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Anaerobic, Nitrate-Dependent Microbial Oxidation of Ferrous Iron

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Enrichment and pure cultures of nitrate-reducing bacteria were shown to grow anaerobically with ferrous iron as the only electron donor or as the additional electron donor in the presence of acetate. The newly observed bacterial process may significantly contribute to ferric iron formation in the suboxic zone of aquatic sediments.

Iron is the most prevalent redox-active metal in the biosphere. In anoxic soils and sediments, ferric iron is reduced to the ferrous state mainly through the activities of various iron-reducing bacteria (15, 18). Widespread and well-studied reoxidation processes of iron(II) are its chemical reactions with molecular oxygen under neutral conditions (21) and its biological utilization as an electron donor by aerobic acidophilic (1, 4, 25) or neutrophilic (5, 6, 12, 13, 25) bacteria. In the absence of oxygen, iron(II) can be chemically oxidized with manganese(IV) oxide (17); however, manganese(IV) oxide is insoluble and is therefore not a diffusible oxidant in aquatic sediments. The anaerobic chemical oxidation of iron with the diffusible oxidants nitrate (2, 22a), nitrite, and nitrous oxide (14, 16) is possible in vitro, but it is doubtful whether such abiological reactions are significant under the conditions of natural habitats (2). Biological, O_2-independent oxidation of ferrous iron with light as the energy source was recently demonstrated in cultures of newly isolated phototrophic bacteria (24); ferrous iron was used as the electron donor for CO_2 fixation (3). Here we demonstrate that the biological oxidation of ferrous iron in the absence of oxygen is also possible by a light-independent, chemotrophic microbial activity with nitrate as the electron acceptor.

Sediment samples for enrichment cultures were taken from town ditches (Bremen, Germany) and a brackish water lagoon (Hiddensee, Baltic Sea, Germany). The denitrifying bacterial strains LP-1, AR-1 (8), and ToN1 (19) were from subcultures that had been kept in our laboratories since the isolation of these bacteria. *Thiobacillus denitrificans* (ATCC 25259) and *Pseudomonas stutzeri* (ATCC 14405) were from the American Type Culture Collection (Rockville, Md.), and *Thiomicrospira denitrificans* (DSM 1251) and *Paracoccus denitrificans* (DSM 1404) were from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

Enrichment and pure cultures were grown in defined anoxic, bicarbonate-buffered (30 mM, pH 7.0) mineral media containing vitamins and trace elements, as described elsewhere (23); media were prepared without sulfide. Unless otherwise indicated, 4 mM $NaNO_3$ was added. Depending on the inoculum source, either freshwater or brackish water medium was used (23). $FeSO_4$ was added as the sole electron donor and as the reductant from an anoxic stock solution to obtain a concentration of 6.6 or 10 mM in the medium. The Fe^{2+} ions reacted with carbonate and phosphate in the medium to form a colorless precipitate. The butyl rubber-sealed culture tubes (20 ml) and bottles (50, 100, and 500 ml) contained a headspace (1/10 of the total volume) of N_2-CO_2 (90:10 [vol/vol]). The inoculum size was 2 to 4% of the culture volume. Cultures were incubated at 15 and 28°C. Iron-oxidizing denitrifying bacteria were isolated via repeated agar dilution series (23) with sodium nitrate and ferrous sulfate (10 mM) in the absence or presence of sodium acetate (1 mM) as an auxiliary substrate.

Iron oxidation and nitrate reduction were quantified in flat 500-ml bottles. The bottles were incubated in a horizontal position to yield a large surface area for the precipitated ferrous minerals. Samples were withdrawn through the stoppers via anoxic syringes while the medium was stirred to allow homogeneous distribution of precipitated iron minerals. Otherwise, the bottles were incubated without agitation. To allow complete separation of withdrawn iron minerals from nitrite, a possible product which interferes with the iron assay, samples were first anoxically mixed with Na_2CO_3 (final concentration, 50 mM) and then centrifuged under N_2. The supernatant was used to determine the concentrations of nitrate and nitrite by high-pressure liquid chromatography (19). The pellet was dissolved in 1 M HCl (30 min at 25°C) for the colorimetric determination of the concentrations of ferrous and ferric iron (3). Ammonium ions were quantified by using the indophenol formation reaction (10).

Ten bottles with media containing iron(II) and nitrate were inoculated with freshwater and brackish water mud samples. After 1 to 2 weeks of incubation, eight enrichment media exhibited yellow-brown precipitates resembling iron(III) oxides. Positive enrichment cultures were transferred repeatedly at intervals of 1 to 2 weeks. Growth at 28°C was faster than at 15°C. Whereas in brackish water enrichment cultures, the formation of ferric iron after four transfers occurred only in the presence of organic substrates such as 1 mM acetate or succinate, freshwater enrichment cultures continued to grow without an addition of organic electron donors. The only organic compounds added to the medium were vitamins; however, their total concentration was as low as 40 μg/liter. The time course of ferrous iron oxidation in a chemolithotrophic freshwater enrichment culture is shown in Fig. 1. Ferrous iron was

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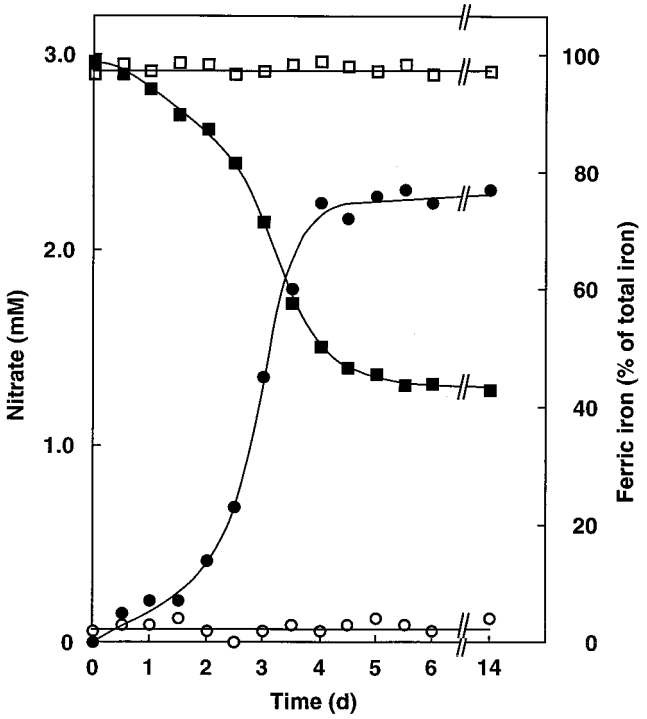

FIG. 1. Anaerobic bacterial oxidation of ferrous iron (10 mmol of $FeSO_4$ was added per liter) with nitrate in a freshwater enrichment culture under lithoautotrophic conditions. ●, ferric iron in growing culture; ○, ferric iron in the control with pasteurized cells; ■, nitrate in growing culture; □, nitrate in the control with pasteurized cells. The shown data are average values from three determinations. The formation of nitrite was not detected in the culture. The enrichment had been grown in 20 consecutive subcultures in mineral medium without an organic substrate before being used as the inoculum for the experiment.

not oxidized in nitrate-free controls in complete medium without inoculum, or with heat-inactivated (10 min at 80°C) cells (Fig. 1). With nitrite (2 mM) in the absence of cells, an initial fast oxidation of ferrous iron (approximately 1 mM within 1 day) was observed, which then continued only very slowly; in 18 days of incubation, only 3 mM ferrous iron was oxidized. The chemical oxidation of ferrous iron with N_2O (50 ml/liter) was not observed.

Attempts to isolate iron-oxidizing, nitrate-reducing bacteria have so far led to the isolation of three different gram-negative strains that are able to oxidize ferrous iron (Table 1). One brackish water isolate and one freshwater isolate (strains HidR2 and BrG1, respectively; inoculum size, 4% [vol/vol]) oxidized 60% of the added ferrous iron within 7 to 10 days if an organic substrate such as acetate was provided at a low concentration (≤1 mM). Without acetate, less than half of the added iron was oxidized within 4 weeks. Another freshwater isolate, strain BrG2, oxidized ferrous iron almost completely within 3 weeks in the absence of organic substrates. The addition of 0.5 mM fumarate allowed iron(II) oxidation within 1 week. A nitrate reducer which oxidized ferrous iron as rapidly as the lithotrophic enrichment culture (Fig. 1) has not been isolated so far. All isolates were able to grow well on various organic acids in the presence of nitrate (Table 1) or oxygen. The main product of nitrate reduction by the enrichment culture and the new isolates was dinitrogen, as demonstrated by gas chromatography (detection by thermal conductivity) of cultures grown under a headspace of He-CO_2 (90:10 [vol/vol]). The formation of ammonium was not detectable. In the lithotrophic enrichment culture, the molar ratio of formed iron(III) to reduced nitrate was 1:0.22, which is in good agreement with the expected stoichiometry of ferrous oxidation according to the following equation: $10\ FeCO_3 + 2\ NO_3^- + 24\ H_2O \rightarrow 10\ Fe(OH)_3 + N_2 + 10\ HCO_3^- + 8\ H^+$. Also, the electron balances in the mixotrophically grown cultures of strains HidR2 and BrG1 (Table 1) are in accordance with this equation. In the culture of strain BrG2 (Table 1) grown under purely lithotrophic conditions, more nitrate disappeared than was theoretically expected from the formation of iron(III) and nitrite; one may speculate that some hitherto-unidentified, oxidized nitrogen species, which eventually form complexes with iron (11), were formed in this culture. The enrichment culture and the isolates never oxidized more than approximately 80% of the added iron. Nevertheless, these cultures formed a vivid yellow-brown to orange, rusty precipitate, and there was no indication of mixed Fe(II)-Fe(III) hydroxides as major products, which would have caused a grayish-green appearance (20). The remaining, nonoxidizable iron(II) was probably due to compact crystalline forms of iron(II) which were not readily accessible as growth substrates.

Reducing equivalents are also required for cell synthesis. However, because of low cell yields, cell synthesis could not be quantified by the analytical methods applied thus far (3). There was no obvious surplus of electrons in the total electron balance (Fig. 1; Table 1) in comparison with the number of electrons of the catabolic reaction (resulting from the above-de-

TABLE 1. Properties of newly isolated iron-oxidizing nitrate-reducing bacteria

Property or test type	Result with strain:		
	HidR2	BrG1	BrG2
Cell morphology			
Shape	Rod	Rod	Slightly curved rod
Length by width (μm)	3.5 by 1	2 by 0.6	1.5 by 0.6
Compounds tested as substrates[a]			
Acetate	+	+	+
Propionate	+	+	+
Lactate	+	+	−
Succinate	+	+	+
Fumarate	+	+	+
Glucose	+	−	−
Fructose	+	−	−
Yeast extract	+	+	−
Quantitative growth experiments			
Incubation time (days)	10	8	21
Acetate added (mM)	1.0	0.5	None
$FeSO_4$ added (mM)	6.6	10.0	10.0
Fe(III) formed (mM)	4.0	5.8	8.3
Electrons derived from donors or donor (mM)[b]	12.0	9.8	8.3
NO_3^- disappeared (mM)	2.3	2.0	4.0
NO_2^- formed (mM)	0.0	0.0	1.2
Theoretical NO_3^- reduction (mM)[c]	2.4	2.0	2.4

[a] The listed compounds were added at concentrations between 5 and 10 mM, and yeast extract was added at 0.5 g/liter; the electron acceptor was nitrate. Symbols: +, utilized; −, not utilized.

[b] Stoichiometrically, 8 mol of electrons are derived per mol of acetate added, and 1 mol of electrons per mol of Fe(II) oxidized to Fe(III). Because of low cell yields, the utilization of electrons for cell synthesis was not considered here.

[c] Stoichiometrically, 1 mM electrons allows the reduction of 0.2 mM NO_3^- to N_2 or of 0.5 mM NO_3^- to NO_2^-. The formation of NO_2^- by strain BrG2 consumed 1.2 mM NO_3^- and 2.4 mM electrons. The remaining 8.3 − 2.4 = 5.9 mM electrons would allow the reduction of 5.9 · 0.2 = 1.18 mM NO_3^-, theoretically yielding a total consumption of 1.2 + 1.18 = 2.38 mM NO_3^-.

scribed equation); also, this result indicated that the portion of electrons used for assimilatory reactions was very small. There was no visible cell turbidity in the culture medium above the precipitated iron minerals. The number of cells which could be seen by light microscopy in the precipitate was much lower than in the case of ferrous iron-oxidizing phototrophic bacteria (3, 24). Determining cell numbers by microscopy was not reliable because the majority of cells were closely associated with the inorganic particles.

The capacity for anaerobic iron oxidation was also tested with some other, previously described strains of denitrifying bacteria. Strains LP-1 and AR-1, which had been originally isolated with aromatic substrates and nitrate (8), oxidized more than half of the ferrous iron in the presence of 1 mM acetate within 1 week. Strain ToN1, which had been isolated with toluene (19), and *Thiobacillus denitrificans* as well as *Pseudomonas stutzeri* oxidized ferrous iron under autotrophic conditions within 8 weeks. In contrast, *Thiomicrospira denitrificans* and *Paracoccus denitrificans* were unable to oxidize ferrous iron within 12 weeks.

Strains BrG1, BrG2, ToN1 and *Thiobacillus denitrificans* were able to oxidize black ferrous sulfide (5 mmol FeS and 10 mM $NaNO_3$ were added per liter). The black color disappeared within 3 weeks, and a beige sediment was formed.

The observed microbial oxidation of ferrous iron with nitrate is in agreement with bioenergetic considerations. At a pH of 7 in the presence of CO_2 and HCO_3^- as a naturally important buffer system, the prevailing forms of ferric and ferrous iron are Fe_2O_3 hydrates and $FeCO_3$, respectively, which under the given conditions constitute a redox pair with a redox potential of around +0.1 V (9, 19, 24, 25). All redox pairs of the nitrate reduction pathway are much more positive ($E^{\circ\prime}$ values: NO_3^--NO_2^-, +0.43 V; NO_2^--NO, +0.35 V; NO-N_2O, +1.18 V; N_2O-N_2, +1.35 V [22]) and hence are energetically favorable electron acceptors for ferrous iron oxidation.

The reduction of nitrate by ferrous iron to various oxidation states of nitrogen ranging from nitrite to ammonia was previously demonstrated to occur as an abiological reaction in bicarbonate-free systems at a pH of ≥7 in the presence of catalytic concentrations of copper ions (≥10 μM [2]). In addition, nitrite and nitrous oxide were chemically reduced by ferrous iron (14, 16). In the bicarbonate-containing medium with extremely low concentrations of added copper (≤0.1 μM) used by us, only the process of biological reduction was significant. Activities such as those exhibited by the enrichment and pure cultures may be responsible for the occurrence of anaerobic ferrous iron oxidation in suboxic zones of aquatic sediments (7) where conditions are considered to be unfavorable for chemical nitrate reduction (2). Since even nitrate reducers which had never previously been grown in iron media exhibited the capacity for ferrous iron oxidation, this activity may be widespread in various aquatic habitats with active denitrification.

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