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Anaerobic and aerobic oxidation of ferrous iron at neutral pH by chemoheterotrophic nitrate-reducing bacteria

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Abstract Nine out of ten anaerobic enrichment cultures inoculated with sediment samples from various freshwater, brackish-water, and marine sediments exhibited ferrous iron oxidation in mineral media with nitrate and an organic cosubstrate at pH 7.2 and 30° C. Anaerobic nitrate-dependent ferrous iron oxidation was a biological process. One strain isolated from brackish-water sediment (strain HidR2, a motile, nonsporeforming, gram-negative rod) was chosen for further investigation of ferrous iron oxidation in the presence of acetate as cosubstrate. Strain HidR2 oxidized between 0.7 and 4.9 mM ferrous iron aerobically and anaerobically at pH 7.2 and 30° C in the presence of small amounts of acetate (between 0.2 and 1.1 mM). The strain gained energy for growth from anaerobic ferrous iron oxidation with nitrate, and the ratio of iron oxidized to acetate provided was constant at limiting acetate supply. The ability to oxidize ferrous iron anaerobically with nitrate at approximately pH 7 appears to be a widespread capacity among mesophilic denitrifying bacteria. Since nitrate-dependent iron oxidation closes the iron cycle within the anoxic zone of sediments and aerobic iron oxidation enhances the reoxidation of ferrous to ferric iron in the oxic zone, both processes increase the importance of iron as a transient electron carrier in the turnover of organic matter in natural sediments.

Key words Iron oxidation · Ferrous iron · Ferric iron · Nitrate reduction · Sediments

Introduction

Iron is one of the most abundant elements in the Earth's crust and is the second most abundant metal. Due to their low water solubility, iron oxides accumulate in aquatic habitats in higher amounts only in sediments, as complex amorphous or crystalline structures (Cornell and Schwertmann 1996).

Ferric iron is reduced by heterotrophic bacteria to ferrous iron (Lovley 1991). Ferrous iron can be reoxidized in the presence of oxygen at low pH by acidophilic bacteria such as *Thiobacillus ferrooxidans* (Blake et al. 1993) or at a pH of approximately 7 by bacteria such as *Gallionella ferruginea* (Hallbeck et al. 1993), both of which obtain growth energy from this redox reaction.

The standard redox potential (E_0) of the Fe^{3+}/Fe^{2+} couple is +770 mV, but the actual redox potential in natural habitats depends strongly on the prevailing pH (Widdel et al. 1993). At pH 7 in the presence of bicarbonate, the redox transition of $FeOOH/FeCO_3$ dominates with an E value of approximately +200 mV.

With this low redox potential, ferrous iron could also serve as an electron donor for redox processes in anoxic habitats. Anoxygenic photosynthetic bacteria that can use ferrous iron as an electron donor have been recently isolated and described (Widdel et al. 1993; Ehrenreich and Widdel 1994). Electrons released at +200 mV could also be used for microbial dissimilatory nitrate reduction. Such a metabolism has been reported recently for mesophilic bacteria (Straub et al. 1996) and for a hyperthermophilic archaeobacterium (Hafenbradl et al. 1996). This process would connect the iron cycle with the nitrogen cycle, and thus, would further increase the importance of iron as an electron acceptor in anoxic microbial habitats. The present study concentrates on cosubstrate-dependent oxidation of ferrous iron by facultatively anaerobic nitrate-reducing bacteria.

Materials and methods

Sources of organisms

For enrichment cultures, various sediment samples from freshwater sources (Vorsee, Mittlerer Buchensee, and Lake Constance, Germany), marine sources (Venice, Italy; Ameland and Groningen, The Netherlands), and brackish-water sources (Hiddensee,

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Germany), or activated sludge of the municipal sewage treatment plant of Konstanz (Germany) were used as inocula.

Media and cultivation methods

For enrichment and cultivation of freshwater and marine bacteria, anoxic bicarbonate-buffered mineral media were used (Widdel and Bak 1992) with 1 mM sulfate as the sulfur source and no reducing agent added. The concentrations of NaCl and $MgCl_2$ applied were adapted to the original habitat of the microorganisms. The respective amounts of NaCl and $MgCl_2 \cdot 6H_2O$ in g per l medium were: 1.0 and 0.4 for freshwater, 7.0 and 1.0 for brackish-water, and 20.0 and 3.0 for marine sediments. After autoclaving and cooling under N_2/CO_2 (80:20, v/v), 1 ml each of the trace element solution SL9 (Tschech and Pfennig 1984) and the 7-vitamin solution (Widdel and Pfennig 1981) were added per liter, and the pH was adjusted to 7.2. For aerobic growth experiments, the same medium was used as above, but it was buffered with 20 mM Hepes instead of bicarbonate at pH 7.2.

Enrichment and isolation

Serum bottles with 120 ml volume were filled anoxically with 50 ml autoclaved medium and were sealed with butyl rubber stoppers. Approximately 5 ml inoculum was added anoxically. Substrates were injected aseptically with syringes to final concentrations of 4 mM $FeSO_4$ and 5 mM nitrate. Each inoculum material was used for enrichment at pH 6.5, 7.0, or 8.0 at two different temperatures, 16 and 30° C. The enrichment cultures were transferred periodically after 2–4 weeks, when a rusty-brown precipitate had formed.

Bacteria enriched in the brackish-water cultures were purified using the agar dilution method (Pfennig and Trüper 1981) with the following modifications: to 3 ml of 3% (w/v) liquid agar solution at 60° C in a 25-ml tube, 8 mM $FeSO_4$ from a sterile stock solution (0.8 M) was added and mixed carefully. Afterwards, 7 ml of prewarmed (42° C) medium with 5 mM nitrate was added, making a final concentration of 4 mM ferrous iron. Tubes of each dilution series were sealed, inoculated, cooled in a water bath, gassed with N_2/CO_2 (80:20, v/v), and incubated at 30° C in the dark. Colonies growing in these tubes were subjected to two agar dilutions before they were transferred into liquid medium.

Pure cultures of freshwater and marine enrichments were isolated on plates. The plates contained the above-described media with 0.01 M Mops as buffer instead of bicarbonate, 1.5% (w/v) agar, 5 mM nitrate, and 3 mM acetate. Inoculated plates were incubated in anoxic jars under an N_2/CO_2 atmosphere (80:20, v/v) at 30° C in the dark. Colonies were transferred under anoxic conditions into 60-ml serum bottles with freshwater medium containing 8 mM $FeSO_4$, 1 mM acetate, and 5 mM nitrate. Only inoculum from bottles showing ferrous iron oxidation was streaked two more times on agar plates to ensure purity and was transferred to liquid medium afterwards.

Purity was checked microscopically after growth in mineral medium with 0.2% yeast extract. Cultures used for growth experiments were checked for contamination by microscopy at regular intervals. To determine the type of flagellation, a silver impregnation stain according to Blenden and Goldberg (1965) was applied.

Growth experiments

Bacteria were grown in 500-ml bottles containing 300 ml medium. Noninoculated bottles treated the same way as inoculated ones did not show ferrous iron oxidation for at least 2 months of incubation at 30° C. After vigorous shaking, samples were taken through the butyl rubber stoppers with syringes that had been preflushed with N_2/CO_2 (80:20, v/v) and were transferred directly into HCl or phosphate buffer for further analysis.

For growth experiments in oxygen gradient tubes, 22-ml tubes were filled anoxically with 4 ml anoxic medium that contained 1.5% (w/v) agarose, 15 mM ferrous iron, and 1 mM acetate. After this layer had solidified under an N_2 atmosphere, a second layer of 5 ml anoxic medium with 0.5% (w/v) agarose and the inoculum were placed on top. After solidification of the second layer under an N_2 atmosphere, 1 ml of sterile liquid medium without agarose was added to prevent drying of the agarose medium. The tube was covered loosely with an aluminum cap and incubated upright at 30° C in the dark on a slowly shaking (80 rpm) rotary shaker. All experiments were performed at least in duplicate.

Analytical procedures

Ferrous iron was quantified photometrically with ferrozine (Stookey 1970). The total iron concentration was measured the same way after reduction of all iron in the sample to the ferrous state with hydroxylamine hydrochloride (Stookey 1970). Ferric iron concentration was calculated as the difference between measured total iron and ferrous iron. For measurements of nitrite-containing samples, the samples were mixed with 1 vol. 500 mM phosphate buffer (pH 7.0) under anoxic conditions, incubated at room temperature for 15 min, and centrifuged in an Eppendorf centrifuge at maximum speed for 5 min. The pellet was resuspended in 1 ml 1 M HCl. With this treatment, nitrite could be removed to avoid chemical oxidation of ferrous iron by nitrite before and during the analysis. With more than 1 mM nitrite in the sample, the procedure had to be repeated at least twice. For iron analysis in gradient tubes, the agarose medium was cut in 2-mm-thick slices with a razor blade and transferred into 1 ml 5 M HCl. After incubation for 15 min in a water bath at 40° C, slices were liquified by this acid treatment, and Fe was measured as described above. Nitrate and nitrite were determined quantitatively by HPLC with a Grom-Sil anion column (Grom, Herrenberg, Germany) and absorption detection at 220 nm. To prevent interaction of iron with nitrite and to protect the column, iron-containing samples were treated with phosphate buffer as described above, and the supernatant was used for nitrate and nitrite determination. The detection limit of this method was approximately 30 μM nitrite.

Acetate was quantified by gas chromatography (GC6000 Vega Series 2; Carlo Erba, Milan, Italy) on a packed column (2 m × 2 mm; 60/80 Carbopack C/0.3% carbowax 20 M/0.1% H_3PO_4) with a flame ionization detector. To protect the column, samples for acetate measurement were prepared as described above. N_2O was quantified by gas chromatography (GC6000 Vega Series 2; Carlo Erba) on a packed column [2 m × 2 mm; 60/80 Carbosieve SII (Supelco, Bellefonte, Pa., USA)] with a thermal conductivity detector.

Ammonium was quantified by flow injection analysis according to Hall and Aller (1992). Protein was measured as described (Peterson 1983). For quantification of the protein content in iron-containing samples, standards for protein measurement were prepared in medium containing the same amount of iron as the samples to account for possible interactions of iron with the protein assay chemicals. Dry mass was calculated from the protein measured using a previously determined correlation between the dry mass and the protein content of several cultures of strain HidR2 with different optical densities.

Oxygen profiles in gradient tubes were measured as described (Revsbech 1989), with oxygen microelectrodes driven by a micromanipulator.

Results

Enrichments

All enrichment cultures except those inoculated with sediment from a Venice channel formed rusty-brown precipitates within 7–10 days of incubation. The precipitates were identified by chemical analysis as ferric iron hydroxides; no ferrous iron oxidation was found in noninoc-

ulated cultures. After the third or fourth transfer, addition of acetate or succinate as organic cosubstrates was required to maintain significant ferrous iron oxidation. Only one enrichment culture (from marine sediment from Groningen) oxidized ferrous iron without addition of an organic cosubstrate, even after repeated transfer for more than 1 year in mineral medium containing only ferrous iron and nitrate. Comparative experiments with all enrichment cultures indicated that ferrous iron oxidation did not differ significantly in rate and extent with incubations at 16 or 30° C, or at pH 6.5 or pH 8.

Characterization of pure cultures

From all ferrous iron-oxidizing enrichment cultures, pure cultures were isolated that oxidized ferrous iron anaerobically in the presence of acetate as organic cosubstrate with nitrate as the electron acceptor. Strain HidR2, isolated from an enrichment culture from a Baltic Sea sediment, was chosen for further characterization.

Cells of strain HidR2 were gram-negative, non-spore-forming rods, 3–6 × 1 μm in size (Fig. 1), and motile by tufts of monopolar flagella. Cells were oxidase- and catalase-positive. With the phase-contrast microscope, small dark inclusions were observed at both cell ends. These inclusions disappeared after treatment with chloroform and were likely to be accumulations of polyhydroxyalkanoates. After growth with excess acetate (> 3 mM acetate), strain HidR2 often formed aggregates of approximately 10–50 cells in batch cultures. In liquid medium with ferrous iron plus acetate and nitrate, the majority of cells were found within flocs of iron hydroxides or siderite as visualized by 4,6-diamidino phenylindole (DAPI) staining, but did not encrust with ferric iron hydroxides. In agar with acetate and nitrate as substrates, strain HidR2 formed white, fluffy colonies. In agar with ferrous iron plus acetate and nitrate, colonies were dark brown and spherical with eroded margins. The temperature range for growth was 5–40° C with an optimum at 33° C; the pH range was 5.5–9.5, with an optimum at pH 7.5. The strain grew anaerobically with nitrate, nitrite, or N_2O, or aerobically with oxygen as an electron acceptor.

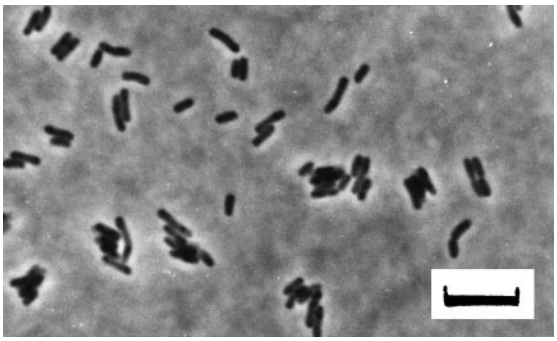

Fig. 1 Phase-contrast photomicrograph of cells of strain HidR2 grown anaerobically with acetate and nitrate (*bar* 10 μm)

No growth was found with sulfate, fumarate, or amorphous $Fe(OH)_3$ (Lovley and Phillips 1986) as the electron acceptor and acetate or succinate as the electron donor. Electron donors tested and oxidized were glucose, fructose, xylose, arabinose, propionate, butyrate, L-lactate, isobutyrate, succinate, ethylene glycol, ethanol, glycine, and yeast extract. Isovalerate, benzoate, methanol, and ammonia were not oxidized.

Anaerobic oxidation of ferrous iron by strain HidR2

During anaerobic incubation in the absence of an organic cosubstrate, strain HidR2 did not grow or oxidize ferrous iron at a measurable rate. Addition of acetate or succinate, for example, significantly enhanced ferrous iron oxidation, and growth became measurable.

Nitrate-dependent anaerobic oxidation of ferrous iron by strain HidR2 with acetate as cosubstrate at 30° C and pH 6.7 is documented in Fig. 2 and is compared with anaero-

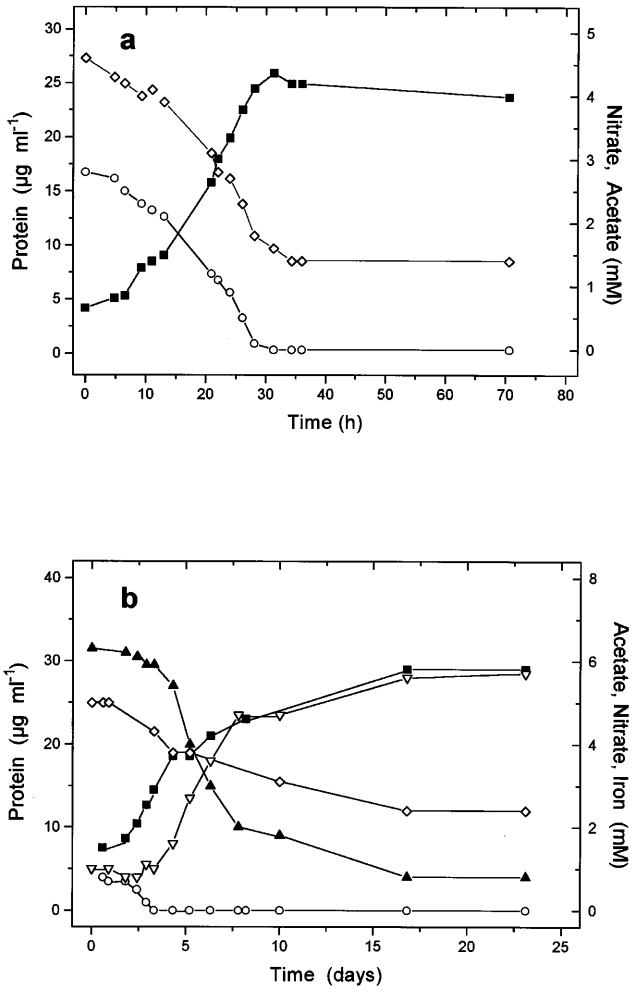

Fig. 2a, b Anaerobic growth and substrate conversion by strain HidR2 in mineral medium containing acetate and nitrate at 30° C and pH 6.7. Nitrite was assayed but was never detected (detection limit, 30 μM). **a** Growth in the absence of ferrous iron; **b** growth in the presence of ferrous iron. ■ protein, ◇ nitrate, ○ acetate, ▲ iron(II), and ▽ iron(III)

Table 1 Stoichiometry of growth and substrate conversion by strain HidR2 grown anaerobically on acetate and nitrate with or without added ferrous iron. The columns represent independent experiments with various additions of acetate and $FeSO_4$. The low cell yield in the experiments with low acetate concentrations (columns 1 and 2) did not allow reliable assay of protein formation; consequently, there is no calculation of dry mass formation, in contrast to experiments with high acetate concentrations (columns 3 and 4). Therefore electrons for cell synthesis were not considered in the balance calculations (columns 1 and 2) (*nd* not determined)

	Presence of $FeSO_4$			Without $FeSO_4$
Acetate supplied and consumed (mM)	0.2	1.1	3.1	2.8
$FeSO_4$ supplied (mM)	6.4	6.4	5.9	–
Fe(III) formed (mM)	0.7	4.9	4.3	–
Nitrate consumed (mM)	0.5	3.1	5.1	3.2
Nitrite formed (mM)	0	0	0	0
N_2O formed (mM)	0.06	0.77	1.6	0
Cell dry mass formed (mg/l)	nd	nd	58	39
Acetate assimilated (mM)[a]	nd	nd	1.2	0.8
Acetate oxidized (mM)[b]	0.2	1.1	1.9	2
Y_s (g dry mass/mol acetate)	nd	nd	31	20
Electrons recovered (%)[c]	106	108	123	100

[a] Acetate assimilated was calculated based on the dry mass formed (<$C_4H_7O_3$>) according to: 17 acetate + 11 $H^+ \rightarrow$ 8 <$C_4H_7O_3$> + 2 HCO_3^- + 4 OH^-

[b] Acetate oxidized was calculated as the amount of acetate consumed minus acetate assimilated.

[c] Electrons recovered represent the electrons needed to reduce the consumed nitrate to N_2 and partially to N_2O when found, divided by the electrons derived from the acetate oxidized (column 4) or from acetate plus ferrous iron oxidized (columns 1–3). Since for columns 1 and 2 no acetate oxidation could be calculated (as indicated), acetate consumed was taken as 100%

bic growth with acetate and nitrate in the absence of ferrous iron. In the presence of ferrous iron, acetate, and nitrate, cells grew exponentially with a doubling time of 45 h. After depletion of acetate, cells doubled slowly once more within approximately 14 days, depending on the initial acetate concentration. In the absence of ferrous iron, cells grew with acetate and nitrate at a doubling time of 11 h. As shown in Table 1, strain HidR2 growing on acetate reduced nitrate to N_2 plus N_2O when grown on acetate and ferrous iron as substrates, as did nearly all other enrichment cultures. No ammonia was formed by strain HidR2 during growth on nitrate, and ammonia was not oxidized aerobically. Control assays with autoclaved cells did not show ferrous iron oxidation or an increase in protein content under the same conditions.

Anaerobic growth with and without ferrous iron is documented in Table 1. Ferrous iron oxidation was never complete, but ceased when approximately 90% of the added ferrous iron was oxidized. This proportion could not be changed by additional pulses of either 1 mM acetate or 5 mM $FeSO_4$, indicating that the remaining ferrous iron was not available to the cells.

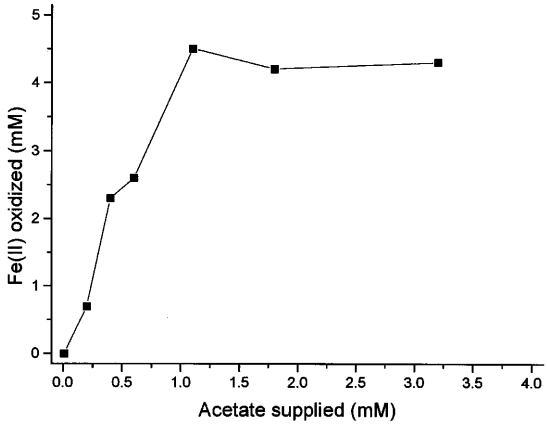

Fig. 3 Ferrous iron oxidation and acetate utilization by strain HidR2 with 8 mM $FeSO_4$ and 5 mM nitrate in the medium and acetate at various concentrations. Incubation was at 30° C in the dark for approximately 3 weeks; pH in the medium was constant at 6.8

The influence of acetate on the extent of ferrous iron oxidation was tested in a series of growth experiments with various acetate concentrations (Fig. 3). The amount of iron oxidized per acetate supplied was stable up to approximately 1 mM acetate, with a ratio of 4 mol ferrous iron oxidized per mol acetate consumed. When more acetate was provided, the extent of iron oxidation did not increase beyond approximately 90% of the total ferrous iron supplied.

Aerobic oxidation of ferrous iron by strain HidR2

Profiles of iron and oxygen formed by the metabolic activity of strain HidR2 in semisolid media with opposing gradients of oxygen and ferrous iron and small amounts of acetate are shown in Fig. 4. Noninoculated tubes were treated and analyzed in the same way as the inoculated ones to allow differentiation of biological and chemical iron oxidation. The tubes were incubated under an air atmosphere for 4 weeks. After incubation, a section with a multilayered stratification of thin and dense layers of ferric iron had formed slightly below the agarose meniscus of the inoculated tubes. The control tubes did not show such a section of multilayered stratification, but formed an orange layer of ferric iron that decreased with depth in the tube and extended further towards the bottom than in the inoculated tubes. Chemical analysis revealed a significant maximum of Fe(III)-oxide precipitation deeper in the agarose medium and at significantly lower oxygen partial pressure than in the uninoculated control tubes, thus confirming the macroscopic impression that Fe(II) was oxidized aerobically by strain HidR2. Obviously, cells of strain HidR2 trapped the major portion of ferric iron in a distinct layer; this is also found in natural sediments.

Control for chemical ferrous iron oxidation

Control assays for chemical ferrous iron oxidation were performed under the same conditions as were the growth

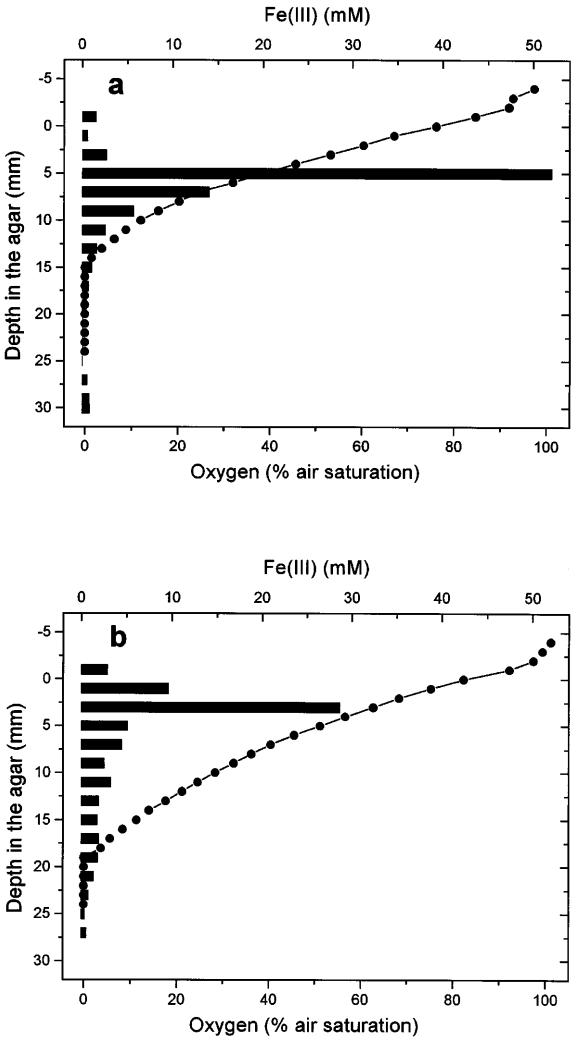

Fig. 4a,b Iron(III) and oxygen profile of agarose oxygen-iron(II) gradient tubes after 30 days of incubation at 30° C on a rotary shaker. **a** Tube inoculated with strain HidR2; **b** uninoculated control. *Circles* oxygen, *bars* iron(III)

experiments with strain HidR2. Since ferrous iron is easily oxidized by molecular oxygen, controls without inoculum were run to check for chemical iron oxidation caused, for example, by molecular oxygen penetrating through the butyl rubber stoppers. These controls proved that there was no detectable ferrous iron oxidation under the conditions provided for incubation times of at least 6 weeks.

Addition of 5 mM nitrite to anoxic medium at pH 6.7 containing 8 mM ferrous iron caused fast ferrous iron oxidation. Approximately 0.8 mM nitrite disappeared in 4 days at a constant rate of 8 μM h^{-1}. With 1 mM nitrite in anoxic media at pH 6.7, no significant iron oxidation was found in the same time period. With 0.3 mM nitrite in the medium, no ferrous iron oxidation was observed within 4 days.

Discussion

In the present report, aerobic and anaerobic ferrous iron oxidation by a denitrifying bacterial strain in the presence of small amounts of an organic cosubstrate at pH 7.2 and 30° C is documented in detail. The high reactivity of ferrous iron towards oxygen at pH 7.2 made controls necessary in order to distinguish chemical from biological processes and to rule out possible interference of nonbiological oxidation with biological processes.

Care had to be taken to avoid penetration of oxygen through stoppers into the culture vessels. Also, nitrite formation had to be checked for during growth in the culture media since nitrite may accumulate during nitrate reduction and is known to react chemically with ferrous iron (Komatsu et al. 1977; Moraghan and Buresh 1977; Christianson and Cho 1983; Brons et al. 1991). Since strain HidR2 oxidized iron in significant amounts only in the presence of an organic cosubstrate, we had to rule out the possibility that the ferric iron formation was due to chemical iron oxidation with nitrite or N_2O that had accumulated from cosubstrate-dependent nitrate reduction. The control experiments proved that:

1. Oxygen penetrating through the butyl rubber stoppers was not responsible for ferrous iron oxidation in inoculated bottles since no ferrous iron oxidation could be detected in noninoculated control bottles during 6 weeks.
2. Nitrite accumulating during growth was not responsible for ferrous iron oxidation found with strain HidR2 because nitrite could never be detected during growth at concentrations higher than 30 μM (which was the detection limit); chemical oxidation of ferrous iron with nitrite could play a significant role only if nitrite accumulated to concentrations of several millimolar, as is known to happen in batch cultures of some denitrifiers.
3. Chemical oxidation of ferrous iron by N_2O could also be ruled out since no significant chemical iron oxidation with N_2O was found at 50 ml N_2O/l (Straub et al. 1996), equivalent to 2 mmol l^{-1}.

Since all experiments for chemical ferrous iron oxidation were performed under the same conditions as were the biological experiments, we conclude that the anaerobic nitrate-dependent ferrous iron oxidation observed must be a biologically catalyzed reaction.

We demonstrated that strain HidR2 is able to use the electrons derived from the oxidation of ferrous iron in its dissimilatory metabolism, thus gaining energy from iron oxidation. This conclusion is based on a measured 55% growth yield increase of strain HidR2 in the presence of ferrous iron and acetate as compared to growth with acetate alone (Table 1). The observation that the cell mass of strain HidR2 still increased during iron oxidation when acetate was depleted (Fig. 2) also supports this conclusion. The cosubstrate acetate is obviously needed by strain HidR2 for cell matter synthesis; this strain cannot grow autotrophically. Nonetheless, acetate is not exclusively assimilated into cell material. Part of the acetate was dissimilated also in the presence of excess ferrous iron; when limiting acetate was provided, acetate and ferrous iron were oxidized simultaneously at a constant ratio of 1:4 (Fig. 3). Obviously, acetate oxidation provides

electrons for acetate assimilation (e.g., at the NADH level) that the cells cannot recruit from ferrous iron oxidation, perhaps due to the lack of a suited reversed electron transport system. The growth yield per mol acetate (14 g dry mass) compared to that per mol acetate plus 4 mol ferrous iron (19 g dry mass) revealed that acetate electrons (arising at an average E_0' of –290 mV) were actually nearly twice as valuable for the cell metabolism (1.75 g per mol electrons) as were those derived from ferrous iron oxidation (1.0 g per mol electrons; with an E' of +200 mV). This agrees well with the overall energy gain calculated for the transport of these electrons to nitrate [average E_0' of the couple 2 NO_3^-/N_2 = +751 mV; all calculations based on Thauer et al. (1977)].

The slower growth of strain HidR2 observed when it grows anaerobically in the presence of ferrous iron is not necessarily related to the iron oxidation metabolism. It may also be caused by toxicity of excess ferrous iron, or by a lower availability of phosphate or other micronutrients, either by complex formation or precipitation of those nutrients with iron (Hughes and Poole 1991).

The biochemistry of the anaerobic iron oxidation still leaves several questions open. The observed energy conservation would necessitate a proton translocation step across the cytoplasmic membrane. We do not know where in the cell ferrous iron is oxidized. If enzymes of ferrous iron oxidation are localized in the periplasm as in the case of nitrite and N_2O reduction (Ferguson 1994), the question arises how ferric hydroxide formed in the periplasm is transported out into the medium. Ferrous iron could also be oxidized outside the cell, e.g., at the outer cell surface. This would require an electron transfer system through the periplasm (Stouthamer 1991; Ferguson 1994), for example by a periplasmic cytochrome. Such an electron carrier has been suggested to be present in acidophilic aerobic iron oxidizers (Blake et al. 1993). Since cells of strain HidR2 do not encrust themselves in iron hydroxides as the phototrophic *Rhodomicrobium vannielii* does (Widdel et al. 1993), ferric hydroxides are obviously formed outside the outer cell envelope.

Indications for anaerobic nitrate-dependent ferrous iron oxidation activity have been obtained earlier by measurements of nitrate and iron distribution gradients in marine and freshwater sediments (Froehlich et al. 1979). This activity is likely to be due to living bacteria since the nitrite concentrations in natural sediments are far too low (≤ 1 μM) to allow significant chemical oxidation with nitrite. Obviously, heterotrophic denitrifying bacteria as described here could be responsible for the iron-oxidizing activities found. This metabolism appears to be widespread among denitrifying bacteria since more than 90% of our enrichment cultures for such a metabolism with various sediments were positive and contained morphologically different bacteria. Furthermore, the above-described type of nitrate-dependent iron oxidation should be expected especially in habitats rich in ferrous iron with additional, but limiting supply of organics, e.g., profundal sediments of oligotrophic waters. Iron accumulates in sediments of these waters due to its low solubility (Stumm and Morgan 1981), and the content of organic carbon in a sediment decreases with increasing water depth.

From growth experiments with strain HidR2 in oxygen/ferrous iron gradient systems, we concluded that this strain is also able to oxidize ferrous iron aerobically in the presence of small amounts of an organic cosubstrate. This conclusion is based on the following observations:

1. The gradient tube inoculated with strain HidR2 exhibited an iron oxidation pattern visually different from that of the control tube, and this was verified by chemical analysis of vertical iron profiles in the tubes.
2. Incubation of the tube inoculated with strain HidR2 resulted in the formation of approximately twice as much ferric iron in the top section.
3. Oxygen was depleted significantly closer to the top of the agarose medium in the inoculated tube.

It is well-known that nearly all known denitrifying bacteria can reduce oxygen and oxidized nitrogen species (Knowles 1982). Therefore, it is not astonishing that strain HidR2 can also oxidize iron aerobically. Considering the widespread ability of denitrifying bacteria to oxidize ferrous iron, it can be assumed that aerobic iron oxidation might also be a common ability of these bacteria. Thus, aerobic iron oxidation would not be limited further to the known autotrophic aerobic iron-oxidizing bacteria such as *Gallionella ferruginea*, and microbial iron oxidation would turn out to be a far more important process in sediments than assumed so far. By providing the electron acceptor for iron reduction, aerobic and anaerobic microbial iron oxidation increases the importance of mineralization of organic material in sediments by iron-reducing bacteria that use a broad variety of substrates (Lovley and Phillips 1986; Lovley 1991). Furture work will focus on the ecological importance of the new iron oxidation process described here.

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