**Sphaerotilus natans** encrusted with nanoball-shaped Fe(III) oxide minerals formed by nitrate-reducing mixotrophic Fe(II) oxidation

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**Abstract**

Ferrous iron has been known to function as an electron source for iron-oxidizing microorganisms in both anoxic and oxic environments. A diversity of bacteria has been known to oxidize both soluble and solid-phase Fe(II) forms coupled to the reduction of nitrate. Here, we show for the first time Fe(II) oxidation by *Sphaerotilus natans* strain DSM 6575ᵀ under mixotrophic condition. *Sphaerotilus natans* has been known to form a sheath structure enclosing long chains of rod-shaped cells, resulting in a thick biofilm formation under oxic conditions. Here, we also demonstrate that strain DSM 6575ᵀ grows mixotrophically with pyruvate, Fe(II) as electron donors and nitrate as an electron acceptor and single cells of strain DSM 6575ᵀ are dominant under anoxic conditions. Furthermore, strain DSM 6575ᵀ forms nanoball-shaped amorphous Fe (III) oxide minerals encrusting on the cell surfaces through the mixotrophic iron oxidation reaction under anoxic conditions. We propose that cell encrustation results from the indirect Fe(II) oxidation by biogenic nitrite during nitrate reduction and that causes the bacterial morphological change to individual rod-shaped single cells from filamentous sheath structures. This study extends the group of existing microorganisms capable of mixotrophic Fe(II) oxidation by a new strain, *S. natans* strain DSM 6575ᵀ, and could contribute to biogeochemical cycles of Fe and N in the environment.

**Introduction**

Iron (Fe) exists in divalent or trivalent states within the biosphere depending on the environmental conditions (Cornell & Schwertmann, 2004). Although the abiotic redox changes between Fe(II) and Fe(III) play an important role in redox processes in the environment, microorganisms also significantly contribute to iron biogeochemical cycling in both oxic and anoxic environments on Earth (Kappler & Straub, 2005; Weber *et al.*, 2006a,b,c), because they are able to utilize both Fe(II) and Fe(III) as electron donor and acceptor, respectively. However, the exact mechanisms of Fe mineral formation during microbial Fe(II) oxidation is barely understood (Benzerara *et al.*, 2011).

Previous studies suggested that the anaerobic microbological Fe(II) oxidation occurs either chemotrophically with nitrate (NO₃⁻) as the electron acceptor (Nealson & Saffarini, 1994; Hafenbradl *et al.*, 1996; Straub *et al.*, 1996; Lack *et al.*, 2002) or phototrophically (Widdel *et al.*, 1993; Ehrenreich & Widdel, 1994; Hegler *et al.*, 2008; Poulain & Newman, 2009), which results in the formation of Fe(III) precipitates under anoxic environments. Hafenbradl *et al.* (1996) reported that the microbiological Fe(II) oxidation coupled with nitrate reduction was achieved using enrichment cultures and pure cultures in the absence of oxygen as a light-independent, chemotrophic microbial process. The appearance of microbiological nitrate-dependent Fe(II) oxidation under anoxic natural conditions may play significant roles in coupling the redox cycling of N and Fe in sedimentary environments (Weber *et al.*, 2001). Furthermore, the anaerobic nitrate-dependent Fe(II) oxidation has important implications for soil and sediment mineralogy and geochemistry.
through the formation of Fe(III) oxides, including a variety of environmentally relevant Fe(III)-bearing minerals such as ferric oxyhydroxide, goethite, haematite, green rust, and magnetite (Straub & Buchholz-Cleven, 1998; Chaudhuri et al., 2001; Lack et al., 2002; Weber et al., 2006a, b, c; Pantke et al., 2012).

In addition to the geochemical importance of the anaerobic microbiological Fe(II) oxidation, it has been widely recognized that the aerobic microbiological Fe(II) oxidation under acidic or neutral pH conditions successfully competes with the chemical iron oxidation (Blake et al., 1993; Emerson & Revsbech, 1994; Blake & Johnson, 2000; Emerson, 2000). Indeed, aerobic Fe(II)-oxidizing bacteria mostly belonging to Betaproteobacteria such as Sphaerotilus/Leptothrix, Gallionella, and Rhodococcus spp. or to some genera in the Alpha- and Gammaproteobacteria (Emerson et al., 2010) have been found in a broad range of environments. Among Fe(II)-oxidizing bacteria, Sphaerotilus natans has been characterized by a sheath-forming bacterium enclosing long chains of rod-shaped cells (Hoeniger et al., 1973). It has been suggested that S. natans is the dominant filamentous bacterium causing bulky biofilm and pipe clogging in waste water treatments due to the formation of sheaths which allow a means of bulky biofilm and pipe clogging in waste water treatments (Hoeniger due to the formation of sheaths which allow a means of bulky biofilm and pipe clogging in waste water treatments

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**Materials and methods**

**Medium and culture conditions**

Iron-oxidizing bacterium S. natans strain DSM 6575T was purchased from the Deutsche Sammlung von Mikroorganismen (DSMZ, Braunschweig, Germany) and was pregrown in CGYA medium (5 g casitone, 10 g glycerol, and 1 g yeast extract L⁻¹) (Nierman & Maglott, 1989) under aerobic conditions at 30 °C for 1 day. Pregrown culture of S. natans was inoculated to basal medium including 4 mM FeCl₂, 4 mM nitrate, and 2 mM pyruvate. The basal medium for Fe(II)-oxidizing bacteria prepared as described by Enrenich and Widdel (Ehrenreich & Widdel, 1994). The composition of the basal medium was as follows: 0.14 g L⁻¹ KH₂PO₄, 0.2 g L⁻¹ NaCl, 0.5 g L⁻¹ MgSO₄·7H₂O, 0.3 g L⁻¹ NH₄Cl, 0.1 g L⁻¹ CaCl₂·2H₂O, 5.4 mg L⁻¹ KH₂PO₄·H₂O, 1 mL L⁻¹ vitamin solution, 1 mL L⁻¹ trace element solution, and 22 mM bicarbonate buffer, pH 6.8–7.2, and the headspace of medium was flushed with N₂/CO₂ (80/20%). The addition of the 5 mM FeCl₂ from anoxic 1 M stock solution to the basal medium led to precipitation of a greenish-white precipitates consisting of Fe(II) phosphate and Fe(II) carbonate (Kappler & Newman, 2004). In order to exclude the presence of background of Fe(II) minerals before abiotic Fe(II) oxidation and Fe(II) mineral precipitation started, the basal medium was filtered using a 0.2-µm filter (MFS-25, Advantec MFS, Inc., Dublin, CA) in an anoxic chamber, leaving a clear solution with 3–4 mM dissolved Fe(II). The medium maintained free of Fe(II) precipitates for several weeks in the absence of Fe(II)-oxidizing bacteria, which allowed to identify biologically precipitated Fe(III)-bearing minerals by strain DSM 6575T. For abiotic Fe(II) oxidation experiment as a control, we added 4 mM FeCl₂ and different concentration of nitrite (0.5, 1, 2, 4 mM) to the basal medium prepared as mentioned above under anoxic condition.

**Analytical methods**

All preparation processes were carried out in an anoxic glove box. For quantification of Fe(II), we used the revised ferrozine protocol for nitrite-containing samples (Klugle & Kappler, 2013); 100 µL of culture suspension was withdrawn with a syringe and dissolved in 900 µL of 40 mM sulfamic acid for 1 h at room temperature. Sulfamic acid reacts with the nitrite present and thus preventing abiotic oxidation of Fe(II) by reactive N species formed during sample acidification. After digestion, 10 µL of the extracts were transferred to 2 mL of...
ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulfonic acid monosodium salt, Catalog No. 82950, Fluka, Buchs, Switzerland) solution (ferrozine (1 g L\(^{-1}\)) in HEPES buffer (50 mM) at pH 7) to make a ferrous complex. The Fe(II)-ferrozine complex was quantified at 562 nm using a UV/Vis spectrometer (Optizen POP, Mecasys, Korea). A separate calibration curve was prepared for the revised ferrozine assay using ferrous ethylene diammonium sulfate tetrahydrate (Catalog No. 44932, Fluka, Buchs, Switzerland) that was dissolved in 40 mM sulfamic acid. In order to quantify nitrate and nitrite in the aqueous medium, 1.0 mL was extracted using a 1.0 mL syringe (Korean Vaccine Co., LTD, Seoul, Korea), filtered through a 0.2-μm syringe filter (MFS-25, Advantec MFS, Inc.) and exposed to air to prevent further reduction of nitrite by Fe(II) (Sørensen & Thorling, 1991). Filtered samples were centrifuged, and the supernatant was withdrawn for nitrate and nitrite analyses. Nitrate and nitrite were determined by ion chromatography (Dionex, CA) equipped with IonPac AG14 guard and AS14 analytical columns (Thermo Scientific, Sunnyvale, CA). Nitrate and nitrite were separated using isotropic conditions consisting of 3.5 mM Na\(_2\)CO\(_3\)/1 mM NaHCO\(_3\) for 15 min. The injection volume was 10 μL, and the flow rate was 1.2 mL min\(^{-1}\). Furthermore, N\(_2\)O and NO were quantified by gas chromatography coupled mass spectrometry (GC-MS) directly from the culture headspace. Pyruvate was quantified by high-performance liquid chromatography (HPLC), which was equipped with a photodiode array (PDA) detector (Varian, Walnut Creek, CA) and a Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA). The mobile phase was 5 mM sulfuric acid with a flow rate of 0.6 mL min\(^{-1}\) for 30 min. Ten microliter of each sample was injected, and the UV detection was performed at 210 nm.

### Analyses of bacterial and mineralogical morphologies

During mineralogical analyses, precipitated mineral residues were collected from the culture medium using syringes at 10 days of incubation and washed three times with deionized water. The washed minerals were dried in the anoxic glove box. X-ray diffraction (XRD) analysis was performed using a Rigaku D/MAX Ultima III high resolution XRD meter (Rigaku, Tokyo, Japan) equipped with monochromatic high-intensity CuK\(_\alpha\) radiation. The generator was operated at 40 kV and 40 mA. The samples were scanned between 2θ = 10° and 70° at a scan speed 1° min\(^{-1}\). Scanning electron microscopic (SEM, XL30-FEG, Philips, Eindhoven, The Netherlands) and transmission electron microscopic (TEM, JEOL JEM-2100, Tokyo, Japan) analyses were performed with samples to ascertain bacterial and mineralogical morphologies. Samples were prepared as described previously by Schadler et al. (2008). One microliter of culture was withdrawn anoxically with sterile syringes at a selected time, centrifuged at 9000 g for 3 min, and washed with distilled water to remove medium salt. For SEM analyses, samples were fixed with 2% glutaraldehyde and 4% formaldehyde in 20 mM KH\(_2\)PO\(_4\) and 80 mM Na\(_2\)HPO\(_4\) buffer solution for 20 min at room temperature. After fixation, the samples were washed three times in phosphate-buffered saline (PBS). Ten microliter of PBS suspended samples were deposited onto silicon wafers, dried in anoxic chamber for 12 h. The grid was washed with PBS for 10 min and immersed into a dehydration dilution series of distilled water. The prepared samples for SEM analyses were coated with a thin layer of Au/Pd [90/10% (w/w)]. The coating thickness was c. 10 nm. For TEM analyses, 10 μL of the samples fixed with 3% glutaraldehyde for 2 h at 4 °C, centrifuged for a few minutes at 3637 g and, subsequently, washed three times in distilled water. The fixed samples were stained in 1% osmium tetroxide (OsO\(_4\)) for 3 h at 4 °C and placed onto carbon-coated 200-mesh copper grids. The images were obtained at 200 kV using a JEOL JEM-JEM-2100 high resolution TEM (JEOL, Tokyo, Japan). The cross-sections were prepared by ultramicrotomy. The cross-sectioned specimens were taken from fixed cells as mentioned above and progressively embedded in epoxy resin (Epoxy, Fluka Chemika, St. Louis, MO). Cross-sections with a nominal thickness of 70 nm were prepared with an EM-UC6 ultramicrotome (LEICA, Wetzlar, Germany) using a diamond knife. After deposition on a copper grid, they were stained with uranyl acetate [2% (w/v)] and lead citrate (2 g L\(^{-1}\)).

### Results and discussion

#### Mixotrophic Fe(II) oxidation by *S. natans* strain DSM 6575\(^T\)

In the previous study, Gaudy & Wolfe (1961) reported that iron-oxidizing bacteria, *S. natans* strain DSM 6575\(^T\), grew as a single cell in the presence of both 0.5% of glucose and peptone, and *S. natans* also showed filamentous growth with sheath formation in the presence of both 0.1% of glucose and peptone (Supporting Information, Fig. S1a and b). However, no additional factors that regulate filamentous growth with sheath formation have been described (Pellegrin et al., 1999). In our result, it was observed that single rod cells of *S. natans* strain DSM 6575\(^T\) were dominant rather than filamentous long-chained cells in the basal medium with pyruvate and nitrate under anoxic conditions (Fig. S1c). It is suggested...
that bacterial culture condition such as either the presence of nitrate or anoxic condition can affect the bacterial morphology of \textit{S. natans}.

Furthermore, Pellegrin \textit{et al.} (1999) confirmed that microaerobic Fe(II)-oxidizing and sheathed filamentous \textit{S. natans} strain DSM 6575\textsuperscript{T} harbored nitrate reductase. The amino acid sequence of nitrate reductase from strain DSM 6575\textsuperscript{T} showed 75.7\%, 62.3\%, 21\% and 20.2\% identities to those from \textit{Leptothrix cholodnii} SP-6 (accession number YP001791710), \textit{Acidovorax delafieldil} (accession number WP005798151), \textit{Paracoccus denitrificans} PD1222 (accession number YP918478), and \textit{Pseudomonas} sp. G-179 (accession number AAC79443) (Fig. S2), respectively. The sequence comparison suggests that nitrate reductase from nitrate-dependent Fe(II) oxidizing bacteria could be quite different structurally from denitrifying bacteria, \textit{P. denitrificans} and \textit{Pseudomonas} sp.

In this study, strain DSM 6575\textsuperscript{T} almost completely consumed the provided pyruvate with decreasing from 2 mM to c. 0.02–0.04 mM by 10 days of incubation in bacterial medium containing either nitrate or nitrate and Fe(II) during 10 days of incubation with a similar metabolizing trend for pyruvate (Fig. 1). However, the amount of consumed pyruvate in the bacterial medium containing both nitrate and Fe(II) as an electron acceptor and donor, respectively, was slightly higher than that in the bacterial medium containing nitrate alone as an electron acceptor (Fig. 1), and the rate of pyruvate consumption for the first 2 days in the presence of both Fe(II) and nitrate was higher than that in the presence of nitrate only, suggesting physiological effect of Fe(II) on the initial anaerobic metabolism of pyruvate. Based on the results, strain DSM 6575\textsuperscript{T} was able to grow heterotrophically with pyruvate and also mixotrophically with pyruvate and Fe(II) by utilizing nitrate as an electron acceptor under anoxic conditions. In addition, strain DSM 6575\textsuperscript{T} hardly oxidized Fe (II) in the absence of pyruvate (Fig. 2a), similar to other bacteria previously described (Straub \textit{et al.}, 1996, 2004; Benz \textit{et al.}, 1998; Lack \textit{et al.}, 2002). Strain DSM 6575\textsuperscript{T} was not able to utilize ferrous iron as a sole electron donor, that is, they need an organic cosubstrate such as...
pyruvate for both growth and Fe(II) oxidation. Muehe et al. (2009) demonstrated that mixotrophic oxidation of ferrous iron with cosubstrate, 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) and contributes to the energy metabolism of bacteria. Considering the fact that lithoautotrophic growth of the pure culture with both Fe(II) and nitrate was reported to be weak, with hardly two doublings in one cultivation period (Weber et al., 2006a, b, c), mixotrophic Fe(II) oxidation with pyruvate is the preferred process for nitrate-dependent Fe(II) oxidation in most environments (Straub & Buchholz-Clevén, 1998; Hauck et al., 2001). Indeed, when both nitrate (4 mM) and pyruvate (2 mM) were present in the bacterial culture as an electron acceptor and as an organic substrate, respectively, strain DSM 6575ᵀ almost oxidized the dissolved Fe(II, 4 mM) within 10 days of incubation while consuming only 0.5 mM for the first 2 days (Fig. 2a). This two-phase Fe(II) oxidation pattern was also previously reported with Acidovorax strain BoFeN1 (Klueglein & Kappler, 2013; Klueglein et al., 2014), Pseudogulbenkiania strain 2002 (Weber et al., 2006a, b, c), P. denitrificans ATCC 19367, and P. denitrificans Pd 1222 (Klueglein et al., 2014). The decrease of Fe(II) in bacterial medium without either nitrate, pyruvate (Fig. 2a), or bacterial inoculation (Fig. 2b) was not observed. As mentioned before, strain DSM 6575ᵀ barely oxidized Fe(II) in the absence of pyruvate (Fig. 2a), indicating that strain DSM 6575ᵀ was likely to depend on the presence of pyruvate for the oxidation of Fe(II). As strain DSM 6575ᵀ oxidized Fe(II) in the bacterial culture containing nitrate and pyruvate during incubation, the provided nitrate (4 mM) was also decreased to c. 0. 3 mM with a small amount of nitrite production in the range of 0.9–1.0 mM at day 10 (Fig. 3). In addition, strain DSM 6575ᵀ consumed more nitrate in the bacterial culture containing pyruvate in the presence of Fe(II) than in the absence of Fe(II) during 10 days of incubation (Fig. 3a). It is assumed that addition of Fe(II) in the presence of pyruvate is likely to provide another electron sources to strain DSM 6575ᵀ for the assimilatory or dissimilatory nitrate utilization with more consumption of pyruvate under anoxic conditions. It should be noted that nitrite accumulation in bacterial culture started at day 1 and reached the maximum concentration, c. 1.0 mM after 6 days (Fig. 3b). Neither decrease of nitrate nor increase of nitrite was observed in the control experiment without bacterial inoculation (Fig. S3). Nitrite was not formed at bacterial culture in the absence of either Fe(II) or pyruvate (Fig. 3b). The accumulation of nitrite in bacterial culture of nitrate-dependent Fe(II) oxidation reaction has been also observed in previous studies (Kappler et al., 2005; Larese-Casanova et al., 2010; Chakraborty et al., 2011). The exact reason that nitrite is accumulated in the mixotrophic Fe(II) oxidation culture has been remained an enigma (Picardal, 2012). However, there is possibility that precipitation of Fe(III) mineral on the nitrite reductase through abiotic Fe(II) oxidation by biogenic nitrite causes inhibition of the nitrite reductase by mineral deposition and leads to nitrite accumulation (Miot et al., 2011; Picardal, 2012). However, further reduced forms of nitrogen species, N₂O and NO, in bacterial cultures amended with dissolved Fe(II), pyruvate, and nitrate, were not detected in the culture headspace (data not shown). It has been recognized that abiotic oxidation of Fe(II) by nitrate at neutral pH occurs at high temperature (75 °C) (Buresh & Moraghan, 1976) or in the presence of green rust (Hansen et al., 1996). Therefore, the abiotic

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**Fig. 3.** Concentration of remaining nitrate (a) and present nitrite (b) in the bacterial culture of *Sphaerotilus natans* strain DSM 6575ᵀ. The bacterial culture was incubated with nitrate alone (●), with nitrate and Fe(II) (○), with nitrate and pyruvate (▲), and with nitrate, Fe(II), and pyruvate (◇), respectively. The 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.
oxidation of Fe(II) by nitrate in this study is negligible. Indeed, it has been known that Fe(II) oxidation by nitrite is more rapid than by nitrate (Picardal, 2012). The question is whether the observed Fe(II) oxidation was an enzymatic reaction or chemical reaction by nitrite produced from the nitrate reduction. Klueglein et al. (2014) reported recently that Fe(II) oxidation is a nitrite-driven, indirect mechanism during heterotrophic denitrification of Acidovorax strain BoFeN1, Pseudogulbenkiania strain 2002, P. denitrificans ATCC 19367, and P. denitrificans Pd 1222. As shown in Figs 2 and 3, fast decrease of Fe(II) after 2 days incubation of strain DSM 6575 was correlated with nitrite accumulation found only in bacterial culture with pyruvate and Fe(II), suggesting abiotic Fe(II) oxidation by nitrite produced during mixotrophic denitrification. However, it does not rule out the possibility of inducible enzymatic reactions by different bacteria. No concrete evidences have been revealed for the enzymatic or abiotic reactions for Fe(II) oxidation under denitrifying conditions (Glasauer et al., 2013; Klueglein & Kappler, 2013; Klueglein et al., 2014).

Cell encrustation and Fe(III) mineral formation

SEM image analyses also showed that single cells of strain DSM 6575 encrusted with Fe(III) mineral crusts at their cell surface in the presence of pyruvate and Fe(II) as the electron donor and nitrate as the electron acceptor (Fig. 4). Interestingly, some of the enlarged Fe(III) oxides seemed to show holes or hollowness within the ball-shape structure (see arrows, Fig. 4c). Fe(III) mineral crusts around the cell surfaces were formed as soon as Fe(II) oxidation occurred (Fig. 4a and b), and bacterial cells were completely encrusted with Fe(III) mineral formed via iron oxidation (Figs 4c, d and 5a, c). TEM analyses revealed the images of bacterial cell cross-section showing the cell interior as well as cell–mineral interfaces (Fig. 5c). It is observed that S. natans cells contained iron mineral in the cellular membrane, and the thickness of the iron mineral layer is c. 30–40 nm (Fig. 5c). The thickness of the mineral layer varies among the cells. In addition, the presence of ball-shaped on the surface of the cells as observed in SEM analyses was confirmed (Fig. 5a and c). However, cell encrustation was not observed in the bacterial culture containing both ferrous iron and nitrite instead of nitrate (Fig. 4b). Several studies have been also demonstrated that Fe(III) mineral precipitation by nitrate-reducing Fe(II) oxidizer starts in the periplasm, continuous on the cell surface, and then terminates in the cytoplasm (Miot et al., 2009; Klueglein et al., 2014).

It has been reported that metabolically diverse Fe(II)-oxidizing bacteria were encrusted mainly by crystalized Fe(III) goethite mineral (Emerson & Moyer, 1997; Kappler & Straub, 2005; Schaedler et al., 2009). Partly or fully encrusted bacterial cells have been also observed in natural environments, such as Fe(II)-rich rivers and springs (Benzerrara et al., 2008; Preston et al., 2011). Moreover, many studies suggest that the encrustation of bacterial cells by Fe(III) oxide minerals has been considered biosignatures or microfossils resulted from the presence of microbial activity in modern and ancient environments (Banfield et al., 2001; Posth et al., 2008; Cosmidis et al., 2013; Glasauer et al., 2013). However, it is unclear whether the Fe(III) oxide mineral formation encrusting the cell surface is beneficial to the bacterial cells for uptaking or diffusing substrate (Hallberg & Ferris, 2004). In our study, the result of consumed pyruvate by strain DSM 6575 indicates that encrusted cells could still utilize pyruvate (Fig. 1), and therefore, substrate may be transported to the encrusted cells. Previously, the results were

![Fig. 4. SEM images of Sphaerotilus natans strain DSM 6575 grown in the presence of Fe(II), NO3, and pyruvate under anaerobic conditions. Samples of S. natans strain DSM 6575 were taken after 1 day (a–b) and 3 days (c–d). The insert (d) shows a close-up image of S. natans strain DSM 6575 with Fe(III) oxide mineral deposition at the cell surface during Fe(II) oxidation under anaerobic conditions.]
reported that encrusted cells were metabolically active (Miot et al., 2009) and even divided (Schaedler et al., 2009). As some of researchers suggested, there are possibilities that cell encrustation can protect cells from UV radiation, predation, and dehydration (Pierson et al., 1993; Phoenix & Konhauser, 2008). Furthermore, Fe(III) oxide mineral crust at the cell surface may play a role in electrons transfer from Fe(II) to the cells via the conductive iron mineral crust, as it was shown in abiotic mineral environment (Schaefer et al., 2011).

In addition, XRD analysis for the Fe(III) oxide minerals formed from the nitrate-dependent Fe(II) oxidation by strain DSM 6575T did not reveal significant signals of crystalline phases (Fig. 5b and d), indicating formation of amorphous or less crystalline Fe(III) oxide minerals. It has been also known that biologically produced amorphous Fe(III) oxide minerals could be an excellent substrate for a diverse Fe(III)-reducing bacteria in environments (Straub & Buchholz-Cleven, 1998; Lovley et al., 2004) and highly reactive to other metal(loids) such as arsenic, uranium, and organic pollutants (Weber et al., 2001; Borch et al., 2009; Hohmann et al., 2009, 2011; Vaughan & Lloyd, 2011; Hitchcock et al., 2012). Thus, this iron species transformation between Fe(II) and Fe(III) by coupling Fe(II)-oxidizing bacteria with Fe(III)-reducing bacteria in the environments could provide important ecological and environmental implications for understanding biogeochemical cycling of Fe.

On one hand, the abiotic reaction of ferrous iron with nitrite in the basal medium without bacterial culture at neutral pH leaded to nano-sized yellowish precipitates (Fig. S4a) and identified as goethite [XRD Power Diffraction File (PDF) number 81-0462] (Fig. S4b). The recent study demonstrated that nitrite reacts abiotically with aqueous Fe(II), resulting in the formation of green-rust-like minerals, which are further oxidized to form goethite as the final product (Kampschreur et al., 2011). In the recent studies, Pantke et al. (2012) reported the formation of green rust during Fe(II) oxidation by the nitrate-reducing Acidovorax sp. strain BoFeNa, and Klueglein et al. (2014) also identified goethite mineral formed by heterotrophic denitrification of Acidovorax strain BoFeN1, Pseudogulbenkiania strain 2002, P. denitrificans ATCC 19367, and P. denitrificans Pd 1222. However, as previously mentioned, the bacterial culture of strain DSM 6575T in the basal medium containing ferrous iron and nitrate showed reddish precipitates of amorphous or less crystalline Fe(III) oxide minerals (Fig. 5b and d).
Considering Fe(II) oxidation under denitrifying conditions and formation of amorphous or less crystalline Fe(III) oxide minerals on the bacterial surface with changing bacterial morphology, we propose that S. natans strain DSM 6575T mediates abiotic Fe(II) oxidation using nitrate as an electron acceptor in the presence of pyruvate and provides inducible enzymatic reaction mechanism for the cell encrustation with amorphous iron oxide minerals on the cell membrane. Further experiments are necessary to identify the corresponding enzymes involved in the iron oxidation reactions and their location in the cells.

In summary, Fe(II)-oxidizing and sheath-forming S. natans strain DSM 6575T was able to oxidize Fe(II) using nitrate as an electron acceptor in the presence of pyruvate and appeared as single cells encrusted by the nanoball-shaped amorphous Fe(III) oxide minerals, which contributes to biogeochemical cycles of Fe and N in anaerobic environment.

**Nucleotide sequence accession numbers**

This Whole-Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AZRA00000000. The version described in this paper is version AZRA01000000.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

*Fig. S1.* Transmission electron microscopic images of *Sphaerotilus natans* grown as single cells in the presence of 0.5% glucose and 0.5% peptone (a), as filamentous cells in the presence of 0.1% peptone and 0.1% glucose (b) under aerobic condition, and (c) single cells in the basal medium with pyruvate under anaerobic condition.

*Fig. S2.* Homologies of nitrate reductase of *Sphaerotilus natans* strain DSM 6575T with other bacterial nitrate reductase.

*Fig. S3.* Concentration of dissolved nitrate in the control experiment without *Sphaerotilus natans* strain DSM 6575T inoculation.

*Fig. S4.* TEM images of goethite formed from abiotic reaction between ferrous iron at 4 mM and nitrite at 1 mM (a) and XRD pattern of abiotically formed goethite from ferrous iron and nitrite (b).