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EXPERIMENTAL ARTICLES

Phylogenetic Composition of Enrichment Cultures of Thermophilic Prokaryotes Reducing Poorly Crystalline Fe(III) Oxide with and without Direct Contact between the Cells and Mineral

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Abstract—Thirty enrichment cultures of thermophilic microorganisms were obtained from Kamchatka terrestrial hydrotherms that reduced insoluble poorly crystalline Fe(III) oxide (ferrihydrite) with and without direct contact between the cells and the mineral. Restricted access to the Fe(III) mineral was achieved by incorporation of ferrihydrite into alginate beads. According to phylogenetic analysis of 22 enrichment cultures by denaturing gradient gel electrophoresis of 16S rRNA gene fragments, *Firmicutes* were predominant among bacteria in all the variants. Microorganisms of the phylogenetic types *Aquificae, Bacteroidetes, Nitrospirae, Planctomycetes, Spirochaetes, Synergistetes*, and *Thermotogae* were also revealed. The archaea revealed belonged to the genera *Desulfurococcus, Pyrobaculum*, and *Thermofilum*. In the case of free access to ferrihydrite, most of the phylotypes belonged to genera known for Fe(III) reduction. In the absence of direct contact with the mineral, together with known iron reducers, organisms for which ability to reduce Fe(III) was unknown were detected. Members of the genera *Carboxydothermus, Thermoanaerobacter*, and *Thermotoga* were detected most often both in the presence and absence of contact with ferrihydrite. These organisms probably possess efficient mechanisms for Fe(III) reduction within the experimental temperature range. Microbial phylogenetic diversity was higher when acetate, rather than lactate, was used as a potential electron donor.

Key words: thermophilic microorganisms, microbial Fe(III) reduction, ferrihydrite, insoluble electron acceptors, phylogenetic analysis

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Microbial dissimilatory Fe(III) reduction is an important biogeochemical process in marine, freshwater, and terrestrial ecosystems. Thermophilic prokaryotes reducing Fe(III) may have played a significant role in the early Earth's biosphere, which was probably characterized by high temperatures and high content of iron compounds [1, 2]. Thermophilic iron reducers do not form a specific phylogenetic group; its presently known members belong to more than 20 bacterial and 6 archaeal genera [2]. Most Fe(III) compounds exhibit extremely low solubility at close to neutral pH. Two fundamentally different physiological strategies for microbial reduction of insoluble Fe(III) oxides are presently known: (i) direct contact between the cell and the mineral by means of the proteins localized outside of the cell membrane or conductive pili and (ii) use of the soluble compounds, which either carry out electron transfer between the cell and the mineral by the shuttling mechanism or increase the solubility of iron [3]. Available data on the strategies of reduction of Fe(III) oxides by thermophilic microorganisms are limited to studies of hyperthermophilic archaea of the genus *Pyrobaculum* [4]. No information is available concerning the physiological mechanisms involved in reduction of insoluble Fe(III) compounds by thermophilic bacteria.

The goal of the present work was to study the effect of the presence or absence of direct contact of microbial cells with poorly crystalline Fe(III) oxide (ferrihydrite) used as the terminal electron acceptor and the phylogenetic composition of thermophilic iron-reducing microbial communities.

MATERIALS AND METHODS

Enrichment cultures. The samples of water and sediments from Kamchatka terrestrial hydrotherms were collected in September 2008. To obtain enrichment cultures of thermophilic iron-reducing anaerobic microorganisms, the sample (10% wt/vol) was added

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Table 1. Characterization of environmental samples from Kamchatka terrestrial hydrotherms used for enrichment cultures of thermophilic iron reducers

Designation	Sampling site	Sample description	*T*, °C	pH
1814	Uzon, Orange field	Black coarse sediment from a small spring	76–80	6.6
1823	Uzon, Eastern field	Gray-black sediment from the Treshchinnyi spring	74–85	6.5
1835	Uzon, Izvilistyi spring	Gray sediment from a hot stream in the runway of a spring with sulfur and iron precipitation along the shores	77–86	6.2–6.5
1850	Uzon, Eastern field	Black sediment from a small spring with white filaments	78	6.2
1860	Uzone, Lake Fumarol'noe	Brown clayey sediment from a shallow spring at the border of the lake	84	6.8
1861	Uzon, Trostnikovyi site	Black sediment from a spring with ochra precipitation	60	6.2
1864	Geyser Valley, hot stream from the Grot geyser	Black sediment from a hot spring with brown bottom and higher plants along the shores	78	7.7

Note: Temperature and pH at the sampling site; pH was measured at the sampling temperature.

to anaerobic bicarbonate-buffered medium containing the following (g/l): NH_4Cl, 0.33; KCl, 0.33; $MgCl_2 \cdot 6H_2O$, 0.33; $CaCl_2$, 0.33; KH_2PO_4, 0.33; $NaHCO_3$, 2.0; yeast extract, 0.2; and vitamin and trace elements solutions, 1 ml/l [5]. The gas phase was 100% CO_2; pH 6.8–7.0 (20°C). Acetate (9 mM) or lactate (14 mM) was used as an electron donor. Poorly crystalline Fe(III) oxide (ferrihydrite) was used as an electron acceptor either as a suspension of free ferrihydrite in the medium (at the final Fe(III) concentration of 90 mM) or as ferrihydrite incorporated into calcium alginate beads (at the final Fe(III) concentration of 70 mM). AQDS (9,10-anhtraquinone -2,6-disulfonate) was added as an exogenous mediator of iron reduction (0.1 mM). Free ferrihydrite and alginate beads with or without ferrihydrite were obtained as described earlier [5, 6]. Medium preparation and cultivation of the enrichments were carried out using the Hungate anaerobic technique [7].

Growth and Fe(III) reduction. Microscopy and enumeration of microbial cells was carried out under a Mikmed 1 light microscope (LOMO, Russia) with phase contrast equipment. The concentration of Fe(II) was determined spectrophotometrically with 2,2-dipyridyl [8] in the sample (0.5 ml) dissolved in 5 ml of 0.6 N HCl. In the case of enrichment cultures with ferrihydrite incorporated into alginate beads, the beads were dissolved in 10 ml of 6N HCl prior to analysis.

Phylogenetic analysis. DNA from enrichment cultures was isolated and purified according to Marmur [9]. Fragments of 16S rRNA genes were obtained by direct amplification of genomic DNA according to the known PCR amplification protocol for obtaining DNA fragments for their subsequent analysis by denaturing gradient gel electrophoresis (DGGE) [10]. The Uni515F universal primer [11] with a GC clamp at the 5' end [10] was used together with either the *Bacteria-specific* Bac-907R primer [12] or the *Archaea-specific* Arch-915R primer [13]. Amplification products were separated by DGGE according to the protocol [14] with the denaturing gradient from 35 to 65% (with the 100% denaturant containing 7 M urea and 40% formamide). DNA fragments from the bands obtained by DGGE were transferred to 20 μl of sterile water by passive DNA diffusion from the gel overnight at 4°C. DNA sequences from the individual bands were determined by enzymatic sequencing on an Applied Biosystems DNA Sequencer 373A using the standard protocol and reagent kit (Fluorescent Dye Cycle Sequencing kit, Perkin-Elmer, United States). Comparison of the sequences with 16S rRNA sequences from the NCBI database was carried out using the BLAST software package [15].

RESULTS AND DISCUSSION

Obtaining iron-reducing enrichment cultures. To obtain enrichment cultures of anaerobic thermophilic iron-reducing microorganisms, seven samples of sediments and water from Kamchatka terrestrial hydrotherms were used (Table 1). Three variants of the medium simulating possible interactions between microorganisms and Fe(III) oxide were used for investigation of the physiological mechanisms of Fe(III) reduction: (i) medium with free ferrihydrite, enabling direct contact between the mineral and the cells; (ii) medium with ferrihydrite incorporated into alginate beads, preventing direct contact between the mineral and the cells; and (iii) medium with ferrihydrite incorporated into alginate beads and supplemented with 0.1 mM 9,10-anthraquinone 2,6-disulfonate (AQDS), where, in the absence of direct contact between the mineral and the cells, electron transfer was possible due to the exogenous soluble mediator. AQDS is a soluble mediator of iron reduction, which is capable of electron transfer from microbial cells to the surface of an insoluble electron acceptor via a cyclic mechanism involving multiple oxidation–reduction [16]. The method of ferrihydrite incorpora-

Table 2. Ferrihydrite reduction by Fe(III)-reducing enrichment cultures of thermophilic microorganisms from Kamchatka hydrotherms

Sample (see Table 1)	Incubation temperature, °C	Fe(II) mmol/l*					
		Cultivation conditions					
		Free ferrihydrite		Ferrihydrite incorporated into alginate beads		Ferrihydrite incorporated into alginate beads + AQDS	
		acetate	lactate	acetate	lactate	acetate	lactate
1814	65	20.3	11.0	6.4	4.9	14.9	23.2
	80	–**	–	–	–	–	–
1823	80	–	–	–	–	–	–
1835	80	–	–	–	–	–	–
1850	65	28.0	14.0	3.6	1.8	20.9	22.0
	80	–	–	–	–	5.0	6.4
1860	80	25.6	24.7	–	–	2.4	3.5
1861	60	30.0	30.0	10.5	4.3	11.6	18.2
1864	65	30.0	30.0	2.5	4.2	6.8	15.6

Notes: * Final maximal Fe(II) concentration developing after 7–30 days of incubation.
** No iron reduction observed, Fe(II) concentration below 0.1 mol/l.

tion into alginate beads has been successfully used earlier for determination of the physiological strategies for Fe(III) reduction in mesophilic iron reducers [6]. Acetate, one of the major metabolites of anaerobic decomposition of organic matter, and lactate, a common fermentation product of thermophilic microorganisms, were used as potential electron donors for Fe(III) reduction. Initially, the number of enrichment cultures was 54. After three sequential transfers (5% vol/vol), 30 enrichment cultures were obtained where change in ferrihydrite color from light-brown to dark-brown, gray or black (indicating formation of ferrous minerals) was visible to the naked eye. Among these enrichments, 10 reduced ferrihydrite with direct contact between the mineral and the cells and 20 without direct contact. Among the latter, ferrihydrite reduction in 12 enrichments occurred in the presence of AQDS (Table 2). The black sediment formed in enrichments with free ferrihydrite possessed magnetic properties. Measurement of the final Fe(II) concentration after 7–30 days of incubation revealed the highest Fe(II) content in the cultures with free ferrihydrite. No enrichment cultures were obtained, which reduced similar amounts of Fe(III) via formation of endogenous mediators. In the absence of direct contact between the cells and ferrihydrite, addition of AQDS stimulated iron reduction significantly.

General characterization of the phylogenetic composition of enrichment cultures. Total DNA was isolated from 22 enrichments with taxon-specific primers for the domains *Bacteria* and *Archaea*, 16S rRNA gene fragments were amplified and separated by DGGE, and 16S rRNA gene sequences for individual bands were determined (Table 3). For further analysis, 86 nucleotide sequences were subdivided into three groups.

(i) Phylotypes exhibiting at least 94% similarity to the sequences of known cultured microbial genera and considered here as members of these genera. The similarity level of 93–94% is the threshold accepted presently for description of new genera of prokaryotes.

(ii) Phylotypes exhibiting 90–92% similarity to the sequences of cultured microorganisms.

(iii) Phylotypes exhibiting less than 90% similarity to the sequences of cultured microorganisms and most closely related to uncultured microorganisms. Phylogenetic analysis revealed that most of the sequences belonged to anaerobic thermophilic bacteria. Among these phylotypes, 81 may be assigned to 18 genera of described cultured bacteria, while 15 were most closely related to uncultured microorganisms (Table 4). Most bacterial phylotypes were found to belong to the phylum *Firmicutes* (61 sequence, 76%). Members of the following phyla were found (the numerals in parentheses indicate the number of sequences): *Aquificae* (1), *Bacteroidetes* (1), *Nitrospirae* (1), *Planctomycetes* (3), *Spirochaetes* (1), *Synergistetes* (2), and *Thermotogae* (10). The most common

Table 3. Phylogenetic composition of Fe(III)-reducing enrichment cultures of thermophilic microorganisms

Fe(III) availability	Electron donor	Designation and GenBank accession no.	Closest relative, GenBank	Similarity, %
Sample 1861, incubation *t*, 60°C				
Ferrihydrite	Acetate	61_1_F_Ac (HM003700)	*Thermoanaerovibrio* sp. strain R101 (FN556061)	95
		61_2_F_Àñ (ÍÌ003701)	*Thermotoga lettingae* (NR_027542)	96
		61_3_F_Ac (HM003702)	Uncultured bacterium clone WC49 (GQ461703)	97
		61_4_F_Ac (HM003703)	Uncultured bacterium clone DTB120 (EF205540)	89
		61_5_F_Ac (HM003704)	Uncultured bacterium clone 071020-ONK-PVA2-6 (FJ037668)	94
		61_6_F_Àñ (ÍÌ003705)	*Moorella glycerini* (NR_029198)	97
		61_7_F_Àñ (ÍÌ003706)	*Thermincola carboxydiphila* (AY603000)	92
	Lactate	61_1_F_L (HM003707)	*Thermoanaerovibrio acidaminovorans* (P001818)	95
		61_2_F_L (HM003708)	*Thermotoga elfii* strain SM-2 (EU276416)	96
		61_3_F_L (HM003709)	*Moorella glycerini* (NR_029198)	97
		61_4_F_L (HM003710)	*Syntrophobotulus glycolicus* (X99706)	97
Ferrihydrite in alginate beads	Acetate	61_1_FG_Ac (HM003726)	*Thermotoga elfii* strain SM-2 (EU276416)	97
		61_2_FG_Ac (HM003727)	Uncultured bacterium clone ELAND_40 (AY858502)	
		61_3_FG_Ac (HM003728)	Uncultured bacterium clone D2 (AY526501)	97
		61_4_FG_Ac (HM003729)	*Caloramator* sp. strain 45B (FM244718)	99
		61_5_FG_Ac (HM003730)	*Mahella australiensis* (NR_ 025758)	93
		61_6_FG_Ac (HM003731)	Uncultured bacterium clone HAW-R60-B-1249d-F (FN436200)	96
	Lactate	61_1_FG_L (HM003732)	Uncultured *Symbiobacterium* sp. clone SHBZ1659 (EU638507)	99
		61_2_FG_L (HM003733)	*Thermotoga elfii* strain SM-2 (EU276416)	92
		61_3_FG_L (HM003734)	*Moorella glycerini* (NR_029198)	98
		61_4_FG_L (HM003735)	*Thermosinus carboxydivorans* (AY519200)	95
		61_5_FG_L (HM003736)	*Caloramator coolhaasii* strain Z^T (NR_024955)	98
Ferrihydrite in alginate beads + AQDS	Acetate	61_1_FGQ_Ac (HM003759)	Uncultured *Symbiobacterium* sp. clone SHBZ1659 (EU638507)	99
		61_2_FGQ_Ac (HM003760)	Uncultured bacterium clone KCL40b_13_24 (FJ638569)	
		61_3_FGQ_Ac (HM003761)	*Thermotoga elfii* strain SM-2 (EU276416)	94
		61_4_FGQ_Ac (HM003762)	Uncultured *Gelria* sp. clone MRE50b25 (AY684072)	94
		61_5_FGQ_Ac (HM003763)	Uncultured bacterium clone TP4_N. (AB330851)	92
		61_6_FGQ_Ac (HM003764)	Uncultured bacterium clone: SwB31fl (AB266943)	96
		61_7_FGQ_Ac (HM003765)	Uncultured bacterium clone BB-LB89 (GQ844365)	89

Table 3. (Contd.)

Fe(III) availability	Electron donor	Designation and GenBank accession no.	Closest relative, GenBank	Similarity, %
		61_8_FGQ_Ac (HM003766)	Uncultured bacterium clone 23c11 (EF515355)	92
		61_9_FGQ_Ac (HM003767)	Uncultured *Symbiobacterium* sp. clone SHBZ1659 (EU638507)	99
	Lactate	61_1_FGQ_L (HM003768)	Uncultured bacterium clone KCL40b_13_24 (FJ638569)	99
		61_2_FGQ_L (HM003769)	*Pelotomaculum thermopropionicum* strain SI (AB035723)	97
		61_3_FGQ_L (HM003770)	*Gelria glutamica* strain TGO (AF321086)	95
1864, incubation *t*, 65°C				
Ferrihydrite	Acetate	64_1_F_Ac (HM003711)	*Thermovenabulum* sp. strain R270 (EU443729)	100
		64_2_F_Ac (HM003712)	*Thermolithobacter ferrireducens* strain JW/KA-2 (AF282254)	100
	Lactate	64_1_F_L (HM003713)	*Carboxydocella ferrireducens* strain 019 (EF092457)	98
Ferrihydrite in alginate beads	Acetate	64_1_FG_Ac (HM003737)	*Thermovenabulum* sp. strain R270 (EU443729)	99
		64_2_FG_Ac (HM003738)	*Carboxydothermus hydrogenoformans* strain Z-2901 (CP000141)	94
		64_3_FG_Ac (HM003739)	Uncultured *Symbiobacterium* sp. clone SHBZ1659 (EU638507)	95
		64_4_FG_Ac (HM003740)	*Thermotoga* sp. strain 1864opik (GQ292554)	98
		64_5_FG_Ac (HM003741)	Uncultured bacterium clone D21R45C97 (FM956859)	91
		64_6_FG_Ac (HM003742)	*Gelria glutamica* strain TGO (AF321086)	95
		64_7_FG_Ac (HM003743)	Unidentified *Planctomyceta* OPB17 (AF027057)	96
	Lactate	64_1_FG_L (HM003744)	*Carboxydothermus siderophilius* strain 1315 (EF542810)	98
		64_2_FG_L (HM003745)	*Carboxydocella ferrireducens* strain 019 (EF092457)	91
Ferrihydrite in alginate beads + AQDS	Acetate	64_1_FGQ_Ac (HM003771)	*Carboxydothermus siderophilus* strain 1315 (EF542810)	99
		64_2_FGQ_Ac (HM003772)	*Thermotoga* sp. strain 1864opik (GQ292554)	98
		64_3_FGQ_Ac (HM003773)	Uncultured bacterium clone D21R45C97 (FM956859)	92
		64_4_FGQ_Ac (HM003774)	*Thermolithobacter ferrireducens* strain JW/KA-2 (AF282254)	97
	Lactate	64_1_FGQ_L (HM003775)	*Fervidobacterium* sp. strain 1445t (EU851047)	98
		64_2_FGQ_L (HM003776)	*Gelria glutamica* strain TGO (AF321086)	95
		64_3_FGQ_L (HM003777)	*Morella glycerini* (NR_029198)	97
1850, incubation *t*, 65°C				
Ferrihydrite	Acetate	50_1_F_Ac (HM003692)	*Thermosulfidibacter takaii* (AB282756)	92
		50_2_F_Ac (HM003693)	*Caldanaerobacter hydrothermalis* strain K67 (EF195126)	98
		50_3_F_Ac (HM003694)	*Thermoanaerobacter tengcongensis* MB4 (AE008691)	98
		50_4_F_Ac (HM003695)	*Thermoanaerobacter uzonensis* strain JW/IW007 (FJ360437)	99

Table 3. (Contd.)

Fe(III) availability	Electron donor	Designation and GenBank accession no.	Closest relative, GenBank	Similarity, %
	Lactate	50_1_F_L (HM003696)	*Caldanaerobacter hydrothermalis* strain K67 (EF195126)	100
		50_2_F_L (HM003697)	*Carboxydothermus siderophilus* (EF542810)	97
		50_3_F_L (HM003698)	*Thermoanaerobacter uzonensis* strain JW/IW015 (FJ360438)	99
Ferrihydrite in alginate beads	Acetate	50_1_FG_Ac (HM003714)	*Thermovenabulum* sp. R270 (EU443729)	99
		50_2_FG_Ac (HM003715)	*Thermodesulfovibrio yellowstonii* DSM 11347 (CP001147)	98
		50_3_FG_Ac (HM003716)	*Moorella glycerini* (NR_029198)	96
		50_4_FG_Ac (HM003717)	Uncultured bacterium clone D2 (AY526501)	96
		50_5_FG_Ac (HM003718)	*Thermoanaerobacter uzoniensis*	97
		50_6_FG_Ac (HM003719)	*Caldicellulosiruptor saccharolyticus* DSM 8903 CP000679	97
		50_7_FG_Ac (HM003720)	Unidentified *Planctomycetales* OPB17 (AF027057)	96
	Lactate	50_1_FG_L (HM003721)	*Caldanaerobacter hydrothermalis* strain K67 (EF195126)	99
		50_2_FG_L (HM003722)	*Thermoanaerobacter tengcongensis* MB4	97
		50_3_FG_L (HM003723)	*Carboxydothermus hydrogenoformans* Z-2901	94
		50_4_FG_L (HM003724)	*Moorella glycerini* (NR_029198)	97
		50_5_FG_L (HM003725)	*Thermoanaerobacter uzonensis* strain JW/IW015	99
Ferrihydrite in alginate beads + AQDS	Acetate	50_1_FGQ_Ac (HM003746)	*Carboxydothermus siderophilus* strain 1315 (EF542810)	99
		50_2_FGQ_Ac (HM003747)	*Fervidobacterium* sp. strain 1445t (EU851047)	98
		50_3_FGQ_Ac (HM003748)	*Gelria glutamica* strain TGO (AF321086)	94
		50_4_FGQ_Ac (HM003749)	Uncultured bacterium clone D2 (AY526501)	93
		50_5_FGQ_Ac (HM003750)	*Thermoanaerobacter uzonensis* strain JW/IW015 (FJ360438)	99
		50_6_FGQ_Ac (HM003751)	*Caldicellulosiruptor saccharolyticus* DSM 8903	97
	Lactate	50_1_FGQ_L (HM003752)	*Carboxydothermus hydrogenoformans* Z-2901 (CP000141)	94
1860, incubation *t*, 80°C				
Ferrihydrite	Acetate	60_1_F_Ac (HM003699)	*Pyrobaculum aerophilum* strain IM2 (AE009441)	99
Ferrihydrite in alginate beads + AQDS	Acetate	60_1_FGQ_Ac (HM003753)	*Thermotoga* sp. strain 1864opik (GQ292554)	98
		60_2_FGQ_Ac (HM003754)	*Pyrobaculum aerophilum* strain IM2 (AE009441)	99
		60_3_FGQ_Ac (HM003755)	*Desulfurococcus kamchatkensis* strain 1221n (CP001140)	98
	Lactate	60_1_FGQ_L (HM003756)	*Anoxybacillus flavithermus* strain WK1 (CP000922)	94
		60_2_FGQ_L (HM003757)	*Pyrobaculum aerophilum* strain IM2 (AE009441)	99
		60_3_FGQ_L (HM003758)	*Thermofilum pendens* strain Hrk 5 (CP000505)	97

Table 4. Genera and uncultured groups of microorganisms revealed in enrichment cultures in the presence and absence of direct contact between the cells and ferrihydrite

Genera and uncultured microbial groups*	Capacity for Fe(III) reduction**	Free ferrihydrite	Ferrihydrite in alginate beads	Ferrihydrite in alginate beads + AQDS
Bacteria, *Firmicutes*				
Anoxybacillus	–	–	–	–/1***
Caldanaerobacter	–	1/1	–/1	–
Caldocellulosiraptor	–	–	1/–	1/–
Caloramator	–	–	1/1	–
Carboxydocella	+	–/1	–/–	–
Carboxydothermus	+	–/1	1/2	2/1
Gelria	–	–	1/–	1/2
Moorella	–	1/1	1/2	–/1
Pelotomaculum	–	–	–	–/1
Syntrophobotulus	–	1/–	–	–
Thermoanaerobacter	+	3/–	1/2	1/–
Thermoanaerovibrio	–	1/1	–	–
Thermolithobacter	+	1/–	–	–
Thermosinus	+	–	–/1	–
Thermovenabulum	+	1/–	2/–	–
Related to *Carboxydocella* (91%)	+	–	–/1	–
Related to *Mahella* (93%)	–	–	1/–	–
Related to *Thermincola* (92%)	+	1/–	–	–
Uncultured *Gelria* sp. clone MRE50b25 (*Firmicutes*)	–	–	–	1/–
Uncultured *Symbiobacterium* sp. clone SHBZ1659	–	–	1/1	2/–
Uncultured bacterium clone WC49 (*Firmicutes*)	–	1/–	–	–
Uncultured bacterium clone DTB120 (*Firmicutes*)	–	1/–	–	–
Uncultured bacterium clone ELAND_40 (*Firmicutes*)	–	–	1/–	–
Uncultured bacterium clone D2 (*Firmicutes*)	–	–	2/–	1/–
Uncultured bacterium clone TP4_N. (*Firmicutes*)	–	–	–	1/–
Uncultured bacterium partial clone D21R45C97 (*Firmicutes*)	–	–	1/–	1/–
Bacteria, *Thermotoga*				
Fervidobacterium	–	–	–	1/1
Thermotoga	+	1/1	2/–	3/–
Related to *Thermotoga* (92%)	+	–	–/1	–
Other phyla of the *Bacteria* domain				
Thermodesulfovibrio	–	–	1/–	–
Related to *Thermosulfidibacter* (92%)	–	1/–	–	–
Uncultured bacterium clone: SwB31fl (*Bacteroidetes*)	–	–	–	1/–

Table 4. (Contd.)

Genera and uncultured microbial groups*	Capacity for Fe(III) reduction**	Free ferrihydrite	Ferrihydrite in alginate beads	Ferrihydrite in alginate beads + AQDS
Uncultured bacterium clone HAW-R60-B-1249d-F (*Planctomycetes*)	–	–	1/–	–
Unidentified *Planctomycetales* OPB17 (*Planctomycetes*)	–	–	2/–	–
Uncultured bacterium clone 23cll (*Spirochaetes*)	–	–	–	1/–
Uncultured bacterium clone 071020-ONK-PVA2-6 (phylum not determined)	–	1/–	–	–
Uncultured bacterium clone KCL40b_13_24 (phylum not determined)	–	–	–	1/1
Uncultured bacterium clone BB-LB89 (phylum not determined)	–	–	–	1/–
		Archaea		
Desulfurococcus	–	–	–	1/–
Pyrobaculum	+	1/–	–	1/1
Thermofilum	–	–	–	–/1

Notes: * For uncultured microorganisms, the phyla are given in parentheses.

** Literature data.

*** The numbers of sequences obtained on acetate and lactate are separated by a slash sign (acetate/lactate).

sequences of bacterial genera belonged to *Carboxydothermus* (7) and *Thermoanaerobacter* (7). Archaeal sequences (5) belonged to the genera *Pyrobaculum, Desulfurococcus*, and *Thermofilum*, all belonging to the phylum *Crenarchaeota*. Species capable of iron reduction are known among the genera *Carboxydothermus*, *Carboxydocella, Thermincola, Thermoanaerobacter, Thermolithobacter, Thermosinus, Thermotoga*, and *Thermovenabulum* (*Bacteria*) and *Pyrobaculum* (*Archaea*). The number of sequences belonging to these genera was 54% of the total number of sequences.

Differences in phylogenetic composition depending on the electron donor. More phylotypes were detected in the enrichments when acetate was used as a potential electron donor for iron reduction, rather than lactate (58 and 28 sequences, respectively) (Tables 3, 4). Enrichment cultures with acetate contained significantly more sequences of uncultured prokaryotes than those with lactate (21 and 2, respectively). Theoretically, lactate is a more preferable substrate in terms of available energy; it may be fermented even in the absence of an external electron acceptor. It is likely that, in natural microbial communities used for enrichments, acetate is a more important intermediate of organic matter decomposition and more microorganisms are able to utilize it. It is also possible that lactate and acetate have different effects on ferrihydrite solubility and formation of soluble Fe(III)–alginate complexes. Such effects are known for a number of organic acids [17].

Differences in phylogenetic composition depending on the presence and absence of contact with the mineral. The number of phylotypes detected in enrichment cultures with free ferrihydrite, ferrihydrite in alginate granules, and ferrihydrite in alginate granules + AQDS were 22, 32, and 32, respectively. In the case of free access to ferrihydrite, the detected microorganisms mostly belonged to the genera for which capacity for Fe(III) reduction has been demonstrated. When direct contact is excluded, together with known iron reducers, organisms were detected for which the ability to reduce Fe(III) has been unknown (Table 4). Both in the absence and in the presence of contact between the cells and the minerals, members of the genera *Carboxydothermus, Thermoanaerobacter*, and *Thermotoga* were revealed most often. These organisms probably possess the most efficient mechanisms of Fe(III) reduction in the temperature range of 60–70°C. The realization of a specific strategy for Fe(III) reduction is probably characteristic of a strain or species, rather than of a genus as a whole. Members of the

genera *Carboxydothermus* and *Thermoanaerobacter* were among the first described iron reducers from terrestrial hydrotherms [5, 18]. It should be noted that, since conditions favoring the organisms able to utilize alginate and AQDS as electron donors or acceptors are potentially created in media containing these compounds, the number of ecological niches in these variants is higher than in the medium with free ferrihydrite. Moreover, alginate beads may promote biofilm development and stimulate growth of microorganisms requiring attachment to solid substrates. In sustainable enrichment cultures with alginate and Fe(III), microorganisms were revealed only remotely related to known cultured species. Thus our results may be used for development of procedures for isolation of prokariotes belonging to new taxa.

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