as described by Schwyn & Neilands (1987).

RESULTS

Fe(II) oxidation in *Acidovorax* sp. BoFeN1 cultures quantified with HCl or with sulfamic acid

Fe(II) concentrations were quantified over time in cultures inoculated with the nitrate-reducing Fe(II) oxidizer *Acidovorax sp.* BoFeN1. When samples were incubated in sulfamic acid instead of HCl for Fe analysis, a lower maximal oxidation rate (approximately 3.4 mM day^{-1} in contrast to 5.1 mM day^{-1}) and a delayed start of Fe(II) oxidation were observed (Fig. 1A). Under these conditions, sulfamic acid reacted with nitrite and thus prevented its reaction with Fe(II) during acidification. Nitrite accumulation in the culture started already at day 1, while acetate but not Fe(II) was consumed, and reached values of up to 3 mM. Acetate was completely consumed and absent in solution already after 2 days (Fig. 1B). Nitrate concentration was stable after day 2 at approximately 2–3 mM and decreased only slightly after that (Fig. 1B). Sterile controls showed neither oxidation of Fe(II) nor nitrate reduction.

Abiotic reactions of nitrite with Fe(II) at acidic pH in the presence and absence of sulfamic acid

As described earlier, the analysis of culture fluids suggested that nitrite was formed during initial nitrate reduction with electrons stemming from acetate oxidation. To test whether the nitrite formed reacts with the Fe(II) during acidic extractions of samples from BoFeN1 cultures with HCl and thus potentially explains the observed oxidation of Fe(II), we set up abiotic experiments with 7–8 mM Fe (II) and 1 mM nitrite under oxic and anoxic conditions (Fig. 2A). We found that oxic incubation of HCl-acidified samples led to a high loss of Fe(II). Under oxic conditions, 2 mM Fe(II) (27%) was consumed within the first minute, whereas during anoxic incubation, 1 mM Fe(II) (14%) was oxidized by 1 mM nitrite within 1 min. At the end of the incubation, after 26 min, 94% of Fe(II) was lost under oxic and 18% under anoxic conditions, respectively.

To confirm the importance of nitrite as an oxidant for Fe (II) and to verify the successful application of the revised sulfamic acid ferrozine assay in BoFeN1 cultures (Fig. 1), we incubated different concentrations of nitrite in Fe(II)-containing medium (without adding BoFeN1 cells) and incubated the samples in HCl or sulfamic acid under both oxic and anoxic conditions (Fig. 2B). We observed that incubating the samples under oxic conditions in 40 mM sulfamic acid instead of HCl preserved 94–99% of the Fe(II) even at the highest nitrite concentrations tested (4 mM), while in HCl, 74–100% of Fe(II) was oxidized in the presence of nitrite concentrations of 0.5–4 mM (Fig. 2B). Based on the abiotic reaction of Fe(II) with NO_2 and NO forming from NO_2^-/HNO_2 with N_2O as the final N product (see equations 1–5), an overall stoichiometry of 2:1 (Fe(II): NO_2^-) would be expected (Bonner & Pearsall, 1982). In our anoxic experiments, we were indeed able to confirm this ratio of approximately 1.9:1 with 0.5 mM nitrite, although at higher nitrite concentrations, the ratios were lower with approximately 1.4:1 (1 mM nitrite), approximately 1:1 (2 mM nitrite), and approximately 0.9:1 (4 mM nitrite) probably due to degassing of reactive NO during incubation. In contrast, in the oxic experiments, virtually all Fe(II) was oxidized at all nitrite concentrations leading to much higher ratios, due to the reoxidation of NO by O_2 thus recycling the oxidant for Fe(II).

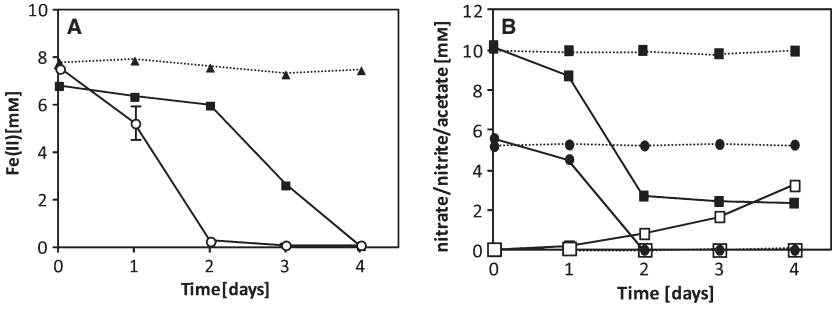

Fig. 1 Oxidation of dissolved Fe(II) in cultures of the nitrate-reducing Fe(II)-oxidizing strain *Acidovorax* sp. BoFeN1. (A) Fe(II) concentrations over time when minerals in the samples were dissolved either with 0.5 M HCl (○) or with 40 mM sulfamic acid (■). For comparison, Fe(II) concentrations in uninoculated controls were analyzed also with sulfamic acid (▲). (B) Representative graphs showing the dissolved concentrations of acetate (●), nitrate (■), and nitrite (□) in inoculated cultures (solid lines) and in sterile controls (dashed lines). Because of slightly different lag phases, the results from four independent experiments showing the same trends are not averaged. Instead, representative data sets are shown.

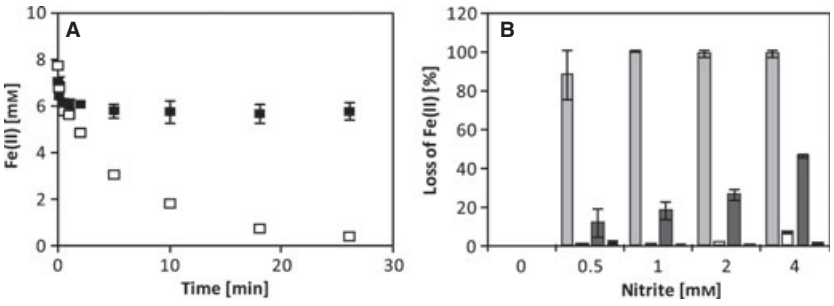

Fig. 2 Abiotic oxidation of Fe(II) by nitrite at acidic pH. (A) Fe(II) concentrations over time of samples containing approximately 7.5 mM Fe(II) amended with 1 mM Na-nitrite. The samples were diluted 1:10 in 1 M HCl and incubated under oxic (□) or anoxic (■) conditions. (B) Loss of Fe(II) with different nitrite concentrations after incubation with 1 M HCl under oxic (light gray) and anoxic conditions (dark gray) in comparison with samples diluted in 40 mM sulfamic acid and incubated under oxic (white) and anoxic (black) conditions. Error bars indicate standard deviation calculated from three independent parallels.

Effect of goethite minerals on abiotic oxidation of Fe(II) by reactive N species at neutral pH

To evaluate the importance of Fe(II) oxidation by nitrite in cultures of BoFeN1, we incubated 7–8 mM Fe(II) with 0.5, 2, 4, and 8 mM nitrite in anoxic growth medium both in the absence and presence of goethite (Fig. 3). These minerals were added because BoFeN1 cultures were shown to produce goethite during Fe(II) oxidation (Kappler *et al.*, 2005). We were interested to test whether the presence of the mineral surface has a catalytic effect on Fe(II) oxidation by nitrite in our cultures as it was shown previously by Sorensen & Thorling (1991). Using again the revised ferrozine assay with sulfamic acid, we found that in the presence of 2 mM goethite, Fe(II) oxidation by nitrite was significantly faster than without goethite. Specifically, we found that under anoxic conditions, complete Fe(II) oxidation was achieved by 8 mM nitrite after 6 days in the presence of goethite, while it took 20 days in the absence of goethite. The maximum rate of oxidation with 8 mM nitrite in the goethite-amended and goethite-free experiments was 3.7 and 1.3 mM day^{-1}, respectively. At lower concentrations of nitrite, Fe(II) oxidation was slower with 0.5 mM day^{-1} (4 mM NO_2^-) and 0.2 mM day^{-1} (2 & 0.5 mM NO_2^-) in the absence of goethite resulting in incomplete Fe(II) oxidation. However, when goethite was added, Fe(II) oxidation was accelerated in the first 2 days (with 2.4, 2.0, and 1.0 mM day^{-1} for 4, 2, and 0.5 mM NO_2^-, respectively) and then leveled off, finally reaching the same amount of oxidized Fe(II) as the equivalent setups without goethite. Interestingly, at some of the higher nitrite concentrations, the oxidation of Fe(II) stopped, although theoretically enough nitrite was present for more Fe(II) oxidation, probably due to either NO degassing or the formation of a stable nitrosyl complex ($Fe(H_2O)_5NO^{2+}$).

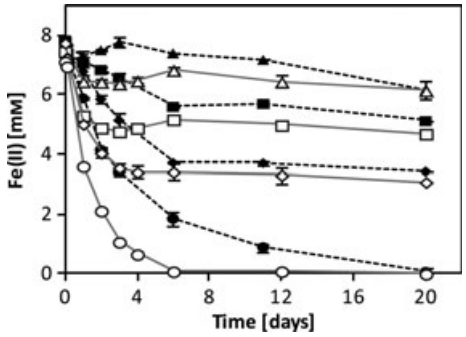

Fig. 3 Abiotic oxidation of Fe(II) by different concentrations of nitrite (● 8 mM, ◆ 4 mM, ■ 2 mM, ▲ 0.5 mM) in medium at approximately pH 7 in the absence (closed symbols, dashed lines) or presence (open symbols, gray lines) of 2 mM goethite. Samples were diluted in 40 mM sulfamic acid before analyzing iron to prevent oxidation of Fe(II) by nitrite during the acidification step. Error bars indicate range of values calculated from two independent parallels. The absence of error bars indicates that the error was smaller than the symbol size.

Growth of BoFeN1 and oxidation of Fe(II) in the presence of N_2O as electron acceptor

To avoid the abiotic reactions of nitrite with Fe(II) and to test whether BoFeN1 has the enzymatic capacity to oxidize Fe(II) (in the absence of nitrite), we set up experiments with acetate/Fe(II) as electron donor and N_2O as electron acceptor because previous experiments (Muehe *et al.*, 2009) had suggested that BoFeN1 can grow with acetate and N_2O (in the absence of Fe(II)). We first inoculated BoFeN1 with acetate and N_2O alone and found that BoFeN1 cannot grow with acetate and N_2O as sole electron acceptor, unless N_2O reduction was induced by adding a small amount of nitrate (100 μM) at the beginning of the experiment (data not shown). With the small amount of nitrate (without N_2O), BoFeN1 showed only poor growth. Nitrite could not be detected at any time point of the experiment in these setups (data not shown). After addition of Fe(II), no significant Fe(II) oxidation was detected, although a significant increase in optical density was observed (Fig. 4), suggesting (i) growth on acetate and N_2O and (ii) that BoFeN1 is unable to enzymatically oxidize Fe(II), at least with N_2O as electron acceptor.

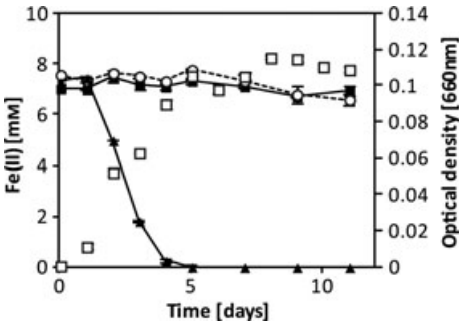

Fig. 4 Concentrations of Fe(II) over time in cultures of *Acidovorax* strain BoFeN1 in the presence of 5 mM acetate and approximately 7 mM Fe(II) amended with 10 mM nitrate (▲) or 5% N_2O and 100 μM nitrate (■). Sterile setups amended with 5 mM acetate, approximately 7 mM Fe(II), 5% N_2O, and 100 μM nitrate are shown for comparison (○). Cell numbers of BoFeN1 were followed photometrically in cultures amended with 5 mM acetate, 5% N_2O, and 100 μM nitrate (□). Error bars indicate standard deviation calculated from three independent parallels. The absence of error bars indicates that the error was smaller than the symbol size.

Only after 11 days of incubation, we observed a slight decrease in Fe(II) both in the sterile and the biologically active setups (Fig. 4) probably due to abiogenic siderite formation and precipitation on the glass wall caused by changes in the CO_2 headspace/bicarbonate buffer ratio during regular N_2O flushing. The same experiment was repeated with 0.5 mM instead of 5 mM acetate, and the same concentration of N_2O as electron acceptor but still no Fe(II) oxidation was observed (data not shown).

Effect of Fe(III) on growth of BoFeN1

The experiments presented so far are in contrast to recent reports that suggested that the oxidation of Fe(II) by nitrate-reducing, Fe(II)- and acetate-oxidizing, mixotrophic bacteria is enzymatically catalyzed and provides a growth benefit, that is, a higher cell number, compared with setups with acetate/nitrate but without Fe(II). Muehe *et al.* (2009) and Chakraborty *et al.* (2011) both demonstrated that cell yields were significantly higher by approximately 27% (Muehe *et al.*, 2009) and approximately 90% (Chakraborty *et al.*, 2011) when cells were grown in the presence of Fe(II)/acetate/nitrate compared with growth without Fe(II) (with acetate/nitrate only). With the CAS siderophore assay, we detected siderophore production in iron-free grown BoFeN1 cultures (data not shown) suggesting a high demand for Fe as nutrient by strain BoFeN1. To determine whether the Fe(II) present did serve as a nutrient for BoFeN1 rather than as an electron source, we incubated BoFeN1 with acetate and nitrate both in the presence and absence of approximately 7 mM of the Fe(III)oxyhydroxide, ferrihydrite (Fig. 5B). We observed rapid growth in both setups with cell numbers approximately 33% higher in the presence of ferrihydrite compared with the setup without Fe(III) amendment. Figure 5A shows that BoFeN1 cannot grow by Fe(III) reduction as already described by Muehe *et al.* (2009) and that the synthesized ferrihydrite contained no Fe(II).

DISCUSSION

Most nitrate-reducing, Fe(II)-oxidizing bacteria are described to be mixotrophic (Benz *et al.*, 1998; Muehe *et al.*, 2009) and couple the oxidation of an organic cosubstrate (e.g., acetate) and Fe(II) to the reduction of nitrate. During the reduction of nitrate to N_2, several reactive N compounds are formed as intermediates that have the potential to abiotically react with Fe(II), thus leading to higher Fe(II) oxidation rates. This is further complicated by the fact that the oxidation of Fe(II) by these bacteria is typically followed by quantification of Fe(II) performed using acidic dissolution of all minerals (in HCl) prior to spectrophotometric quantification (Braunschweig *et al.*, 2012). As the reactions of the reactive N compounds with Fe(II) are even faster at acidic pH, it is expected that this abiotic contribution to the observed oxidation rates is par-

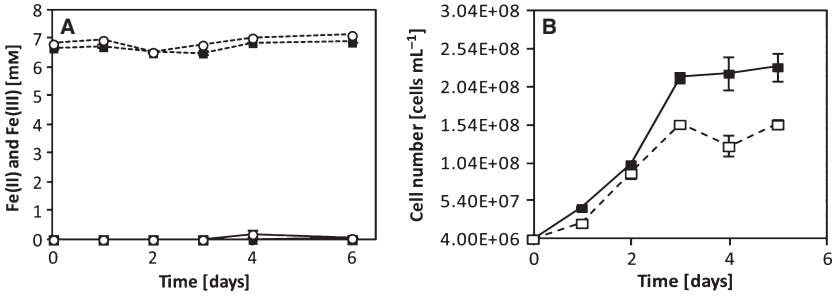

Fig. 5 Fe(III) reduction (dashed lines) and Fe(II) production (solid lines) over time by strain BoFeN1 (A) in cultures amended with 5 mM acetate and approximately 7 mM ferrihydrite (■) or in sterile controls (○). Error bars indicate standard deviation from two parallels. (B) Cell numbers of strain BoFeN1 over time in cultures inoculated with 10 mM nitrate, 5 mM acetate and approximately 7 mM ferrihydrite (■) or inoculated with 10 mM nitrate, and 5 mM acetate without Fe(III) amendment (□). Error bars indicate standard deviation calculated from two parallels. The absence of error bars indicates that the error was smaller than the symbol size.

ticularly relevant during the acidification step. Our results presented above indeed show that both the oxidation of Fe(II) at neutral pH during incubation of the microbial cultures and the oxidation of Fe(II) during the acidic extraction step are relevant and even question the existence of an enzymatic Fe(II) oxidation step by BoFeN1. In the following sections, we discuss the various mechanisms of abiotic Fe(II) oxidation during the extraction step as well as in the microbial cultures before acidification.

Fe(II) oxidation during acidic iron extraction in nitrite-containing samples

During the acidic dissolution of Fe(III) minerals formed during Fe(II) oxidation, the nitrite present forms nitrous acid by protonation (equation 1) that in turn shows fast self-decomposition into NO_2 and NO (equation 2) at low pH values. This was already recognized by Nelson & Bremner (1970b). Both NO_2 and NO are very-reactive N species that can react with Fe(II) according to equations 3 and 4, particularly at acidic pH during the Fe mineral dissolution and thus oxidize some of the Fe(II) to Fe(III) (Bonner & Pearsall, 1982). Additionally, even more Fe(II) was found to be oxidized when the extraction was performed under oxic conditions. This suggests that molecular oxygen present can react with the NO (that forms from the reaction of NO_2 with Fe^{2+}, see equation 3) and is recycling the very reactive N species (probably NO_2) (equation 6) (Van Cleemput & Samater, 1996; Holleman & Wiberg, 2001). This NO_2 can in turn oxidize more ferrous iron leading to an almost complete loss of Fe(II) in solution, despite the fact that only small amounts of nitrite were present at the beginning. This mechanism is confirmed in our abiotic Fe(II) oxidation experiments where we found that 90–95% of 8 mM Fe(II) was oxidized under oxic incubation with only 1 mM nitrite present.

$$2NO + O_2 \rightarrow 2NO_2 \quad (6)$$

Here, we present a revised Fe analysis protocol using sulfamic acid instead of HCl that to a very large extent solves these problems of nitrite being present during acidic dissolution of the Fe minerals. The sulfamic acid is a moderately strong acid (pK_a = 1.3), which reacts with nitrite to form N_2 and sulfuric acid (equation 7).

$$HNO_2 + (H_2N)HSO_3 \rightarrow H_2SO_4 + N_2 + H_2O \quad (7)$$

Granger & Sigman (2009) showed that 40 mM sulfamic acid at a pH between 1.6 and 1.8 was sufficient to consume 500 μM nitrite within a few seconds. In our experiments, 40 mM sulfamic acid was sufficient to remove high nitrite concentrations up to the mM range. With 4 mM nitrite present under oxic conditions, the loss of Fe(II) was below 10%. Based on the data obtained with this revised protocol, for future analyses of Fe(II) and Fe(III) in cultures of nitrate-reducing, Fe(II)-oxidizing bacteria, we suggest anoxic extraction of samples using sulfamic acid. After this dissolution step, the samples can be stored oxically, and also the ferrozine assay can be performed under oxic conditions.

Using sulfamic acid instead of HCl allowed the determination of correct temporal Fe(II) data for a culture of the nitrate-reducing, Fe(II)-oxidizing strain BoFeN1. With this revised protocol, BoFeN1 showed a slower oxidation rate and a delayed oxidation start, clearly differing from already published data (Kappler *et al.*, 2005; Muehe *et al.*, 2009). When reviewing the literature, we found that other nitrate-reducing, Fe(II)-oxidizing strains were shown to also accumulate nitrite during growth. While in some studies, the problem of rapid oxidation of Fe(II) by nitrite at acidic pH was circumvented by an initial anoxic centrifugation step followed by a direct ferrozine assay of the supernatant without acidification, most studies did not consider the abiotic Fe(II) oxidation during acidic Fe extraction (Table 1). Therefore, the Fe(II) concentrations over time published for cultures of these strains are probably also influenced by the abiotic Fe(II) oxidation process during acidic extraction, and the true Fe(II) oxidation rates will probably differ when using sulfamic acid instead of HCl. We thus suggest to reanalyze Fe(II) oxidation rates for these strains with the revised ferrozine method presented here to obtain accurate, temporal Fe(II) data for these strains.

Fe(II) oxidation by nitrite at neutral pH in cultures of strain BoFeN1 — importance of mineral surface catalysis

In addition to the importance of Fe(II) oxidation by nitrite during acidification of culture samples for Fe mineral extraction and Fe quantification, we evaluated the role of Fe(II) oxidation by nitrite under neutral pH conditions. To this end, we first quantified homogeneous abiotic oxidation of ferrous iron by nitrite directly in culture medium and found Fe(II) oxidation rates of approximately 0.2–1.3 mM Fe(II) per day at nitrite concentrations relevant for our microbial cultures. Both Van Cleemput & Baert (1983) and Buresh & Moraghan (1976) already showed that at neutral pH (between pH 6–8), only a very low concentration of nitrous acid is present (due to the pK_a of 3.3), and its self-decomposition is very slow. Nevertheless, they showed that even at neutral pH, the nitrite can already react to a small extent with ferrous iron.

However, the abiotic Fe(II) oxidation rate of approximately 0.2–1.3 mM Fe(II) per day observed at the nitrite concentrations relevant for our BoFeN1 cultures do not explain the overall oxidation rates of up to 3.4 mM day^{-1} in the BoFeN1 cultures determined with the revised ferrozine assay using sulfamic acid. This would still suggest that

Table 1 Overview of isolated nitrate-reducing, Fe(II)-oxidizing strains or environmental samples (including sediments) with nitrate-dependent Fe(II) oxidation capacity that has been published in the last years. In some cases, approaches to prevent abiotic Fe(II) oxidation by nitrite during sampling/analysis are described

Name bacterial strain	Author (year of publication)	Nitrite accumulation	Approach to prevent abiotic Fe(II) oxidation by nitrite	Samples for Fe(II) and Fe(III) analysis diluted in
Isolate HidR2	Benz *et al.*, (1998)	No	Anoxic centrifugation	500 mM phosphate buffer/pellet in 1 M HCl
Thiobacillus denitrificans DSMZ 739	Bosch *et al.*, (2012)	Yes	No	1 M HCl
Acidovorax strain 2AN	Chakraborty *et al.*, (2011)	Yes	Anoxic centrifugation	Pellet in 0.5 M HCl
Lake sediment	Hauck *et al.*, (2001)	Not measured	No	1 M HCL
Acidovorax strain BoFeN1	Kappler *et al.*, (2005	Yes	No	HCl
Isolate FW33AN	Senko *et al.*, (2005)	Yes	No	0.5 M HCl
Sediment & water samples	Straub *et al.*, (1996)	Not shown but stated in text	First dilution in Na_2CO_3 & Anoxic centrifugation	Pellet in 1 M HCl
Isolates BrG1, 2, 3	Straub *et al.*, (2004)	Not stated	No	0.7 M Na-acetate buffer pH 5
Enrichment culture	Weber *et al.*, (2001)	Yes	Anoxic centrifugation	Pellet in 0.5 M HCl
Pseudogulbenkiania strain 2002	Weber *et al.*, (2006a, b)	Yes	No	0.5 M HCl or directly in ferrozine

there is a significant microbial (enzymatically catalyzed) contribution to Fe(II) oxidation. It has to be considered, however, that the oxidation rate of 0.2–1.3 mM Fe(II) per day has been determined for homogeneous oxidation, that is, in the absence of cell surfaces or mineral surfaces that can potentially act as surface catalysts. Coby & Picardal (2005) suggested that Fe(II) sorption on microbial cell surfaces enhances abiotic Fe(II) oxidation by nitrite, thus leading to the formation of cell encrustations. Additionally, Tai & Dempsey (2009) and Sorensen & Thorling (1991) described a catalytic effect of Fe(III) minerals on Fe(II) oxidation by nitrite. They suggest that initial binding of Fe^{2+} ions to the iron mineral surface forms reactive Fe(II) surface species, thus facilitating the reaction with nitrite.

In our experiments, the oxidation of dissolved Fe(II) by nitrite in the presence of goethite was almost twice as fast as in the absence of the mineral. Goethite was used for our experiments because BoFeN1 was shown to produce goethite as the product of Fe(II) oxidation (Kappler *et al.*, 2005). Our observations lead to the following envisioned scenario for the mechanisms and sequence of acetate and Fe(II) oxidation by strain BoFeN1. Initially, the microbes oxidize acetate enzymatically reducing nitrate with the formation of nitrite as intermediate. During this initial phase, while nitrite is built up, almost no Fe(II) oxidation takes place. This sequence of acetate oxidation first, followed by later Fe(II) oxidation were demonstrated already experimentally (Kappler *et al.*, 2005). During this phase, homogeneous abiotic Fe(II) oxidation dominates, probably leading to the formation of green rust as intermediate mineral phase as found recently by Pantke *et al.* (2012) followed by goethite formation. Kampschreur *et al.* (2011) indeed recently demonstrated that NO_2^- reacts abiotically with aqueous Fe(II) forming green-rust-like minerals. As soon as the nitrite can interact with Fe(II) bound to the formed goethite (and green rust) minerals, the reaction proceeds faster. The intermediate green-rust-like minerals will be oxidized further by nitrite, and more goethite is formed as the final Fe(III) mineral product. The formation of goethite after the oxidation of lattice-bound Fe(II) in green rusts was also shown by Hansen *et al.* (1994).

Theoretically, the rates of abiotic Fe(II) oxidation at different nitrite concentrations in growth medium determined in the presence of goethite can be compared with the nitrite concentrations measured and rates observed in BoFeN1 cultures. However, this has to be done with caution because due to the high reactivity of the nitrite building up initially during acetate oxidation coupled with nitrate reduction, the experimentally determined nitrite concentrations in the cultures probably do not represent the amount of nitrite that is actually formed (because some of the nitrite will rapidly react with the Fe^{2+} present, and this will thus not be quantified analytically). The accumulation of nitrite during Fe(II) oxidation and Fe(III) mineral precipitation was suggested to be a result of encrustation of the nitrite reductase in the periplasm. As a consequence, the further reduction of nitrite is slowed down by inhibition of the enzymatic activity leading to nitrite accumulation (Miot *et al.*, 2011). These authors provided support for such mechanisms by their observed encrustation of protein-like structures in the periplasm of BoFeN1. There are additional reasons why a stoichiometric analysis of acetate, Fe(II), Fe(III), nitrite and nitrate concentrations at the different time points of culture incubation has to be performed with caution: First, some of the acetate removed from solution will be assimilated by the bacteria to build cell mass (between 20 and 80% according to Russell & Cook (1995)) and will therefore not lead to nitrate reduction. Second, it is unclear why approximately 2 mM nitrite accumulate in the aqueous phase without nitrate consump-

tion between days 2 and 4. Our initial thought was that internal storage of nitrate and reduction at a later stage could explain this observation. However, a rough calculation of cell-internal concentrations required (with 5×10^6 cells mL^{-1} and assuming a cell volume of approximately 1 μm^3) yields a nitrate concentration of approximately 400 M in the cell, a concentration unrealistically high, and we therefore exclude this possibility. Because currently we cannot explain the discrepancy between the nitrate and nitrite data, this suggests that maybe the quantification of nitrate and nitrite in the presence of high Fe(II) concentrations is associated with potential analytical errors.

In our opinion, an exact stoichiometric balancing of electrons is therefore not easily possible for the mixotrophic strain BoFeN1. However, we suggest the following rough stoichiometric estimate for the reactions between nitrate, nitrite, acetate, and Fe(II): based on the fact that in our experiments, 8 mM of nitrate was metabolized by BoFeN1, theoretically up to 8 mM nitrite was produced (assuming that all nitrate molecules were reduced with two electrons in an initial step to nitrite and that not all acetate was oxidized to CO_2 but assimilated to a significant extent into biomass). At the end of the experiment, we could still measure 3 mM nitrite remaining in the medium. That means, up to 5 mM nitrite could have reacted abiotically with the Fe(II) within 2 days (between days 3 and 4) at rates fully explaining the experimentally determined Fe(II) oxidation rates in BoFeN1 cultures.

Stimulation of growth of BoFeN1 by Fe(II) and Fe(III)

The observed rapid abiotic reaction of nitrite with Fe(II) even at neutral pH in growth medium raises the question whether BoFeN1 can indeed enzymatically oxidize Fe(II) or whether the nitrite produced during reduction of nitrate with electrons stemming from acetate oxidation is mainly or even exclusively responsible for the observed Fe(II) oxidation. To circumvent the oxidation of Fe(II) by nitrite and to determine whether BoFeN1 can indeed oxidize Fe (II), we cultured BoFeN1 with the alternative electron acceptor N_2O and acetate as electron donor including trace amounts of nitrate which is needed for growth on N_2O as electron acceptor. The addition of nitrate is necessary because without preceding intermediates, such as NO_2^- or NO, the nitrous oxide gene cluster is not expressed (Soohoo & Hollocher, 1990; Arai *et al.*, 2003). A similar observation has been made for *Pseudomonas aeruginosa*, a strain which was shown to be unable to grow on N_2O as sole electron acceptor without the addition of small amounts of nitrate, and our data suggests a similar regulation for BoFeN1. When Fe(II) was added to growing N_2O/acetate cultures, we did not see any oxidation of Fe (II) under these conditions. As the N_2O concentration was high enough to theoretically allow oxidation of both acetate and Fe(II) (in particular in the 0.5 mM acetate experiments, see methods section), our results question the ability for enzymatic Fe(II) oxidation by BoFeN1. Whether this suggests that BoFeN1 is completely unable to fuel electrons from Fe(II) oxidation into denitrification or whether it means that electrons from enzymatic Fe(II) oxidation can be used only for reduction of nitrate/nitrite but not for reduction of N_2O remains open.

In previous experiments with strain BoFeN1 and another *Acidovorax* strain, increased growth yields have been observed when grown with Fe(II)/acetate/nitrate compared with acetate/nitrate (Muehe *et al.*, 2009; Chakraborty *et al.*, 2011). Based on our N_2O experiments and the results from their experiments, we hypothesized that the addition of Fe(II) is rather a nutritional effect than a benefit by gaining electrons from Fe(II) oxidation. This hypothesis was supported by our experiments that showed that increased cell numbers can be achieved by addition of the poorly crystalline Fe(III) oxyhydroxides, ferrihydrite, instead of Fe(II). As BoFeN1 cannot reduce Fe(III) this suggests that the iron, that is present as trace metal in the solutions (7.5 μM) added to the growth medium, is limiting. The production of Fe(III)-chelating and Fe(III)-mobilizing siderophores in iron-free grown cultures is also an indication for the high demand of iron by BoFeN1. A positive effect of Fe(III) addition on bacterial growth in general (Dehner *et al.*, 2010) and on denitrification (Baalsrud & Baalsrud, 1954; Pintathong *et al.*, 2009) has been demonstrated before. These authors observed increased nitrate utilization when the medium contained concentrations in the μM range of Fe^{3+}. A possible explanation is the high need of iron for the metalloenzymes, for example, cytochrome cd_1, involved in the denitrification pathway (Tavares *et al.*, 2006). Our data in combination with these observations from the literature suggest that supplying BoFeN1 with iron needed for its enzymes increases the numbers of cells produced during acetate oxidation with nitrate as electron acceptor, and Fe(II) is not used as electron donor.

CONCLUSIONS

In summary, the observations made for (i) the oxidation of Fe(II) by nitrite during acidic Fe extraction, (ii) growth and Fe(II) oxidation experiments with N_2O as electron acceptor, and (iii) the abiotic Fe(II) oxidation by nitrite in growth medium at neutral pH in the presence of goethite suggests that enzymatic Fe(II) oxidation by BoFeN1 was overestimated in its importance. Similar observations might be relevant for other nitrate-dependent Fe(II) oxidizers described in the literature as well. Of course, we cannot rule out the possibility that BoFeN1 and/or the other nitrate-reducing Fe(II) oxidizers described in the literature can, at least to same part, contribute to the observed Fe (II) oxidation by a direct enzymatic Fe(II) oxidation. It

has to be considered that some experiments have been performed under growth conditions where an organic cosubstrate would be necessary for sustained growth (e.g., in some of our recent studies with BoFeN1, see Pantke *et al.* (2012)), while studies with other nitrate-reducing Fe(II) oxidizers have been performed under non-growth conditions (Chaudhuri *et al.*, 2001; Lack *et al.*, 2002; Senko *et al.*, 2005). But even under non-growth conditions, internally stored carbon stemming from pre-growth in the presence of organic compounds could have lead to nitrite formation and thus indirect Fe(II) oxidation. Therefore, further studies are necessary to completely answer the question whether BoFeN1 and other nitrate-reducing Fe (II) oxidizers can perform enzymatic Fe(II) oxidation coupled with nitrate reduction or if it should be considered a biologically induced, abiotic side effect by denitrification of BoFeN1 in an iron(II)-rich environment.

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