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Fe(III) as an electron acceptor for H_2 oxidation in thermophilic anaerobic enrichment cultures from geothermal areas

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Abstract Six sustainable enrichment cultures of thermophilic H_2-oxidizing microorganisms utilizing Fe(III) as an electron acceptor were obtained from geothermally heated environments located on two continents (America, Eurasia) and on islands in the Northern (Iceland) and Southern (Fiji) hemispheres, demonstrating the wide distribution of these microorganisms. The main products of amorphic Fe(III) oxide reduction were magnetite and siderite. The observed temperature range for Fe(III) reduction in growing cultures was from 55°C to 87°C, extending the known limits for growth of Fe(III)-reducing microorganisms producing extracellular magnetite to nearly 90°C.

Key words Fe(III) reduction · Iron reduction · Extreme thermophilic anaerobes · Hydrogen oxidation · Magnetite · Siderite

Introduction

The use of ferric iron as a main or alternative electron acceptor by microorganisms has important environmental implications, and may be involved in the evolution of microbial life (Lovley 1991; Nealson and Saffarini 1994). Microbial Fe(III) reduction has been intensively studied in mesobiotic marine and freshwater anoxic sediments and submerged soils (for review see Lovley 1995), but little is known about microbial reduction of Fe(III) in thermobiotic ecosystems. There is geological evidence for the hydrothermal origin of some Proterozoic magnetic ores (Gow et al. 1994). Furthermore, the discovery of fine-grained magnetite in deep subsurface samples suggests activity of Fe(III)-reducing thermophiles in such environments (Gold 1992). The first thermophilic Fe(III) reducer reported was the aerobic archeon *Sulfolobus acidocaldarius*, which reduces ferric iron with elemental sulfur (Brock and Gustafson 1976). Recently, the obligately anaerobic *Bacillus infernus*, able to reduce Fe(III) with formate and lactate in the temperature range of 40°C–65°C, was isolated from a deep terrestrial subsurface environment (Boone et al. 1995). We report here microbial reduction of ferric iron coupled to oxidation of molecular hydrogen at temperatures up to 87°C in various stable enrichment cultures.

In geothermally heated environments, molecular hydrogen could be one of the most important donors for Fe(III) reduction, since H_2 originates not only from anaerobic decomposition of organic matter, but also from geochemical processes.

Materials and methods

Sixteen samples from geographically distant thermobiotic environments collected at different times were enriched for H_2-oxidizing, Fe(III)-reducing thermophiles (Table 1). Slurries (10% v/v) from each site were inoculated in anaerobic medium supplied with H_2 (100% in gas phase) as a potential electron donor and amorphic Fe(III) oxide (90 mmol of Fe(III) per liter) as an electron acceptor, and incubated at 65°C or 78°C in the dark. The medium contained (in g/l of deionized water): 0.33 KH_2PO_4, 0.33 NH_4Cl, 0.33 KCl, 0.33 $MgCl_2 \cdot 2H_2O$, 0.33 $CaCl_2 \cdot 2H_2O$, 2.0 $NaHCO_3$, and 0.1 yearst extract (BBL) as well as 10 ml vitamin solution (Wolin et al. 1963) and 1 ml trace elements solution; pH was adjusted to 7.0 (25°C). No reducing agent was added to the medium. The trace elements solution contained (mmol/l): 2.0 $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 2.0 Na_2SO_4, 1.0 $CoCl_2 \cdot 6H_2O$, 1.0 $NiCl_2 \cdot 6H_2O$, 0.5 $MnCl_2 \cdot 4H_2O$, 0.5 $ZnSO_4 \cdot 7H_2O$, 0.5 Na_2SeO_3, 0.1 $Na_2MoO_4 \cdot 2H_2O$, 0.1 $Na_2WO_4 \cdot 2H_2O$, 0.1 H_3BO_3, and 0.01 $CuCl_2 \cdot 2H_2O$. The amorphic Fe(III) oxide was prepared by neutralizing a solu-

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Table 1. Environmental samples used for enrichment of H_2-oxidizing, Fe(III)-reducing thermophiles[a]

Location	Sample description	Temperature of sampling site (°C)	pH of sampling site[b]	Designation
Iceland, Fludiv	Sediment, hot spring	90	8.3	I5
Iceland, Hveradge	Sediment, hot spring	85	8.5	I10
Russia, Kamchatka, Geysers Caldera	Sediment, hot spring	72	8.4	K44
New Zealand, Waimangu	Sediment, small water pool	87	7.5	N1a
New Zealand, Waimangu	Sediment, small water pool	73	7.4	N2a
New Zealand, Rotorua	Sediment, hot spring	82	6.9	NA
New Zealand, Rotorua	Sediment, small water pool	80	7.5	NB
Fiji, Vanua Levu Island	Heated soil	96	8.1	F3
Fiji, Vanua Levu Island	Sediment, small water pool	98	8.2	F4
Fiji, Vanua Levu Island	Cyanobacterial mat	65	6.5	F8a
Fiji, Vanua Levu Island	Heated soil	99	6.5	F8d
USA, Yellowstone, Heart Lake	Algal mat	40	8.1	Y1
USA, Yellowstone, Calcite Spring	Sand	60	7.2	Y5
USA, Yellowstone, Calcite Spring	Sediment, black outflow	65	7.5	Y6
USA, Yellowstone, Calcite Spring	Sediment, white filaments	72	7.7	Y7
USA, Yellowstone, Octopus Spring	Cyanobacterial mat	65	8.2	Y10

[a] Dates of collection: Iceland – August, 1992; Kamchatka – September, 1993; New Zealand – December, 1993; Fiji – February, 1995; Yellowstone National Park – September, 1995.
[b] pH at the temperature of the sampling site, determined at the sampling site.

tion of $FeCl_3$ with 10% (w/v) NaOH. The modified Hungate technique was used for media preparation and culturing (Ljungdahl and Wiegel 1986). As a control, the samples were inoculated in the same medium but omitting H_2 (gas phase 100% N_2). A main potential electron donor in these controls was yeast extract (100 mg/l).

Results and discussion

After 7 days of cultivation, ferrous iron, measured after extraction of HCl-soluble Fe(II) with 2,2′-dipyridyl (Balashova and Zavarzin 1980), was detected under both enrichment conditions, i.e., incubations with and without H_2; the concentration of Fe(II) varied from 4.7 to 44.1 mmol/l. The Fe(II) concentration, however, was significantly higher in the enrichments incubated under an H_2 atmosphere. Reduction of amorphic Fe(III) oxide in sterile noninoculated medium incubated at the same temperatures was not detected. In ten enrichments (I5, I10, K44, N1a, NA, F3, F4, F8d, Y6, Y7) incubated under H_2, the nonmagnetic brown amorphic Fe(III) oxide was converted to a black solid material, which was attracted to a magnet. Without H_2 present, no formation of a magnetic precipitate was observed.

After seven subsequent transfers (10% v/v) of enrichments positive for formation of magnetic precipitate, Fe(III) reduction was maintained in six of these cultures (Table 2). Usually, Fe(III) oxide was reduced to magnetic material within 21–60 h. X-ray diffraction analysis indicated that the precipitate formed during reduction of amorphic Fe(III) oxide contained magnetite (Fe_3O_4) as well as siderite ($FeCO_3$). No significant Fe(II) accumulation was detected in the enrichment cultures incubated without H_2 (gas phase 100% N_2). There was no substantial Fe(III) reduction if the cultures under a H_2 gas atmosphere were treated with heat (135°C, 30 min) prior to incubation (Table 2). Neither growth nor Fe(III) reduction were observed in cultures incubated with oxygen (20% in gas phase) or chloramphenicol (100 µg/ml). The cell-free filtrate (10% v/v) of actively growing, Fe(III)-reducing cultures did not reduce amorphic Fe(III) oxide.

The observed temperature range for reduction of amorphic Fe(III) oxide in enrichment cultures was from 55°C to 87°C. Fe(II) production was not detected at 50°C or below, or at 93°C or above. The temperature optima for the reduction of Fe(III) in different cultures varied from 64°C to 76°C (Fig. 1). The occurrence of more than one distinct temperature optimum in a single enrichment culture suggests the presence of different Fe(III)-reducing, H_2-oxidizing thermophiles in the sampled sites.

Quantitative analysis of H_2 oxidation and Fe(III) reduction for the enrichment culture K44 indicated that, for each

Table 2. Concentration of Fe(II) produced in stable enrichment cultures[a] incubated with and without H_2 and in autoclaved (135°C, 30 min) cultures[b]

Sample	Incubation temperature (°C)	Fe(II), mmol/l		
		$+H_2$	$-H_2$[c]	$+H_2$, heat treated
K44	65	18.2	4.0	3.6
Y6	65	11.3	3.8	3.5
Y7	65	17.7	4.2	3.7
I10	78	18.1	3.0	2.5
F3	78	16.1	4.4	4.2
F8d	78	14.6	2.7	3.8

[a] After seven subsequent transfers of initial enrichment.
[b] Incubation 7 days; mean of three independent determinations is presented.
[c] Gas phase: N_2 (100%).

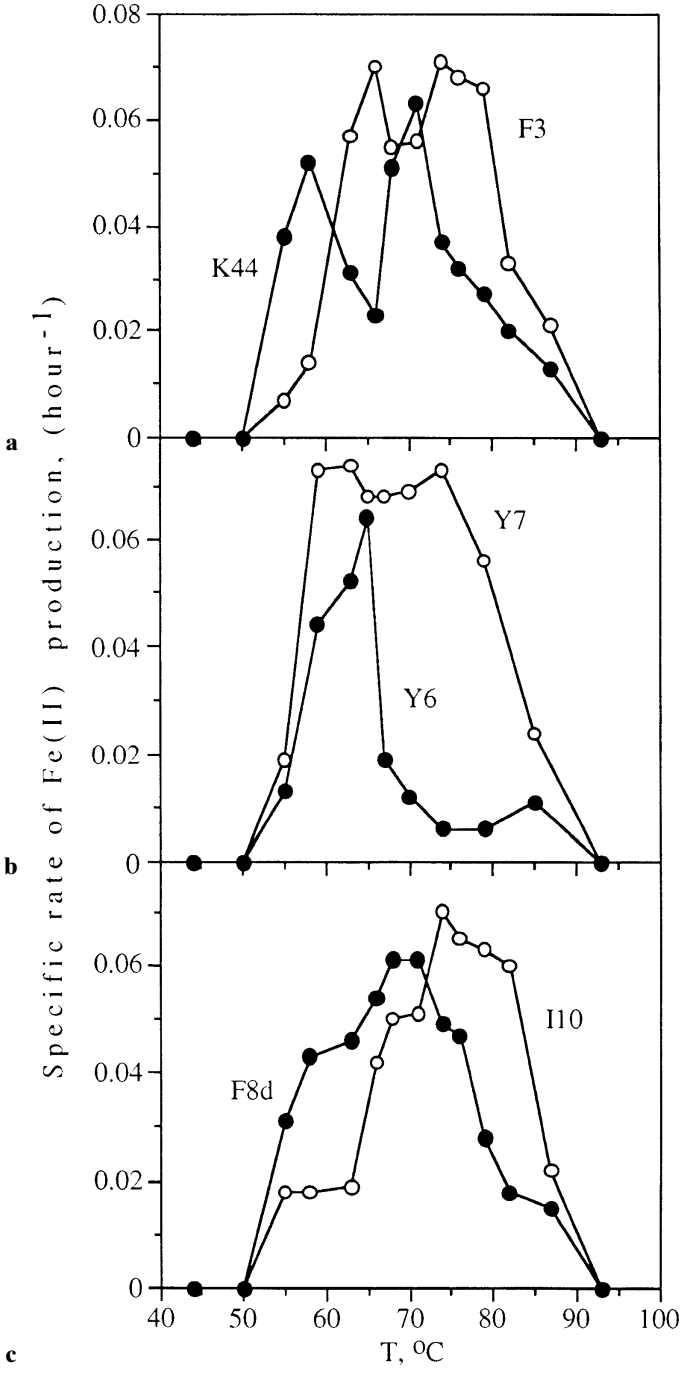

Fig. 1a–c. Effect of temperature on the specific rate of Fe(II) production in the enrichment cultures. **a** Kamchatka K44 (*open circles*) and Fiji F3 (*solid circles*), **b** Yellowstone Y6 (*solid circles*) and Y7 (*open circles*), and **c** Iceland I10 (*open circles*) and Fiji F8d (*solid circles*). Anaerobic medium supplied with H_2 (100% in gas phase) and anmorphic Fe(III) oxide [90 mmol Fe(III) per liter], pH at 65°C = 7.7

mole of H_2 consumed, 2.31 ± 0.53 mol (mean ± standard deviation for five cultures) of Fe(II) was produced (Table 3). This ratio corresponds to a stoichiometric reaction of: $H_2 + 2Fe(III) = 2H^+ + 2Fe(II)$.

All enrichment cultures contained mixed populations of 2–3 morphological types of rods of different sizes, motility, and type of sporulation. No single dominant microorganism could be revealed by light microscopy in any enrichment. The total cell numbers did not exceed 1.5×10^7 cell/ml at any growth stage. Due to attachments of microorganisms to the iron oxide, cell counts were difficult to perform and thus this number is probably an underestimation.

Table 3. Stoichometry of H_2 oxidation and Fe(II) production by K44[a]

Culture[b]	H_2 consumed (μmol)	Fe(II) produced (μmol)	Fe(II)/H_2
1	108	186	1.72
2	124	227	1.83
3	98	233	2.38
4	101	299	2.96
5	87	233	2.68

[a] Hydrogen was analyzed by gas chromatography as described by Slobodkin and Bonch-Osmolovskaya, 1994.
[b] Initial concentration of H_2 was 171 μmol (3.73% in gas phase). Initial concentration of Fe(III) was 900 μmol.

The data presented here unequivocally indicate the presence of microorganisms able to reduce Fe(III) with the oxidation of H_2 in thermobiotic terrestrial freshwater ecosystems. Sustainable enrichment cultures were readily established with samples from sediments of hot springs, small water pools, and geothermally heated soils, located on two continents (America, Eurasia) and on islands in the Northern (Iceland) and Southern (Fiji) hemispheres, suggesting wide distribution of these microorganisms. Furthermore, the temperature optima of Fe(III) reduction above 60°C show that these microorganisms are indeed thermophiles. The observed variations in the temperature optima suggest the occurrence of different thermophilic Fe(III) reducers.

Several mesophilic Fe(III) reducers have been reported to oxidize H_2, either coupled to iron reduction with chemolithoheterotrophic growth (Balashova and Zavarzin 1980; Lovley et al. 1989, 1995; Caccavo et al. 1992, 1994; Rosselo-Mora et al. 1994) or without cell growth (Coleman et al. 1993; Coates et al. 1995). However, to date, no pure cultures of a thermophilic microorganism able to reduce Fe(III) coupled to the oxidation of H_2 have been described. Although the growth occurred concurrently with Fe(III) reduction, thus suggesting energy conservation during this process, the results of this study do not allow us to conclude whether the Fe(III)-reducing microorganisms in the enrichments conserved energy from the H_2 oxidation. In several instances thermophiles have not been shown to produce energy while oxidizing H_2 with elemental sulfur or thiosulfate as electron acceptors (Adams 1990; Fardeau et al. 1994). The presented results, however, demonstrate the biogenic formation of magnetite and siderite from amorphic Fe(III) oxide under anaerobic conditions at temperatures of up to around 90°C.

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