Magnetite Formation by the Novel Fe(III)-reducing Geothrix fermentans Strain HradG1 Isolated from a Hydrocarbon-Contaminated Sediment with Increased Magnetic Susceptibility

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Accepted author version posted online: 05 Apr 2013.Published online: 08 Aug 2013.

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Magnetite Formation by the Novel Fe(III)-reducing *Geothrix fermentans* Strain HradG1 Isolated from a Hydrocarbon-Contaminated Sediment with Increased Magnetic Susceptibility

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Surface sediments at the former military base Hradcany, Czech Republic, heavily contaminated with hydrocarbons, were remediated over years by air-sparging. The sediments show a strong magnetic enhancement at the groundwater fluctuation zone. Here we describe the isolation of a new Fe(III)-reducing and magnetite-producing bacterial strain *Geothrix fermentans* HradG1from this magnetic and redox-dynamic layer. This isolation underlines that the genus *Geothrix* is a relevant group of bacteria in hydrocarbon-contaminated environments that undergo dynamic oxic-anoxic redox fluctuations. The Fe(III)-reducing metabolic activity of these organisms potentially leads to changing magnetic soil properties that can potentially be used to identify biogeochemical hotspots.

Keywords: biodegradation, biomineralization, groundwater, iron reduction, molecular ecology

Introduction

Dissimilatory Fe(III)-reducing bacteria (DIRB) can use Fe(III), either in dissolved form or as Fe(III) (oxyhydr)oxide minerals, as electron acceptor under anoxic conditions (Konhauser et al. 2011; Lovley et al. 1987; Roden and Lovley 1993; Weber et al. 2006). These organisms play a key role in biogeochemical cycling in general, mineral transformation and degradation of organic compounds in many soils and sediments (Borch et al. 2010; Gadd 2010; Vaughan and Lloyd 2011). Due to the high abundance of iron in the environment and efficient Fe cycling involving both abiotic and biotic processes, iron redox transformation can support a vast microbial population of Fe(III)-reducers in soils and aquifers (Lambais et al. 2008; Lin et al. 2007; Lipson et al. 2010). The best-studied phylogenetic groups known for Fe(III) reduction are from the genera *Geobacter* and *Shewanella* (Richter et al. 2012), which can reduce Fe(III) using a variety of different organic and inorganic electron donors. Most of our current knowledge about the mechanisms and ecological importance of microbial Fe(III) reduction stems from studies focusing on these two genera although Fe(III) reduction is much more widespread in nature and is represented by many different types of bacteria (Li et al. 2011).

One of the few exceptions that were described in the literature in addition to *Geobacter* and *Shewanella* is *Geothrix fermentans* (Coates et al. 1999), which can also reduce Fe(III) under anoxic conditions. It belongs to the Acidobacteria phylum, of which only a few strains have been cultured so far including *Holophaga foetida* (Anderson et al. 2012). This group of Gram-negative bacteria consists of slowly growing, versatile oligothrophs. From the unique group *Geothrix* only one cultivated strain has been described in the literature (Coates et al. 1999), isolated from a hydrocarbon-contaminated aquifer. This strain, named *Geothrix fermentans* H-5, is able to couple the reduction of Fe(III) to the oxidation of long-chained carbon molecules such as palmitate.

One particularly interesting and unique feature of *Geothrix fermentans* strain H-5 is its capability to excrete both an Fe(III)-solubilizing chelator and several electron shuttling molecules (Bond and Lovley 2005; Nevin and Lovley 2002) including riboflavin (Mehta-Kolte and Bond 2012), showing that flavin production is not limited to the *Shewanella* group. These redox-active electron shuttles lead to extracellular electron transfer and enable the bacteria to reduce Fe(III) minerals without direct contact.

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Mehta-Kolte and Bond (2012) showed that *Geothrix fermentans* strain H-5 excretes also an electron shuttle with a relatively positive redox potential [∼0.3 V versus standard hydrogen electrode (SHE)] and requires much higher ambient redox potentials to reach its maximal rate of respiration as compared to *Geobacter* (Marsili et al. 2010). This indicates that *Geothrix* might be more competitive in high redox potential environments, such as sediments limited in electron donor or oxic-anoxic transition or fluctuation zones. Culturing studies and 16S rRNA gene sequence data indicated that *Geothrix*-like organisms are Fe(III)-reducers that are frequently abundant at high numbers especially in uranium- and hydrocarbon-contaminated soils (Abed et al. 2002; Rooney-Varga et al. 1999; Zachara et al. 2004).

The presence of Fe(III)-reducing *Geothrix* strains at hydrocarbon-contaminated sites is particularly relevant since it was noticed that during remediation of such sites, the mixed-valent iron mineral magnetite (Fe_3O_4) was formed (Rijal et al. 2010). Magnetite, which can be used as biomarker for example in the case of magnetotactic bacteria (Jimenez-Lopez et al. 2010), is a strong ferrimagnetic mineral, which can indirectly be traced by measuring magnetic susceptibility (MS) (Machel and Burton 1991; Maher 2009). The connection between hydrocarbon contaminations and increased MS might be due to microbial activity. It was suggested that Fe(III)-reducing and Fe(II)-oxidizing bacteria indigenous at such sites can cycle the bioavailable iron and subsequently form magnetic iron minerals including magnetite, thus increasing MS (Porsch et al. 2010). Therefore, measuring MS at such contaminated sites was suggested to facilitate the localization of highly contaminated areas and biogeochemical hotspots or even to provide a detailed spatial picture of the extent of contamination (Perez-Perez et al. 2011; Rijal et al. 2012). Whether other *Geothrix* strains besides the one isolated and described before (Coates et al. 1999) are capable of both hydrocarbon degradation and Fe(III) reduction, and whether they are able to produce magnetite, is currently unknown.

The goal of the present study therefore was to isolate a representative Fe(III)-reducing strain from a hydrocarbon-contaminated redox-dynamic field site where increased MS was observed. Furthermore, we intended to determine whether the isolated strain can form magnetite during Fe(III) reduction and thus might explain the increased MS observed. Finally, we characterized the novel isolated *Geothrix fermentans* strain HradG1 to compare it to the only other cultivated *Geothrix fermentans* strain and facilitate further studies with this environmentally relevant group of bacteria.

Material and Methods

Field Site

The present study was performed with sediment from the former military base Hradcany (50°37′14″ N/14°44′17″ E), situated about 100 km northeast of Prague, Czech Republic. This site was heavily contaminated with petroleum hydrocarbons composed mainly of jet fuel, diesel and gasoline with an estimated total volume of 7,100 tons (Machackova et al. 2012). Long-term remediation was applied from 1997 until 2008 in-

cluding air-sparging and nutrient application, resulting in an enhanced natural degradation of the contaminants by the indigenous bacterial community (Machackova et al. 2008). A side effect observed as a consequence of the air-sparging was a fluctuation of the groundwater table leading to periodically changing oxic-anoxic redox zones. At a stage where the unsaturated zone was considered as cleaned-up, the groundwater was still heavily contaminated (personal communication with the company performing the remediation). Hydrocarbons were probably transported upwards with the rising water table, thus acting as electron donor for microorganisms (Leahy and Colwell 1990). At this groundwater fluctuation zone (GFZ) an increased magnetic susceptibility was measured and suggested to be a consequence of bacterial activity (Rijal et al. 2010).

Isolation of a New Fe(III)-Reducing Microorganism

Strain HradG1 was isolated from Hradcany sediment collected via drilling in January 2011 (Rijal et al. 2010). The sediment was taken from sampling site S1 near groundwater well GS47 (Rijal et al. 2010) from a depth of 2.40 m. One g of wet soil was suspended in 9 ml of anoxic bicarbonate-buffered freshwater mineral media (Hegler et al. 2008) and amended with 5 mM ferrihydrite and a mixture of 5 mM lactate and 5 mM acetate as electron donors for enrichment of Fe(III)-reducers. The enrichment culture was incubated for several weeks at 20°C under anoxic conditions. Ferrihydrite as Fe(III) source was synthesized according to the protocol given by Raven et al. (1998), washed four times with MilliPore water, deoxygenated and autoclaved.

After complete reduction of the added ferrihydrite (after approx. 12 weeks), enrichment cultures were transferred with 10% inoculum into fresh culture media until reduction rates were similar in subsequent cultures. Morphological diversity of the microbial cells in the enrichment culture was monitored in each transfer by fluorescence microscopy after staining of the cells using Live/Dead stain (Invitrogen, Carlsbad, CA). After 12 successive transfers of the enrichment, a dilution series up to 10^{-6} was prepared (Straub et al. 2005). The highest dilution that showed complete Fe(III) reduction contained one dominating type of morphology and a 16S rRNA gene clone library was constructed. After confirming purity, strain HradG1 was further characterized.

Cultivation of Strain HradG1 and Use of Various Electron Acceptors and Donors

Serum bottles (58 mL vol) were washed with 1 M HCl and distilled water prior to sterilization by autoclaving. 25 mL of filtered freshwater medium (Hohmann et al. 2010) were filled anoxically into each bottle [headspace N_2/CO_2 (90/10, v/v)] and bottles were sealed with butyl stoppers and crimped. Bottles were amended with anoxic and sterile solutions of electron donors and acceptors and inoculated with 10% of a HradG1 culture grown fermentativly on 10 mM fumarate. All cultures were incubated at 28°C in the dark. Standard cultivation of strain HradG1 was in LML-medium (described by Myers and Myers 1994) at pH 7.0 with 10 mM fumarate

and 5 mM ferrihydrite as electron acceptor or fermentativly with 10 mM fumarate with no additional electron donor.

Acetate, lactate, fumarate, nitrate, butyrate, nitrite, formiate and succinate were added from stock solutions prepared from sodium salts. H_2 was added directly to the headspace of a sealed and crimped serum bottles. Petroleum was purchased in analysis quality from Merck (Darmstadt, Germany). Palmitate and petroleum were first sorbed to Amberlite XAD7 resin prior to addition to the medium to allow a slow supply into the growth medium by desorption from the XAD resin as described by Morasch et al. (2001). LB (lysogeny broth) medium contained 5 g/L yeast extract, 10 g/L peptones and 10 g/L NaCl. Growth under microoxic conditions was tested in gradient tubes (technique described by Emerson and Moyer 1997) with and without Fe(II)-source. High purity low-melt agarose was used for preparation of the agar gradient tubes.

Microscopy

For Scanning Electron Microscopy (SEM) cells from iron-free and iron-containing cultures were fixed with 2.5% glutaraldehyde at 4°C overnight (Schadler et al. 2008). The samples applied to poly-l-lysine coated glass cover-slips, washed with PBS and successively dehydrated using a series of ethanol dilutions (30%, 70%, 95%, 2 × 100% dried on molecular sieve). After critical point drying in CO_2 using a Polaron 3000 critical point dryer, the samples were mounted on SEM-stubs using conductive carbon pads. The samples were sputter-coated with 8 nm platinum and examined with a LEO Modell 1450 VP at 5 kV.

For confocal laser scanning microscopy (CLSM) wet samples were stained with Syto9 (Invitrogen, Carlsbad, CA), targeting DNA, and with a WGA Alexa Fluor 555 (WGA-A555) conjugate, targeting extracellular polymeric substances (EPS) (Lawrence et al. 2007) (Invitrogen, Carlsbad, CA), mounted on a glass slide and examined in sequential mode with a CLSM (SPE, Leica, Germany). Samples were excited using 488 nm (Syto9) and 561 nm (WGA-A555) lasers. Detection wavelength ranges were: syto9 492–520 nm, WGA-A555 575–620 nm and reflection 482–492 nm. Blind deconvolution (AutoQuant[TM] as part of the Leica software package LAS AF) was applied to the datasets to reduce background noise and to achieve the optimum spatial resolution. The 2D images represent maximum-intensity projections of the 3D datasets.

16S rRNA Gene Analysis

Total DNA was extracted from 2.5 ml of a ferrihydrite-acetate/lactate-grown culture of strain HradG1 with the UltraClean[TM]Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Almost full-length 16S rRNA genes were amplified from the DNA extracts with general bacterial 16S rRNA gene primers GM3F (3′-AGAGTTTG–ATCMTGGCTCAG-5′) (Muyzer et al. 1995) and 1392R (3′-ACGGGCGGTGTGTRC-5′) (Lane 1991). Reaction mixtures contained 1× PCR buffer with 1.5 mM of $MgCl_2$ final concentration (Promega, Madison, WI), 200 μM dNTP mix (New England Biolabs, Ipswich, MA), 200 nM of each primer, 1.25 U Taq DNA-Polymerase (Promega, Madison, WI) and

10 ng /μL of DNA extract as a template in a total volume of 25 μL. The following thermocycling program was used: preheating to 70°C after which the template was added; initial denaturation at 95°C for 5 min; 25 cycles of denaturing (95°C for 1 min), annealing (44°C for 1 min) and elongation (72°C for 3 min) and a final elongation at 72°C for 10 min. For clone library construction, products of PCR reactions were combined and purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI).

PCR products were ligated in the pDRIVE cloning vector (QIAGEN, Hilden, Germany) and transformed into *Escherichia coli* (DH5α) electro-competent cells. The vector was isolated using the QIAprep kit (QIAGEN, Hilden, Germany) and sequenced with M13 forward and reverse primers by GATC Biotech (Konstanz, Germany). For comparative phylogenetic analysis, sequences from eight 16S rRNA gene clones of strain HradG1 were assembled and trimmed using Geneious version 5.6.5 (Biomatters, http://www.geneious.com). Almost full-length sequences were aligned online using the SINA aligner (Pruesse et al. 2012) from the SILVA rRNA database project (Pruesse et al. 2007). Sequences were analyzed with the ARB software package (version 5.2) (Ludwig et al. 2004) as recommended by Peplies et al. (2008) using an updated version of the SILVA SSURef 111 non-redundant database (all sequences classified as *Geothrix* spp. in the SILVA SSUParc 111 database were added) (Pruesse et al. 2007).

The alignment was manually refined taking into account the secondary structure information of the 16S rRNA gene. Trees were constructed from 163 almost full-length sequences using neighbor joining, maximum parsimony, and maximum likelihood algorithms implemented in ARB and a 50% positional conservation filter created for the phylum *Acidobacteria*. Partial sequences were added to the tree using the ARB parsimony tool. A multifurcation was introduced where the tree topology could not be unambiguously resolved based on the different treeing methods and the underlying data set. For better clarity, only a selected subset of the sequences used for treeing is shown in the figure (Figure 1).

Analytical Techniques

For quantification of Fe(II) and Fe(III), 100 μL of culture suspension were withdrawn anoxically with a syringe and dissolved in 900 μL of 0.5 M HCl for 1 h at room temperature. After Fe mineral dissolution, dissolved Fe(II) and Fe(III) were quantified by the ferrozine assay (Kappler and Brune 2002; Stookey 1970). Total Fe was determined by reducing the sample with hydroxylamine hydrochloride (10% w/v in 1M HCl) prior to the addition of the ferrozine reagent. Fe(III) was calculated by subtracting the amount of Fe(II) from the amount of total Fe. The ferrozine-Fe(II) complex was quantified using a microtiter plate reader at 562 nm (FlashScan 550, Analytik Jena, Germany). All iron measurements with the ferrozine assay were carried out in triplicates. For quantification of cell growth as a function of temperature, optical density (OD) was quantified at 660 nm (SPEKOL 1300, Analytik Jena, Germany) at four different temperatures varying between 13 and 37°C.

Mineral identity was examined with a μ-XRD-device (Bruker D8 Discover X-ray diffraction instrument, Bruker AXS GmbH, Germany) equipped with a Co K_α X-ray tube and operating at 30 kV/30 mA. The EVA$^{®}$ 10.0.1.0 software was used to identify the containing mineral phases using the PDF-database licensed by ICDD (International Centre for Diffraction Data). For measuring magnetite production over time, bulk magnetic susceptibility (κ) was measured with a KLY-3 Kappabridge (AGICO, Czech republic) as described by Porsch et al. (2010). Total flavins were quantified with a spectrofluorometer (HORIBA FluoroMax$^{®}$-4) at an excitation wavelength of 440 nm and an emission wavelength of 525 nm. Anoxic samples were centrifuged (10 min, 8000 rpm), the supernatant filtered (0.22 μm, cellulose acetate, Fisher Scientific, Germany) and exposed to oxygen (air) for several hours prior to measurement. Riboflavin (Fluka, St. Louis, MO) standards were prepared at concentrations ranging from 0.001 to 5 μM. Total flavin concentrations were calculated from a calibration curve.

Nucleotide Sequence Accession Numbers

16S rRNA gene sequences from *Geothrix fermentans* strain HradG1determined in this study have been deposited in GenBank under accession numbers HF559174-HF559181.

Results

Isolation and Phylogeny of Strain HradG1

The hydrocarbon-contaminated field site in Hradcany underwent fluctuating groundwater levels and oxic-anoxic redox cycles during remediation leading to a zone of enhanced magnetic susceptibility. Incubation of sediment from this zone in growth medium with ferrihydrite and a mixture of lactate/acetate as electron donor and thirteen successive transfers (10% inoculum) including one dilution series up to 10^{-6} led to the isolation of a novel Fe(III)-reducing strain. Comparative sequence analysis of the 16S rRNA gene indicated that strain HradG1 belongs to the species *Geothrix fermentans*, in the genus *Geothrix* within the *Acidobacteria* phylum. The closest cultivated relative is *Geothrix fermentans* strain H-5 isolated by Coates and co-workers from a hydrocarbon-contaminated aquifer (Coates et al. 1999).

Sequence identity is 98% to *Geothrix fermentans* strain H-5 and 95% to *Holophaga foetida*. Sequencing of eight clones containing the 16S rRNA gene of the isolate revealed micro-heterogeneities of the 16S rRNA in seven of this eight clones. Single nucleotide substitutions were found at 1–3 different positions of the 16S rRNA gene resulting in a similarity between all clones of >99%. For phylogenetic tree calculations all seven unique sequences were used but only one representative sequence is shown in the tree (Figure 1).

Cell Morphology of Strain HradG1

Strain HradG1 generally showed smooth, non-motile, non-spore-forming cells without flagella. Cultures appeared white or rose growing fermentatively. Cell morphology of strain HradG1 changed significantly with culture conditions and age. Cells in cultures grown fermentatively with fumarate or with lactate/fumarate were non-motile rods (length of 1–2 μm and a width of 0.3 μm), appearing as single cells or short chains of two or three cells (Figure 2). Cells grown on ferrihydrite/lactate, nitrate/lactate or aged cultures grown fermentatively were thin and formed hair-like chains of cells, several μm in length and approx. 0.1 μm width, which clumped together forming biofilm-like structures and disintegrated again after a few days. Using a scanning electron microscope we observed that strain HradG1 forms large cell-mineral aggregates when the poorly soluble Fe(III) mineral ferrihydrite was present (Figures 2 A,B). To exclude artifacts from sample drying (Dohnalkova et al. 2011), we examined samples in their original, hydrated stage with a confocal laser scanning microscope. Fluorescent labeling of *Geothrix fermentans* strain HradG1 grown on 7 mM ferrihydrite and 10 mM glucose showed again cell-iron-mineral aggregates (Figures 2 C,D), and in particular a significant amount of exopolysaccharides (EPS) associated with the cells and minerals.

Metabolic Versatility of Strain HradG1

To determine the metabolic versatility of strain HradG1, we tested a series of different simple organic acids but also fatty acids and a hydrocarbon mixture as electron donor in combination with different electron acceptors (Table 1). We found that strain HradG1 can reduce solid-phase Fe(III) [ferrihydrite, $Fe(OH)_3$] with a variety of organic acids including lactate, fumarate, succinate, citrate, and also glucose and glycerol as electron donor. With acetate as electron donor, strain HradG1 was not able to completely reduce 7 mM of ferrihydrite and stopped at ~20%. Fastest reduction of 7 mM ferrihydrite was observed with 10 mM fumarate as electron donor within 16 days, whereas with other electron donors, such as lactate, ferrihydrite reduction was slow and, depending on the type of electron donor, required up to a couple of weeks to months for complete Fe(III) reduction. A variety of other electron donors including butyrate, yeast extract, H_2, proline, ethanol and formiate were tested but found not to be utilized by strain HradG1 with ferrihydrite as electron acceptor (Table 1). Although isolated from a hydrocarbon-contaminated field site, strain HradG1 was not able to couple oxidation of palmitate and petroleum to ferrihydrite reduction even during incubations of several weeks (Table 1).

Strain HradG1 can also grow fermentativly (without additional electron acceptor) using fumarate or citrate. In addition to the Fe(III) mineral ferrihydrite, strain HradG1 was able to use alternative electron acceptors, such as the dissolved Fe(III)-complexes Fe(III)-citrate and Fe(III)-NTA, with fumarate as electron donor. With lactate as electron donor strain HradG1 could use nitrate and fumarate but not Mn(IV) [added as Mn(IV) oxide] or nitrite as electron acceptor. There was no growth under oxic conditions in LB-medium or on LB-plates. However, strain HradG1 was able to grow under microoxic conditions in gradient tubes [technique described by Emerson and Moyer (1997)] with and without
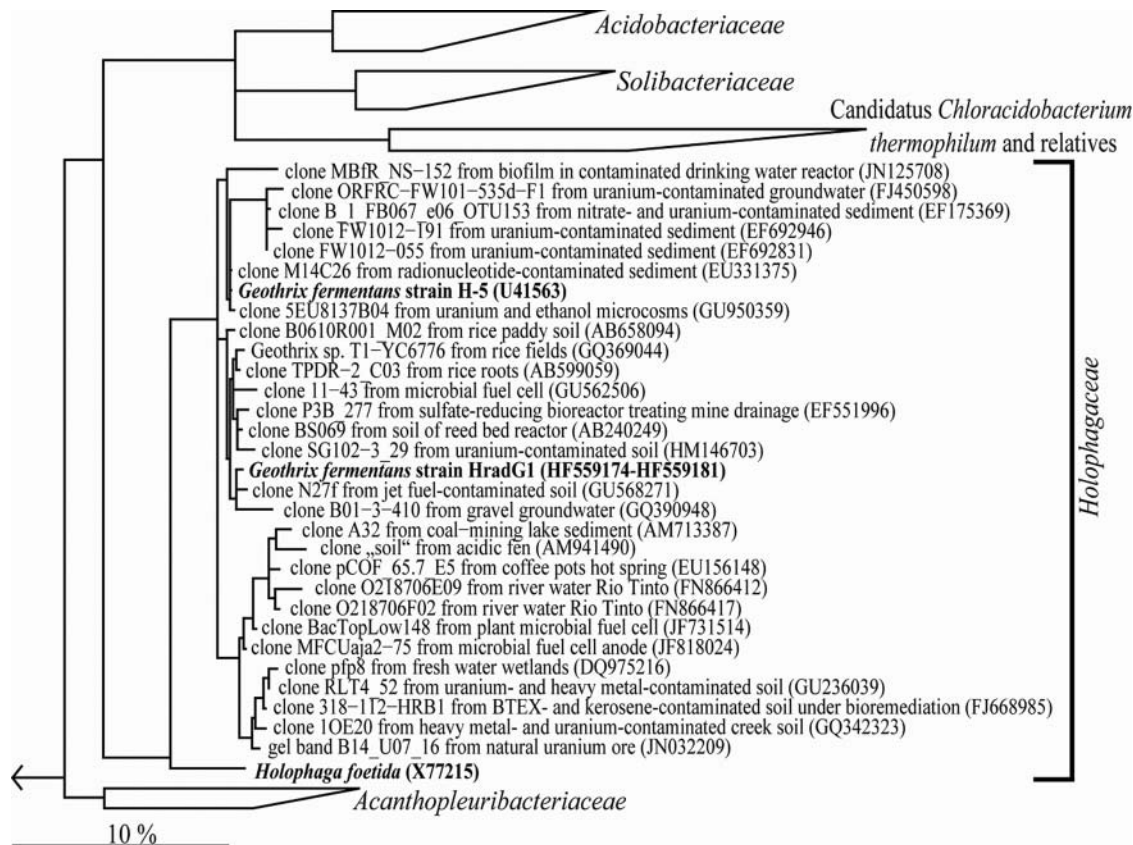
Fig. 1. Phylogenetic affiliation of *Geothrix fermentans* strain HradG1 to selected sequences of the phylum *Acidobacteria* based on comparative 16S rRNA gene analysis. Branching orders that were not supported in all calculation methods are shown as multifurcations. Cultivated species are highlighted in bold. The bar represents 10% estimated phylogenetic divergence.

Fe(II)-source probably using in both cases the agarose as electron donor but not oxidizing the Fe(II). Strain HradG1 fermented 10 mM fumarate fastest at temperatures between 20 and 38°C with optimal growth at 28°C and at circumneutral pH (data not shown). Fastest growth and iron reduction was observed in LML medium. Although fermentative growth of strain HradG1 was also possible in minimal medium [prepared after the recipe provided by Mehta-Kolte and Bond (2012)], there was only incomplete Fe(III) reduction using ferrihydrite in this medium.

Fe(III) Reduction and Magnetite Formation by Strain HradG1

The sediment collected from the Hradcany field site used to isolate strain HradG1, showed increased magnetic susceptibility (MS) values suggesting the presence of magnetite or other strong magnetic minerals (Rijal et al. 2010). We identified the minerals formed by strain HradG1 during Fe(III) reduction and found that strain HradG1 reduced the poorly crystalline Fe(III) mineral ferrihydrite [$Fe(OH)_3$] with 10 mM fumarate almost completely within 15 days (Figure 3). Measuring MS over time in ferrihydrite-fumarate cultures showed increasing MS indicating magnetite formation (Figure 4A). After a slow increase in the first nine days the major increase in MS is rather rapid between day 9 and day 12 and levels off after day

16. XRD analysis of the mineral product after 19 days showed diffraction reflexes indicative for magnetite (Figure 4B). Strain HradG1 is also able to reduce dissolved Fe(III)-citrate with a significantly faster rate than ferrihydrite (Figure 3). Following up on a previous study by Mehta-Kolte and Bond (2012) who demonstrated flavin production for strain H-5, we analyzed whether strain HradG1 also produces and releases flavins during iron reduction in minimal media. Although slow iron reduction was observed in minimal media using 7 mM ferrihydrite and 10 mM fumarate, strain HradG1 produced no flavins under these conditions (data not shown).

Discussion

The isolated strain HradG1 belongs to the genus *Geothrix*, which until now contained only one cultivated member. *Geothrix*-related 16S rRNA gene sequences have been found in many hydrocarbon- and uranium-contaminated soils and aquifers worldwide. However, its ecological relevance cannot be fully evaluated due to a lack of a variety of isolated cultures available for physiological comparison, which is required to fully understand its importance in the environment. *Geothrix fermentans* strain HradG1 is the second cultivated strain of this species and as its closest relative strain H-5 (Coates et al. 1999) it also is capable of Fe(III) reduction and utilization
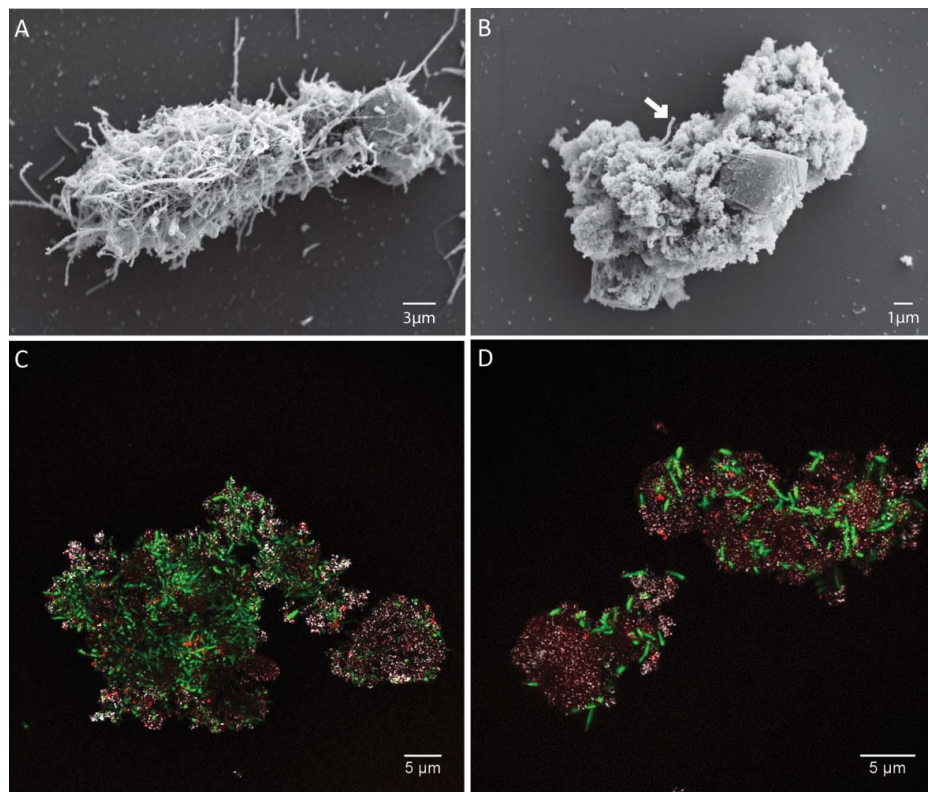
Fig. 2. Scanning electron micrographs of the isolated strain *Geothrix fermentans* HradG1 grown with 10 mM nitrate and 20 mM lactate (A) or 7 mM ferrihydrite and 10 mM fumarate (B). The white arrow in (B) marks a bacterial cell of strain HradG1 associated with iron minerals. CLSM images of *Geothrix fermentans* strain HradG1 grown with 7 mM ferrihydrite and 10 mM glucose (C & D). Green fluorescence (Syto9) represents cells, red (WGA-A555) exopolysaccharides and white is the reflection of minerals.

of a variety of organic acids. However, strain HradG1 shows some phylogenetic and physiological differences compared to the existing strain H-5. The results presented in this study indicate that this new species may be an important member of the bacterial community in hydrocarbon-contaminated environments.

Comparison of Strain HradG1 to **Geothrix Fermentans** *H-5* and other *Acidobacteria*

Analysis of the 16S rRNA genes of strain HradG1 showed 98% similarity to strain *Geothrix fermentans* H-5 (Coates et al. 1999). The obtained clones of strain HradG1 contained several single nucleotide exchanges at different positions of the
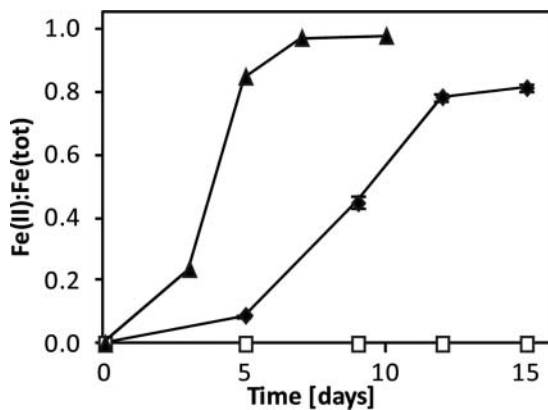
Fig. 3. Fe(III) reduction over time by *Geothrix fermentans* strain HradG1 in cultures amended with 7 mM of the poorly soluble Fe(III) mineral ferrihydrite and 10 mM fumarate (♦), with 7 mM dissolved Fe(III)-citrate and 10 mM fumarate (▲) or a sterile control (□). The data represent the 0.5 M HCl extractable iron fraction. Error bars indicate the range of concentrations of two parallels. The absence of error bars indicates that the error was smaller than the symbol size.

Table 1. Substrates and electron acceptors tested for growth of *Geothrix fermentans* strain HradG1

Electron acceptor: (concentration in mM)	Electron donors utilized by strain HradG1 (concentration in mM)	Electron donors not utilized by strain HradG1 (concentration in mM)
Ferrihydrite (7)	Succinate (10)	Butyrate (10)
	Lactate (10)	Palmitate (1)
	Acetate[a] (10)	Ethanol (10)
	Fumarate (10)	Proline (10)
	Glucose (10)	Formiate (10)
	Glycerol (10)	Petroleum (2 μl/ml)
	Citrate (10)	Yeast extract (1 g/L)
		H_2 (0.45 mol/L in headspace)

Electron donor: (concentration in mM)	Electron acceptors utilized by strain HradG1 (concentration in mM)	Electron acceptors not utilized by strain HradG1 (concentration in mM)
Fumarate (10)	Ferrihydrite (7)	
	Fe(III)-Citrate (7)	
	Fe(III)-NTA[a] (7)	
Lactate (20)	Nitrate (10)	Mn(IV)O$_2$ (5)
	Fumarate (10)	Nitrite (10)

Fermentation:	Compounds utilized by strain HradG1 (concentration in mM)	
	Fumarate (10 mM)	
	Citrate (10 mM)	

Other growth:		
	O$_2$[b]	O$_2$[c]

[a]Only incomplete reduction (∼20%) after at least 30 days of incubation.
[b]Grown microaerophilicaly in gradient tubes described by Emerson and Moyer (1997) without iron(II) source.
[c]Tested in liquid LB media and LB-plates.
All cultures were incubated in LML medium at 28°C in the dark for at least 30 days. If not stated otherwise the extent of reduction of the iron(III) source was 80% or higher.

16S rRNA gene. The microheterogeneities in the 16S rRNA sequences of HradG1 may indicate the presence of several 16S rRNA operons in the genome. Divergent rRNA genes have been shown to be expressed under different environmental or culture conditions (e.g., temperature, growth rate), most likely as an adaptation to optimize ribosome functioning and improve bacterial niche fitness (Jensen et al. 2009; Lopez-Lopez et al. 2007; Rudner et al. 1993). Alternatively, Taq DNA polymerase errors may have contributed to an artificial sequence divergence. Acinas et al. (2005) recommend to report sequence
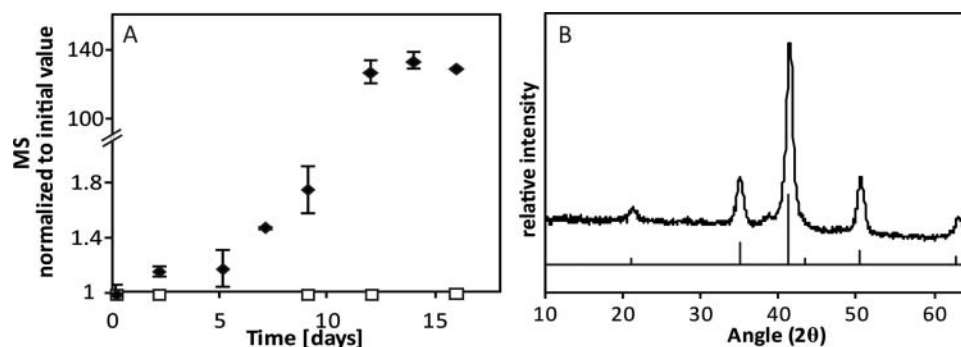
Fig. 4. A) Change in magnetic susceptibility over time in cultures of *Geothrix fermentans* strain HradG1 growing with 7 mM ferrihydrite and 10 mM fumarate (♦) or a sterile control (□). Error bars indicate standard deviation calculated from three parallels. Please note the changing MS values on the y-axis after 10 days. B) X-ray diffractogram of the mineral product after 19 days of strain HradG1 growing with 7 mM ferrihydrite and 10 mM fumarate. For comparison the specific reflections of magnetite are shown.

diversity at a similarity cutoff of 99%, at which all 16S rRNA gene variants obtained in this study would be merged into one ribotype. In previous studies, both Korotkevych et al. (2011) and Kabelitz et al. (2009) found *Geothrix fermentans*-related sequences in soil from the Hradcany field site, which are very similar (99.5% and 97.1%) to the sequence of our isolated *Geothrix fermentans* strain (see phylogenetic tree in Figure 1: acc. numbers GU568271 and FJ668985).

The finding of many *Geothrix*-like sequences at different field sites (see phylogenetic tree in Figure 1) indicates that these bacteria may play a role in the carbon- and iron-cycle of hydrocarbon- and uranium-contaminated environments. The isolation of the first *Geothrix* strain as Fe(III)-reducer by Coates et al. (1999), showed that Fe(III) reduction is also occurring within the *Acidobacteria*. *Geothrix fermentans* strain H-5 is one of the rare exceptions of Fe(III)-reducers known, which do not belong to the families *Geobacter* or *Shewanella*, which today still represent the best studied Fe(III)-reducing bacteria.

The phylum *Acidobacteria* is quite abundant and sometimes represents up to 50% of obtained 16S rRNA gene sequences in soils or sediments and the major groups within the *Acidobacteria* differ up to 22% in their 16S rRNA gene (Barns et al. 2007; Quaiser et al. 2003; Rawat et al. 2012). Despite this high diversity and abundance, physiological data about this group is very limited (Eichorst et al. 2011; Mannisto et al. 2012). Only a few cultured members are known, often slowly growing oligotrophs, which can utilize a variety of simple and complex organic compounds. *Holophaga foetida* can degrade aromatics anaerobically and produces volatile sulfur compounds via methyl group transfer from aromatic compounds to sulfide forming e.g., methanethiol or dimethylsulfide (Anderson et al. 2012). *Acanthopleuribacter pedis*, however, isolated by Fukunaga et al. (2008), is a marine, strictly aerobic bacterium.

Our new isolate *Geothrix fermentans* strain HradG1 is able, as his closest relative *Geothrix fermentans* strain H-5, to reduce poorly soluble and dissolved Fe(III) compounds coupled to the oxidation of different electron donors. Also similar to strain H-5, it can grow by fermentation of fumarate or citrate. However, there are also some distinct differences in growth and metabolic capabilities compared to strain H-5, e.g., strain HradG1 cannot use yeast extract alone or palmitate as electron donor. In contrast, strain HradG1 can utilize glucose and glycerol, which are not utilized by strain H-5 (Coates et al. 1999). A major metabolic difference between these two *Geothrix* strains is the ability of strain HradG1 to grow under microoxic conditions in agar tubes, probably using the agar in the solidified medium as electron source.

It has to be noted that due to the very slow growth and low substrate utilization of strain HradG1 under certain incubation conditions, we tested growth with some electron donors (glycerol, citrate, succinate) for only one or two successive transfers. Therefore, for these substrates we cannot rule out that internally stored carbon from the fermenting pre-culture supported growth as well. However we assume that carbon storage is only of minor importance (if at all), since we did not see any reduction of ferrihydrite without the addition of extra electron donor.

Strain HradG1 was shown to grow best in complex media amended with peptone and yeast extract. In defined minimal media fermentation of fumarate was possible but reduction of ferrihydrite was observed only to a lower extent and was always incomplete but could be enhanced by the addition of yeast extract and/or peptone. Based on our observations, we conclude that strain HradG1 has some unknown auxotrophic requirements. As a consequence, routine cultivation of strain HradG1 was done in the complex medium containing yeast extract. This in turn prevented us from analysis of the synthesis and release of flavins under these conditions; flavins are redox-active compounds that were found in cultures of strain H-5 functioning as electron shuttles in Fe(III)-mineral-reducing cultures (Mehta-Kolte and Bond 2012).

Since the yeast extract added to our medium already contains high amounts of flavins (Masuda et al. 2010), we had to test cultures growing with minimal media without yeast extract for the production, release and extracellular presence of flavins. However, in this minimal medium we could not detect any flavins in the culture supernatants although it has to be kept in mind that iron reduction was not optimal in this medium and therefore flavin production might have been below the detection limit. Further studies are required to determine whether *Geothrix fermentans* strain HradG1 has similar abilities as strain H-5 concerning the production of electron shuttles or iron chelators.

Ecological Advantage of Geothrix at the Field Site in Hradcany with Hydrocarbon Contamination and Fluctuating Redox Conditions

The isolation of a *Geothrix fermentans* strain from the Hradcany field site raises the question to which extent the environmental conditions at our field site are suitable or even favorable for *Geothrix*. One reason might be that *Geothrix fermentans* is able to use a diversity of organic compounds as electron donors and is therefore able to perform Fe(III) reduction under changing geochemical conditions in the sediment when complex hydrocarbons are degraded via several intermediates that are available in the sediment during the progress of hydrocarbon degradation. Another reason might be that the sediment at this site experienced air-sparging and nutrient amendment over a long time (approximately 12 years).

Air-sparging is a process where air is regularly pumped into the subsurface to i) oxygenate the sediments and stimulate aerobic hydrocarbon degradation, and ii) to enhance evaporation of volatile compounds especially within the water-saturated zone (Johnson 1998). The input of high volumes of gas during air-sparging led to a bulk water flow upwards during the time of pumping, meaning that several times a day the groundwater table changed significantly. This groundwater fluctuation led to a massive change in sediment properties and the bacterial community.

Kabelitz et al. (2009) showed an increased biomass production over the time of remediation at this exact field site in sediment samples taken approximately at the groundwater table. Sequence-based analysis showed that there was an increase of several bacterial phyla including *Acidobacteria*-like sequences after 2.5 years of remediation. At the start of the remediation in 1997 there were no *Acidobacteria* detectable, suggesting that air-sparging and/or nutrient supply promoted the growth of microorganisms belonging to the

Acidobacteria phylum. Similarly, Militon et al. (2010) could also show an increase of *Acidobacteria*-like sequences over time in an air-sparged soil mesocosm with hydrocarbon contamination. Oxygenation and changing groundwater tables due to air-sparging lead to changing oxic and anoxic zones.

This challenges microorganism to deal with alternating redox conditions. Mehta-Kolte and Bond (2012) showed recently that *Geothrix* is capable of living under changing redox conditions because it excretes an electron shuttle with a relatively high redox potential that helps to transfer electrons even under relatively oxidizing conditions. The isolation of our *Geothrix* strain HradG1 from a dynamic oxic-anoxic groundwater fluctuation zone supports their hypothesis and we conclude that in particular our *Geothrix fermentans* strain might have an advantage in air-sparged soils where oxygen is regularly pumped into the soil, leading to daily changing oxic-anoxic zones, compared to more strictly anaerobic Fe(III)-reducers such as *Geobacter*. This conclusion is in particular based on the fact that our strain HradG1 can grow under microoxic conditions in contrast to *Geothrix fermentans* strain H-5, which was described to be strictly anaerobic.

Magnetite Formation and its Potential as Indicator for Microbial Activity

In this study we could show by X-ray diffraction analysis that *Geothrix fermentans* strain HradG1 produces magnetite as the main mineral product of Fe(III) reduction. Magnetic susceptibility (MS) measurements in *Geothrix fermentans* strain HradG1 culture bottles also suggest that analyzing MS over time is a useful tool to follow magnetite formation in the cultures as suggested previously by Porsch and co-workers (2010).

Following MS over time showed that the first increase in MS is rather slow but MS increases rapidly between day 9 and 12. After reaching the highest value at day 12, MS did not increase further but slowly decreased, which can have two reasons: i) increasing magnetite particle size could lead to a lower volume specific MS and would lead to a decrease in bulk MS if particles grow over the superparamagnetic to single-domain grain-size transition (Piepenbrock et al. 2011), or ii) a further microbial reduction of magnetite to siderite may occur that would also cause a decrease of MS (Zachara et al. 2002).

High MS values can directly be linked to the formation of ferrimagnetic iron minerals such as magnetite and maghemite (Maher 1986) or in some cases also greigite (Fe_3S_4) (Dearing 1994). In the environment, these minerals can either have a geological or an anthropogenic source (Maher 2009) or they are produced by iron-metabolizing bacteria. Microbial Fe(III) reduction and the concomitant release of soluble Fe(II) can lead to the formation of magnetite and has been described for many Fe(III)-reducers in culture (Amstaetter et al. 2012; Piepenbrock et al. 2011; Zachara et al. 2002). There is also evidence that in the environment the metabolic activities of Fe(III)-reducers are an important source for changing magnetic soil properties (Gibbs-Eggar et al. 1999) because they cannot only form but also dissolve magnetite (Kostka and Nealson 1995). Magnetite formation and transformation in soils or sediments is important because biogenic magnetite can reduce inorganic contaminants, such as uranium and chromium (Crean et al. 2012; Veeramani et al. 2011), and therefore helps to detoxify and immobilize these harmful substances (Gorski et al. 2010).

The major contaminants in Hradcany are petroleum hydrocarbons, which were released over a time span of several decades. The coincident appearance of hydrocarbons and magnetite has already been noticed several years ago (Elmore et al. 1987; Machel and Burton 1991; Rijal et al. 2012).

Although geological and geochemical processes could also be important for the magnetite accumulation, bacterial activity probably plays a major role. Hydrocarbons can provide electrons for both aerobic and anaerobic bacteria (Baelum et al. 2012; Rooney-Varga et al. 1999). Fe(III) can be used as electron acceptor by some of these bacteria and form magnetite during Fe(III) reduction (Zachara et al. 2002). The Fe(II) produced, in turn, can function as electron donor for Fe(II)-oxidizing bacteria also producing magnetite (Dippon et al. 2012; Jiao et al. 2005).

At our field site in Hradcany, Rijal et al. (2010) showed that the ongoing fluctuation of the groundwater table led to an increase in magnetic parameters of the soil, probably because of an increased bacterial activity and they identified the responsible mineral as magnetite using temperature-dependent MS characteristics. Here we present the isolation of the Fe(III)-reducing strain *Geothrix fermentans* HradG1 from this field site which is capable of producing magnetite during Fe(III) reduction and may have contributed directly to the increased MS measured in the field. Our study therefore suggests that measuring magnetic susceptibility in soils or sediments is a useful tool not only to observe magnetite formation at contaminated sites but also indicates the potential activity of Fe(III)-reducing bacteria at these sites.

Description of Geothrix fermentans HradG1

Geothrix fermentans strain HradG1 was isolated from a hydrocarbon-contaminated soil at Hradcany, Czech Republic. Strain HradG1 can use Fe(III) as electron acceptor in form of dissolved Fe(III) complexes or poorly soluble Fe(III) minerals. The main mineral product of Fe(III) reduction is magnetite. Fumarate, succinate, lactate, acetate, citrate, glucose and glycerol can serve as electron donor. Alternative electron acceptors are nitrate or fumarate. Growth is possible by fermentation of fumarate or citrate or under microoxic conditions in gradient tubes using agarose as electron donor. Optimum growth was observed at 28°C and circumneutral pH. The strain HradG1 has been deposited at the DSMZ.

Acknowledgments

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