

## Anaerobic Biooxidation of Fe(II) by *Dechlorosoma suillum*

J.G. Lack, S.K. Chaudhuri, R. Chakraborty, L.A. Achenbach, J.D. Coates

Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA

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### ABSTRACT

Anaerobic microbial oxidation of Fe(II) was only recently discovered and very little is known about this metabolism. We recently demonstrated that several dissimilatory perchlorate-reducing bacteria could utilize Fe(II) as an electron donor under anaerobic conditions. Here we report on a more in-depth analysis of Fe(II) oxidation by one of these organisms, *Dechlorosoma suillum*. Similarly to most known nitrate-dependent Fe(II) oxidizers, *D. suillum* did not grow heterotrophically or lithoautotrophically by anaerobic Fe(II) oxidation. In the absence of a suitable organic carbon source, cells rapidly lysed even though nitrate-dependent Fe(II) oxidation was still occurring. The coupling of Fe(II) oxidation to a particular electron acceptor was dependent on the growth conditions of cells of *D. suillum*. As such, anaerobically grown cultures of *D. suillum* did not mediate Fe(II) oxidation with oxygen as the electron acceptor, while conversely, aerobically grown cultures did not mediate Fe(II) oxidation with nitrate as the electron acceptor. Anaerobic washed cell suspensions of *D. suillum* rapidly produced an orange/brown precipitate which X-ray diffraction analysis identified as amorphous ferric oxyhydroxide or ferrihydrite. This is similar to all other identified nitrate-dependent Fe(II) oxidizers but is in contrast to what is observed for growth cultures of *D. suillum*, which produced a mixed-valence Fe(II)–Fe(III) precipitate known as green rust. *D. suillum* rapidly oxidized the Fe(II) content of natural sediments. Although the form of ferrous iron in these sediments is unknown, it is probably a component of an insoluble mineral, as previous studies indicated that soluble Fe(II) is a relatively minor form of the total Fe(II) content of anoxic environments. The results of this study further enhance our knowledge of a poorly understood form of microbial metabolism and indicate that anaerobic Fe(II) oxidation by *D. suillum* is significantly different from previously described forms of nitrate-dependent microbial Fe(II) oxidation.

## Introduction

Anaerobic bio-oxidation of Fe(II) has only recently been identified and very little is known regarding the ubiquity and diversity of organisms capable of this metabolism. Previous studies have shown that anoxic Fe(II) oxidation is mediated by anoxygenic phototrophs [20, 46] as well as various nitrate-respiring [5] or perchlorate-respiring organisms [6, 10, 14, 29], and even by a hyperthermophilic archaeobacterium [18]. The end product of anoxic Fe(II) biooxidation is generally amorphous Fe(III) oxide [ $\text{Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}(\text{am})$ ] or ferrihydrite [5, 6, 18, 42, 46]; however, this may be a function of the medium chemistry and growth conditions of the various organisms. Amorphous Fe(III) oxide is representative of metal oxides in the natural environment and is a precursor to many natural forms of crystalline Fe(III) oxides such as goethite and hematite [15, 40, 48].

As part of a study on the metabolic diversity of organisms capable of growth by the anaerobic respiration of perchlorate, we isolated a novel organism, *Dechlorosoma suillum* strain PS, from a swine waste lagoon [1, 14, 29] and demonstrated the ubiquity of *Dechlorosoma* species in diverse environments including swine waste lagoons, petroleum-contaminated soil, river sediments, pristine soil, aquifer sediments, and even Antarctic soils [1, 14] (L.A. Achenbach, unpublished). Physiological characterization revealed that *D. suillum* rapidly oxidized Fe(II) with nitrate or chlorate as the electron acceptor under strict anaerobic conditions [10, 29]. Recently, we demonstrated that in contrast to previous studies on nitrate-dependent biooxidation of Fe(II), oxidation by *D. suillum* in growth culture experiments resulted in the formation of a broad range of crystalline iron minerals, including magnetite, which constituted as much as 25% of the original Fe(II) in the culture under the growth conditions tested [10]. This has important implications regarding the biogenic origin of banded iron formations in the Precambrian era of Earth's early history and also on the use of the presence of biogenic magnetite as a specific indicator of the historical activity of dissimilatory Fe(III)-reducing bacteria [10].

Nothing is currently known of the biochemistry involved in the anaerobic biooxidation of Fe(II). The results of our previous studies indicated that all perchlorate reducing bacteria contain *c*-type cytochrome(s) and that these cytochromes are involved in the biological oxidation of Fe(II) [6, 10, 14]. However,

similar studies have not been performed on other known anaerobes capable of Fe(II) oxidation. In the aerobic acidophilic Fe(II) oxidizer *Thiobacillus ferrooxidans*, it has recently been proposed that the pathway for electron transfer between ferrous iron and oxygen involves a *c*-type cytochrome that transfers electrons from Fe(II) to rusticyanin, which in turn passes them on to a *c*<sub>4</sub> cytochrome and then to cytochrome oxidase [3]. The product of the *cyc2* gene, a high-molecular-weight cytochrome *c*, is likely responsible for the first step in the pathway, the transfer of electrons from Fe(II) to rusticyanin [2]. The reversible loss of Fe(II) oxidative ability by *T. ferrooxidans* in an event called phenotypic switching [38] is thought to be the result of reversible transposition of an insertion sequence into the *resB* gene [7], whose product, a putative protein involved in cytochrome *c*-type biogenesis, is proposed to be essential for Fe(II) oxidation but not for sulfur oxidation in *T. ferrooxidans*. Similarly to *T. ferrooxidans*, many of the known perchlorate-reducing bacteria including *D. suillum* are also capable of the anaerobic oxidation of sulfide [9, 14, 29]. However, in contrast to *Thiobacillus* species, elemental sulfur is the end product of this metabolism, and no oxyanions of sulfur such as sulfite or sulfate are produced [14]. Whether *cyc2* and *resB* genes are present in *D. suillum* or are involved in the oxidation of Fe(II) or sulfide by the perchlorate reducing bacteria or any other anaerobic Fe(II) oxidizer has yet to be determined.

The goal of the current study is to complete a more in-depth analysis of anaerobic Fe(II) oxidation by *Dechlorosoma suillum* and get a better understanding of the environmental variables which control this metabolism.

## Materials and Methods

### Medium and Culture Conditions

*Dechlorosoma suillum* strain PS was maintained in anoxic, defined freshwater medium previously described [6] with acetate (10 mM) as the sole electron donor and chlorate (10 mM) or nitrate (10 mM) as the sole electron acceptor using standard anaerobic culturing techniques [4, 22, 30]. Anoxic medium was prepared under a headspace of N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v) by boiling to remove dissolved O<sub>2</sub> prior to dispensing under an N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v) gas phase into anaerobic pressure tubes or serum bottles and sealing with thick butyl rubber stoppers. Freshly prepared medium was sterilized by autoclaving at 121°C for 15 min and culture incubations were carried out at 35°C in the dark.

## Cell Suspension Preparation

Cells of *D. suillum* strain PS were grown anaerobically in 500 mL volumes with acetate (10 mM) as the electron donor and oxygen from air (101 kPa), chlorate, or nitrate (10 mM respectively) as the electron acceptor. After dense growth, cells were harvested by centrifugation at 4°C under an N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v) headspace. Cell pellets were washed twice and resuspended in 1 mL of anoxic bicarbonate buffer (2.5 g L<sup>-1</sup>) and sealed in a 10 mL serum vial with a thick butyl rubber stopper under a headspace of N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v). Anaerobic washed cell suspensions were used immediately after preparation. All experiments were performed in triplicate.

## Sediment Studies

Anaerobic iron-containing sediments were collected from Pohic Bay, MD, and a hydrocarbon-contaminated aquifer in Hanahan, SC. Samples were sealed under an anoxic headspace of N<sub>2</sub>:CO<sub>2</sub> (95:5 v/v) and stored at 4°C prior to use. All experiments were performed in triplicate with the respective sediments using strict anaerobic techniques. Serum vials (35 mL) were amended with 20 g sediment and 1–2 mL of sterile, anoxic water under an N<sub>2</sub>-CO<sub>2</sub> (95:5 v/v) headspace and sealed with thick butyl rubber stoppers. Nitrate was added from a sterile anoxic stock to give a final concentration of 10 mM when needed. The inoculum size was 10% (vol/wt) of anaerobically grown cells with nitrate (10 mM) as the sole electron acceptor. All samples were incubated at 37°C in the dark. At regular time intervals subsamples (1.0 g) were collected and assayed for Fe(II).

## Analytical Techniques

Acetate concentrations were analyzed by HPLC with UV detection at 210 nm (Shimadzu SPD-10A, Shimadzu Scientific Instruments,

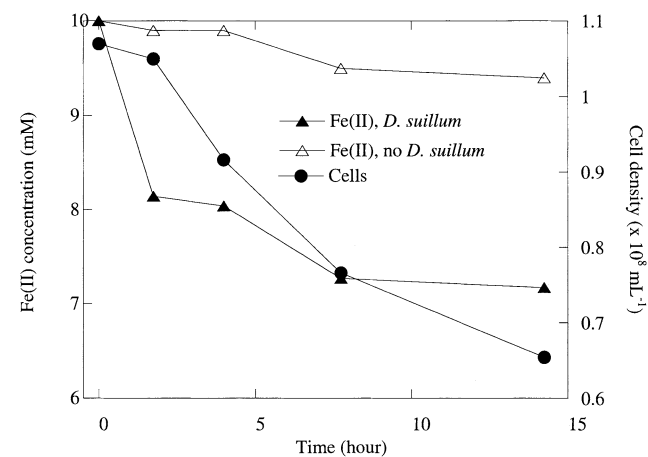


Fig. 1. Fe(II) oxidation and cell density changes in cultures of *D. suillum* with FeCl<sub>2</sub> (10 mM) and nitrate (10 mM) as the sole electron donor and acceptor respectively. CO<sub>2</sub> was the sole carbon source. The depicted results are the average of triplicate determinations.

Columbia, MD) using an HL-75H<sup>+</sup> cation exchange column (Hamilton #79476, Hamilton Company, Reno, NV). The eluent was 0.016 N H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4 mL min<sup>-1</sup>. Chlorate, chloride, nitrate, and nitrite concentrations were analyzed by ion chromatography with conductivity detection (Shimadzu CDD-6A, Shimadzu Scientific Instruments) using IonPac AS9-HC with suppressed conductivity by ASRS-II in a recycle mode (Dionex #51786, Dionex Corporation, Sunnyvale, CA). The eluent was 9 mM sodium carbonate at a flow rate of 1.0 mL min<sup>-1</sup>. Growth of cultures on soluble electron acceptors was determined by direct cell counts and by increase in optical density at 600 nm. Concentrations of HCl-extractable Fe(II) were determined colorimetrically by the ferrozine assay at 562 nm [27]. X-ray diffraction (XRD) analysis of the biologically-produced Fe(III)-oxides was determined as previously described [10]. Briefly samples were collected and centrifuged under an N<sub>2</sub> gas phase and washed twice prior to overnight drying in an anoxic glove bag containing a headspace of N<sub>2</sub>-H<sub>2</sub> (95-5). The dried samples were mounted and analyzed using a Philip Analytical X-Ray Diffractometer (model PW1710) using nickel-filtered CuKα radiation.

## Results and Discussion

### Fe(II) Oxidation by *D. suillum*

Previously we reported on the ability of *D. suillum* to oxidize Fe(II) with nitrate or chlorate as the electron acceptor [10]. Our results suggested that growth was not coupled to anaerobic Fe(II) oxidation by *D. suillum*. As previously published [10], the addition of a small amount (0.1 mM) of acetate as a potential carbon source resulted in a cell density increase; however, this was only observed concomitant with acetate oxidation. No Fe(II) oxidation occurred until after the acetate was depleted. Once acetate depletion occurred, Fe(II) oxidation was initiated and the Fe(II) was oxidized rapidly throughout the stationary phase of the growth culture [10]. In support of this, if acetate was omitted from the medium in growth culture experiments with Fe(II) as the sole electron donor and either nitrate (10 mM) (Fig. 1) or chlorate (10 mM) (data not shown) as the electron acceptor, Fe(II) oxidation again occurred rapidly; however, cell density declined throughout the 15 h incubation (Fig. 1). As previously observed, no Fe(II) oxidation was observed in the absence of a suitable electron acceptor or if the cells were omitted. This result supports our previous findings that *D. suillum* does not grow lithoautotrophically with Fe(II) as the sole electron donor [10]. Similar results were previously obtained with the closely related perchlorate reducer *Dechloromonas agitata* strain CKB [6].

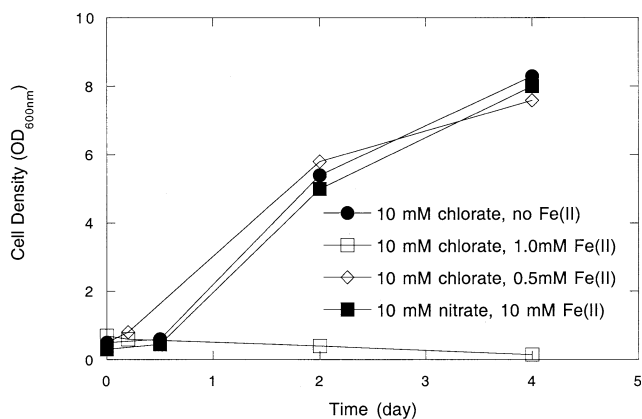


Fig. 2. Growth of *D. suillum* on acetate (5 mM) in the presence of varying concentrations of Fe(II) with either nitrate or chlorate as the sole electron acceptor. The depicted results are the average of triplicate determinations.

Interestingly, with chlorate as the electron acceptor, Fe(II) concentrations of 1 mM or higher were completely inhibitory to the growth of *D. suillum* on acetate and no Fe(II) oxidation occurred (Fig. 2). In contrast, no inhibition of growth or Fe(II) oxidation was observed with nitrate as the electron acceptor even at Fe(II) concentrations as high as 25 mM (Fig. 2). A similar inhibition to growth was observed with *Dechloromonas agitata* strain CKB (data not shown). As *D. agitata* does not grow by nitrate reduction [6], the effects of Fe(II) on nitrate cultures could not be determined. The selective toxicity of Fe(II) to the growth of perchlorate-reducing bacteria when grown with chlorate as the electron acceptor suggests that Fe(II) may interfere with the reductive pathway of chlorate and may potentially interact abiotically with transient intermediates such as chlorite formed during the reductive process [14, 35]. Such an interaction would potentially scavenge electrons away from the electron transport chain and “short circuit” the reductive pathway.

#### Aerobic Oxidation of Fe(II)

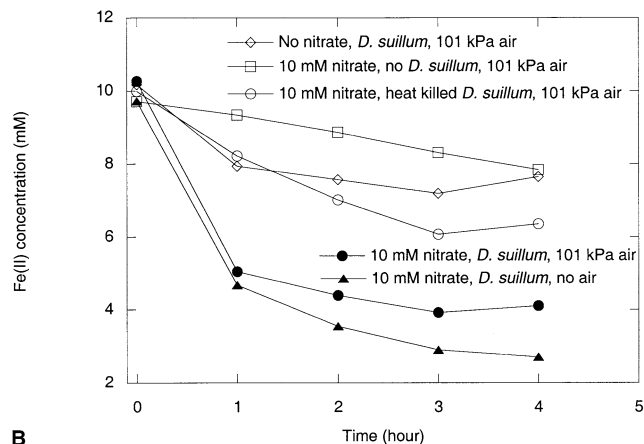
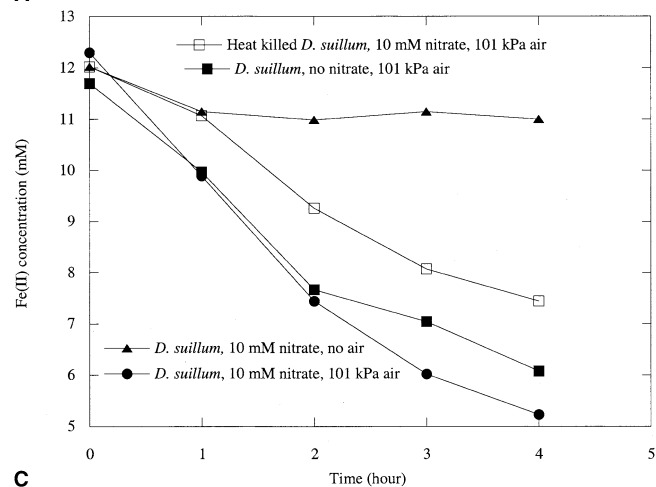
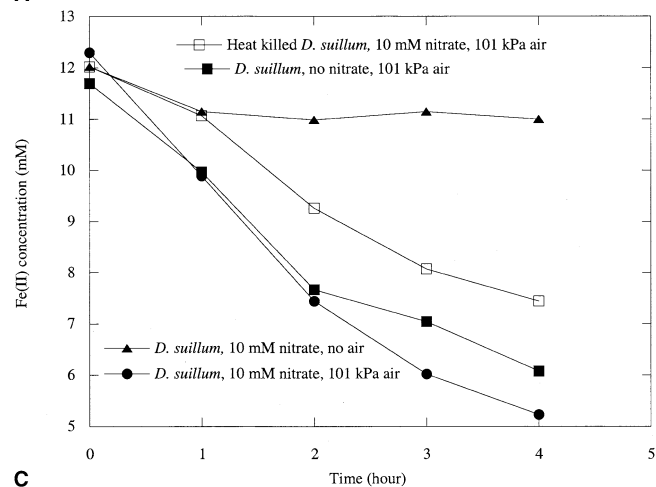
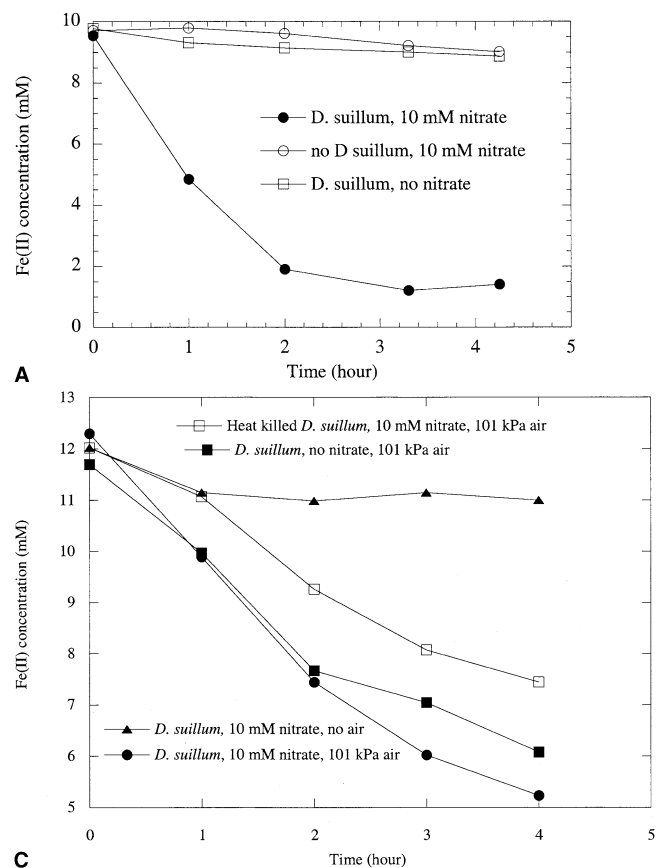
When acetate–nitrate grown cells of *D. suillum* were used to prepare anaerobic washed whole-cell suspensions in a non-growth bicarbonate buffer (pH 6.8) amended with FeCl<sub>2</sub>, the Fe(II) was rapidly oxidized with either chlorate (data not shown) or nitrate (Fig. 3a) as the electron acceptor. Greater than 87% of the initial Fe(II) added (10 mM) was oxidized within 3 h of addition of the cells. If either the electron acceptor or the cells were omitted, no significant oxidation of the Fe(II) occurred (Fig. 3a). If a

similar experiment was performed with oxygen as the electron acceptor in the washed whole-cell suspensions, the Fe(II) was slowly oxidized throughout the incubation and after 4.5 h almost 50% of the initial Fe(II) was oxidized (Fig. 3b). The rate and extent of Fe(II) oxidation was identical to that of the heat-killed control and the uninoculated control, suggesting that the observed Fe(II) oxidation was the result of abiotic reaction with dissolved O<sub>2</sub> in the medium. In contrast, if chlorate (data not shown) or nitrate (Fig. 3b) was present in addition to the O<sub>2</sub>, Fe(II) oxidation occurred rapidly and greater than 70% of the original Fe(II) added was oxidized in the same time frame. This result indicates that the anaerobically grown cells of *D. suillum* required chlorate or nitrate as a suitable electron acceptor for the biooxidation of Fe(II) even under aerobic conditions. Conversely, if a similar experiment was performed with acetate–oxygen grown cells of *D. suillum* the results were reversed (Fig. 3c). As such the rate of Fe(II) oxidation by washed whole-cell suspensions of aerobically grown cultures was significantly greater than that in abiotic controls with oxygen as the sole electron acceptor and was not enhanced by the addition of nitrate (Fig. 3c). In contrast, the washed whole-cell suspensions of aerobically grown cultures did not oxidize Fe(II) with nitrate as the sole electron acceptor in the absence of oxygen (Fig. 3c).

This result supports the finding of previous studies on Fe(II) oxidation in which it was suggested that Fe(II)-oxidizing nitrate reducers are also capable of Fe(II) oxidation coupled to aerobic respiration [5, 41]. However, it demonstrates that for cells of *D. suillum* the coupling of Fe(II) oxidation to a particular electron acceptor is dependent on the prior growth conditions of the organism and the induction of an enzymatic respiratory system.

#### Oxidation of the Fe(II) Content of Natural Sediments

In addition to being able to oxidize FeCl<sub>2</sub>, *D. suillum* could also oxidize the Fe(II) content of natural sediments. When an active anaerobic culture of *D. suillum* was inoculated (10%, vol:wt) into anoxic sediments collected from either the Hannahan aquifer, SC, or the Potomac River, MD, amended with nitrate (10 mM), Fe(II) oxidation rapidly occurred (Fig. 4). No Fe(II) oxidation occurred in the unamended samples (Fig. 4). Some Fe(II) oxidation was observed in the absence of *D. suillum* if the sediments were amended with nitrate (data not shown). This was probably the result of the activity of



**B**

Fig. 3. Fe(II) oxidation coupled to alternative electron acceptors by washed whole cell suspensions of *D. suillum* (a) acetate-nitrate grown cells with nitrate as the electron acceptor, (b) acetate-nitrate grown cells with oxygen as the electron acceptor, in the presence and absence of nitrate, and (c) acetate-oxygen grown cells with oxygen as the electron acceptor, in the presence and absence of nitrate. All results are the average of triplicate determinations.

indigenous nitrate-dependent Fe(II) oxidation. This was expected as previous studies have demonstrated that this metabolism is widespread in the environment [5, 32, 41, 43] and several perchlorate-reducing bacteria closely related to *D. suillum* and capable of nitrate-dependent Fe(II) oxidation have previously been isolated from the Potomac River sediments [13, 14].

The fact that the Fe(II) content of natural sediments can be readily biooxidized has important implications regarding the environmental significance of nitrate-dependent Fe(II) oxidation. Despite the fact that dissolved Fe(II) is by far the most common water quality problem associated with groundwater [8], in most reduced environments soluble Fe(II) represents only a small fraction of the total Fe(II) in the environment [12, 21, 26]. Environmental ferrous iron including the Fe(II) produced as a result of microbial Fe(III) reduction may also be bound to the surfaces of partially reduced crystalline Fe(III) oxides [36, 37] or be present as insoluble carbonate, sulfide, or mixed-valence iron phases such as siderite ( $\text{FeCO}_3$ ), pyrite ( $\text{FeS}_2$ ), or magnetite ( $\text{Fe}_3\text{O}_4$ ). Previous studies on nitrate-dependent Fe(II) oxidation using biogenic solid-phase Fe(II) demonstrated that Fe(II)-oxidizing enrichment

cultures could rapidly oxidize the end products of microbially reduced hydrous ferric oxide and the Fe(II) content of microbially reduced subsoils with nitrate as the sole electron acceptor [45]. However, microbially produced solid-phase Fe(II) may only be a fraction of the total solid-phase Fe(II) content in the environment, especially in environments in which nitrate is present as nitrate is inhibitory to microbial Fe(III) reduction [11, 12, 25]. A significant fraction of soil solid-phase Fe(II) may also be present in siliceous or sulfide mineral phases such as biotite ( $[(\text{Mg}, \text{Fe}^{2+})_3(\text{Si}_3\text{Al})\text{O}_{10}(\text{OH})_2]\text{K}$ ) or marcasite ( $\text{FeS}_2$ ). Our previous studies on anaerobic Fe(II) oxidation by *D. suillum* demonstrated that washed anaerobic whole-cell suspensions of *D. suillum* rapidly oxidized the Fe(II) content in various insoluble iron minerals including almandine ( $\text{Fe}_3\text{Al}_2(\text{SiO}_4)_3$ ) and staurolite ( $(\text{Fe}, \text{Mg}, \text{Zn})_2\text{Al}_9(\text{Si}, \text{Al})_4\text{O}_{22}(\text{OH})_2$ ) [10]. Both the rate and extent of Fe(II) oxidation was different for the various minerals tested in those studies and was probably due to differences in bioavailability of the Fe(II) in the mineral matrices [10]. The extent to which biogenic or abiotic mineral phases support nitrate-dependent Fe(II) oxidation remains to be determined.

### End Products of Fe(II) Oxidation

Recently we demonstrated that Fe(II) oxidation by growth cultures of *D. suillum* resulted in the production of a mixed Fe(II)–Fe(III) green/gray precipitate known as green rust which, after aging, results in the formation of various crystalline iron phases including maghemite and magnetite [10]. In contrast, previous studies on nitrate-dependent Fe(II) oxidation indicated that the Fe(II) was completely oxidized to an amorphous ferric oxyhydroxide or ferrihydrite [5, 42, 43]. Interestingly and in contrast to our previous studies with growth cultures, Fe(II) oxidation by anoxic washed whole-cell suspensions of *D. suillum* in a non-growth bicarbonate buffer (30 mM) with either nitrate or chlorate as an electron acceptor also resulted in the production of an orange/brown precipitate within 1 to 4 h. No Fe(II) oxidation or orange/brown precipitation occurred in the anaerobic buffer if the cells were omitted or heat-killed. X-ray diffraction (XRD) analysis of the orange/brown precipitants showed a broad absorbance peak indicative of an amorphous, noncrystalline structure (data not shown).

The fact that *D. suillum* produced different end products of Fe(II) oxidation under growth (green rust [10]) and non-growth conditions (amorphous ferrihydrite) is of interest. Several previous studies have demonstrated that the type and crystallinity of iron-based solids formed under abiotic conditions are dependent on several environmental parameters including pH and concentration of carbonate/bicarbonate, chloride, phosphate, nitrate, and sulfate [16, 19, 23, 24, 33, 34, 39, 47], which might suggest that the observed difference is due to the difference in the growth culture medium versus the bicarbonate buffer used in the cell suspension experiments. However, this is unlikely to be the explanation for the observed results in this instance because rapid (10 min) air oxidation of the Fe(II) content of anoxic uninoculated growth culture medium in which green rust can be biogenically formed by an active culture of *D. suillum* [10] results in the formation of an orange/brown precipitate similar to that observed in non-growth cell suspension experiments. This result indicates that Fe(II) can be oxidized to either green rust or amorphous ferrihydrite in the growth culture medium although the chemical content is identical. An alternative explanation for our observed results is that the different end products of Fe(II) oxidation by *D. suillum* under the conditions tested is a function of the rate of oxidation by a washed cell suspension (4 h) versus a growth culture (5 days). This is

supported by previous studies on abiotic Fe(II) oxidation which demonstrated that the rate of oxidation can have a significant effect on the end products formed [17]. In these studies it was demonstrated that rapid chemical oxidation of Fe(II) led to the formation of ferrihydrite while lower rates of oxidation resulted in the formation of green rust or magnetite [17].

### Significance

Anaerobic biooxidation of Fe(II) was only recently identified and very little is currently known regarding the ubiquity and diversity of organisms capable of this metabolism or of the biochemistry involved. Preliminary investigations indicate that nitrate-dependent Fe(II) oxidation is widespread in the environment [43] and that several known nitrate respirers are capable of the anaerobic oxidation of Fe(II) with nitrate as the sole electron acceptor [42]. However, only a few organisms have been described that can couple nitrate-dependent Fe(II) oxidation to growth, and the majority of these are lithoheterotrophs requiring an organic carbon co-substrate for growth [5, 43]. In addition, most probable number enumeration studies of Fe(II)-oxidizing nitrate reducers indicate that lithoheterotrophic Fe(II)-oxidizing bacteria may account for as much as 0.8% of the total nitrate-reducing community in environmental samples and appear to be substantially more prevalent than strictly lithoautotrophic Fe(II) oxidizers in the same sample [5, 43]. As such, the true environmental significance of this metabolism has yet to be determined and the organisms

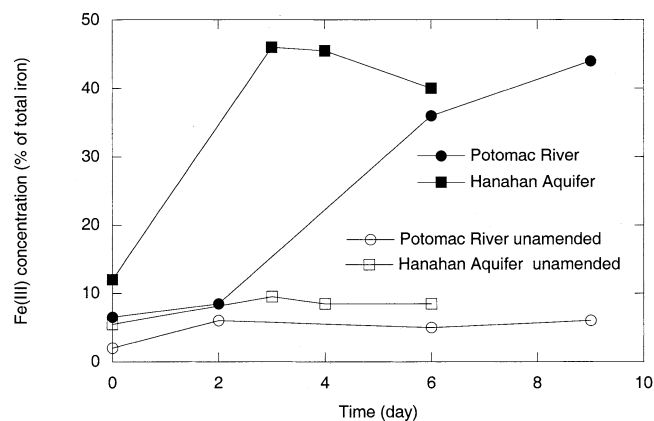


Fig. 4. Nitrate-dependent biooxidation of the Fe(II) content of natural sediments by anaerobic cultures of *D. suillum*. Depicted results are the average of triplicate determinations.

mainly responsible for anaerobic Fe(II) oxidation in the environment have yet to be identified. Our previous studies demonstrated that several perchlorate-reducing organisms are also capable of anaerobic Fe(II) oxidation [6, 10, 14, 29]. These studies demonstrated that the type strain species of the two dominant perchlorate-reducing genera in the environment, *Dechloromonas* and *Dechlorosoma*, are both capable of this metabolism [6, 10, 14, 29]. The ubiquity of both of these genera in a broad diversity of environments further indicates the widespread capability of Fe(II) biooxidation [14, 31]. Although the true environmental role of perchlorate-reducing bacteria has yet to be determined, it is unlikely that the ubiquity of these organisms is related to their ability to grow by dissimilatory perchlorate reduction. This is because perchlorate in the environment is predominantly the result of anthropogenic activity while these organisms have been found in several pristine environments not known to have had any prior contact with perchlorate or chlorate [14]. Previous studies have demonstrated that these organisms are in general very versatile and can use a broad range of alternative electrons donors including hydrogen [44], simple organic acids and alcohols [6, 14, 28, 29, 35], aromatic hydrocarbons [13], hexoses [28], reduced humic substances [6, 13], Fe(II) [6, 10, 14, 29], and hydrogen sulfide [6, 9, 14]. As such, the selective pressures for these organisms in the environment may be based on the diversity of their metabolic capabilities rather than any individual metabolism.

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