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Pond Sediment Magnetite Grains Show a Distinctive Microbial Community

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Abstract Formation of magnetite in anaerobic sediments is thought to be enhanced by the activities of iron-reducing bacteria. *Geobacter* has been implicated as playing a major role, as in culture its cells are often associated with extracellular magnetite grains. We studied the bacterial community associated with magnetite grains in sediment of a freshwater pond in South Korea. Magnetite was isolated from the sediment using a magnet. The magnetite-depleted fraction of sediment was also taken for comparison. DNA was extracted from each set of samples, followed by PCR for 16S bacterial ribosomal RNA (rRNA) gene and HiSeq sequencing. The bacterial communities of the magnetite-enriched and magnetite-depleted fractions were significantly different. The enrichment of three abundant operational taxonomic units (OTUs) suggests that they may either be dependent upon the magnetite grain environment or may be playing a role in magnetite formation. The most abundant OTU in magnetite-enriched fractions was *Geobacter*, bolstering the case that this genus is important in magnetite formation in natural systems. Other major OTUs strongly associated with the magnetite-enriched fraction, rather than the magnetite-depleted fraction, include a *Sulfuricella* and a novel member of the Betaproteobacteria. The existence of distinct bacterial communities associated with particular mineral grain types may also be an example of niche separation and coexistence in sediments and soils, which cannot usually be detected due to difficulties in separating and concentrating minerals.

Keywords *Geobacter* · *Sulfuricella* · Magnetite · Operational taxonomic units · 16S bacterial ribosomal RNA

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Introduction

Magnetite is a naturally occurring magnetic mineral that is widely distributed around the world [1, 2]. While there is no doubt that its formation follows on from iron reduction (from Fe III to Fe II) by bacteria, it has been unclear whether the next stage—combination of Fe II with more Fe III in sediment to produce magnetite—mostly occurs biotically associated with bacterial cells or abiotically without needing biological assistance [3, 4].

In recent years, a number of possible candidate bacteria for magnetite formation in sediments have been identified. When

grown in culture, these can form magnetite crystals either externally or internally to their cells [5, 6]. Of particular interest has been *Geobacter*—a common bacterium in anaerobic sediments—which reduces iron and uses the electrons to oxidize organic compounds in the sediment [7]. *Geobacter* has recently been found to form natural mutualistic "batteries" within sediment, allowing increased energy generation through oxidation activity in anaerobic sediments [8]. If magnetite is indeed produced extracellularly by *Geobacter* in natural sediment environments, it may itself facilitate this activity by conducting electrons [9]. Other magnetite formers include known examples of magnetotactic bacteria (e.g., *Magnetospirillum gryphiswaldense*), which use the magnetite grains within themselves to orient relative to the Earth's magnetic field.

However, the prevalence and importance of these various magnetite formers in actual sediments are unclear. There is a need to investigate the evidence for physical association between magnetite in sediment and bacteria which may be playing a role in its formation. Demonstrating that the bacterial taxa which are known to produce magnetite in culture are consistently abundant in association with magnetite crystals in sediment (and less abundant on non-magnetite particles in the same sediment) would strengthen the case that they are actually important in nature.

Using culture-independent methods, it may also be possible to discover novel magnetite-associated bacteria without being able to culture them. Although of course this does not in itself prove that they have the metabolic capabilities to form magnetite, finding a consistent association is the first step towards to identifying novel candidate magnetite formers.

In a more general way in bacterial ecology, being able to compare the whole bacterial community of mineral particles of magnetite, against the community in the "magnetite-depleted" sediment left behind, may go some way towards understanding how bacterial diversity is structured. Since culture-independent methods first became possible, it has been an ecological puzzle as to how tens or hundreds of thousands of species of bacteria are able to coexist in a gram of soil or sediment [10, 11]. One hypothesis is that they coexist through being partitioned between a myriad of separate niches, preventing out-competition [12, 13]. One of the many possible ways in which niches could be distinct is through specialization on different types of sediment/soil particles, due to the distinct physical or chemical properties of these particles. Magnetite is unusual in that it can readily be separated from all other mineral particles in sediment, but it could be broadly representative of many other minerals in terms of whether or not it has a distinct bacterial community. Thus, analyzing magnetite particles may provide a general glimpse into the world of bacterial ecology and the ways in which bacterial communities in general are structured.

This study was structured around testing three general hypotheses relating to the topics set out above.

1. That certain bacteria known to form magnetite in culture, such as *Geobacter*, will be especially abundant on sedimentary magnetite grains, due to their important role in magnetite formation in sediments.
2. That other previously uncultured bacterial forms will be especially abundant on sedimentary magnetite grains, inviting further investigation of their potential role as magnetite formers.
3. That there will be diverse communities of bacteria that are distinct between magnetite grains and grains of non-magnetic minerals, suggestive of a potential axis of niche differentiation and coexistence in soils and sediment.

Methods

Sampling and Redox Potential Measurement

We sampled a freshwater pond on the campus of Seoul National University, in a hilly area south of Seoul, South Korea (37°27′38.3″N, 126°57′07.5″E). The pond was formed artificially in a pre-existing upland stream course, more than 30 years ago. It is about 0.25 Ha in area, and mostly around 1 m deep, fringed by aquatic vegetation and trees, with a small stream supplying water and sand grade sediment from the granite hills above.

The sediment in the pond was sampled at 4 points, spaced in a line 1 m apart, in about 50 cm water depth. Samples were all taken on the same day in late June (early summer). At each sampling point, the upper 5 cm of sediment was cleared away, and a scoop of sediment at 5–15 cm depth was taken, using a trowel. Redox potential was measured a few weeks later during the same summer in which sampling was carried out. A portable platinum electrode connected to a pH meter was used to measure soil redox potential [14]. The measured value was standardized to a hydrogen reference electrode by adding 244 mV to each value [15]. Two measurements of redox conditions in the sediment at water depth 24 cm showed 311–310 mV vs. standard hydrogen electrode (SHE) (sediment 0–6 cm depth) and 341–347 mV vs. SHE (sediment below 6 cm) confirming at least moderately reducing conditions.

Magnetic Separation

At the laboratory, 200 g portions of each sediment sample were placed in glass beakers, which were then filled with distilled water and gently shaken, while a powerful rare earth magnet with 0.4 tesla magnetic force was placed against the edge of the beaker. Fine-grade black materials stuck to the side of the

beaker and were removed using a clean spatula. The black magnetic material removed from each beaker was placed into a 50-ml Falcon tube and again suspended by shaking in distilled water, while a magnet was placed against the side of the tube, allowing magnetic material to stick to its side. This process was repeated several times until no more black material could be obtained. The purified magnetic material obtained at the end of the process was removed for DNA extraction.

The magnetically-depleted sediment left in the original Falcon tube after several shaking phases and passes with the magnet was yellowish (in contrast with the grayish color of the original mixed sediment) and sand-to-silt grade. This was taken for DNA extraction as the "magnetically depleted" sediment fraction.

DNA Extraction, PCR Reaction, and Sequence Analysis

DNA was extracted from each magnetically-enriched sample of black material, and also extracted separately from each magnetically-depleted sample, using the Power Soil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA), following manufacturer's instructions. DNA was PCR amplified for the V3 region of bacterial 16S rRNA using universal bacterial primer, 338 F (5′-XXXXXXXX-YY-GTACTCCT ACGGGAGGCAGCAG-3′) and 533R (5′-XXXXXXXX-YY-TTACCGCGGCTGCTGGCAC-3′) while X and Y each denotes barcode sequence and adaptor sequence. PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and quantified by PicoGreen (Invitrogen) with spectrofluorometer (TBS 380, Turner Biosystems, Inc., Sunnyvale, CA, USA). The same amount (250 ng) of purified PCR product from each sample was pooled together in a tube and sent for sequencing. Paired end sequencing (2×150 bp) was conducted by a HiSeq2000 Illumina sequencing machine at Celemics (Seoul, Korea). All sequence data are available at NCBI Sequence Read Archive (SRA) with accession no. SRP049281.

Two paired sequences were assembled by using PANDAseq [16], and further sequence processing was performed following the Miseq SOP (http://www.mothur.org/wiki/MiSeq_SOP) in Mothur [17]. Sequences were classified using EzTaxon-e database as a template [18]. For operational taxonomic unit (OTU)-based analysis, distances between sequences were calculated and sequences which have over 97 % similarity were merged into an OTU. Each sample was subsampled to 5227 reads to calculate unweighted UniFrac matrix. A non-metric multidimensional scaling (NMDS) based on unweighted UniFrac distance was plotted to show difference between two proportions using PRIMER6 software. ANOSIM and MRPP with 999 permutations were performed to check the significance of the difference between magnetite-depleted and magnetite-enriched fractions using "vegan" package in R.

Structural Microstructural Characterization of the Magnetic Fraction

Additional samples of the black magnetic material were taken from the pond sediment and checked mineralogically to validate that they were indeed mostly magnetite. They were washed with alcohol several times to remove any organic material which could interfere with the crystallography. The gross structure of the magnetically-enriched fraction was then observed by X-ray diffraction (XRD, D8 advance by DAVINCI) by using $Cu_{K\alpha}$ radiation with 2θ in the range of 10–80° at scanning step of 0.02° par min. The detailed crystallographic structure and microstructure was observed by high resolution TEM (HRTEM) (JEOL-2100). The growth mode and surface morphological variations were observed by scanning ion microscope (SIM) images (CORBRA-FIB, Orsay physics). Imaging was performed at 23 pA of beam. The Focussed ion beam (FIB) was used to cut the cross section using 2.5 nA and 139 pA of beam current. Images were taken as 45° tilted against cross sectioned wall.

Quantitative PCR Analysis

Sediment from 4 point samples was taken for quantitative PCR (qPCR) analysis to measure relative abundance of bacterial 16S rRNA gene copy of the magnetically-enriched fraction, compared to the bulk sediment which had not been subject to the magnetic depletion/enrichment process. 0.5 g of sediment from each bulk and magnetic fraction was used for DNA extraction using the same DNA isolation kit described above.

To estimate the bacterial biomass, we performed qPCR using CFX96 (Bio-Rad, Hercules, CA) and SYBR Green as a detection system (Bio-Rad, USA). Each reaction in 20 μl contained the specific primer set for bacteria: 341 F(5′-CCTA CGGGAGGCAGCAG-3′)-797R(5′-GGACTACCAGGGTC TAATCCTGTT-3′) [19, 20]. The amplification followed a 3-step PCR for all targeted genes: 44 cycles with denaturation at 94 °C for 25 s, primer annealing at 64.5 °C for 25 s, and extension at 72 °C for 25 s. Two independent real-time PCR assays were performed on each soil DNA extract. The standard curves were created using 10-fold dilution series of plasmids containing the bacterial 16S rRNA gene from environmental samples.

Results

Presence and Microstructure of Magnetite in the Magnetic Black Fraction

Figure 1 shows the typical X-ray diffraction pattern of the magnetically-enriched fraction of pond sediment. The peak

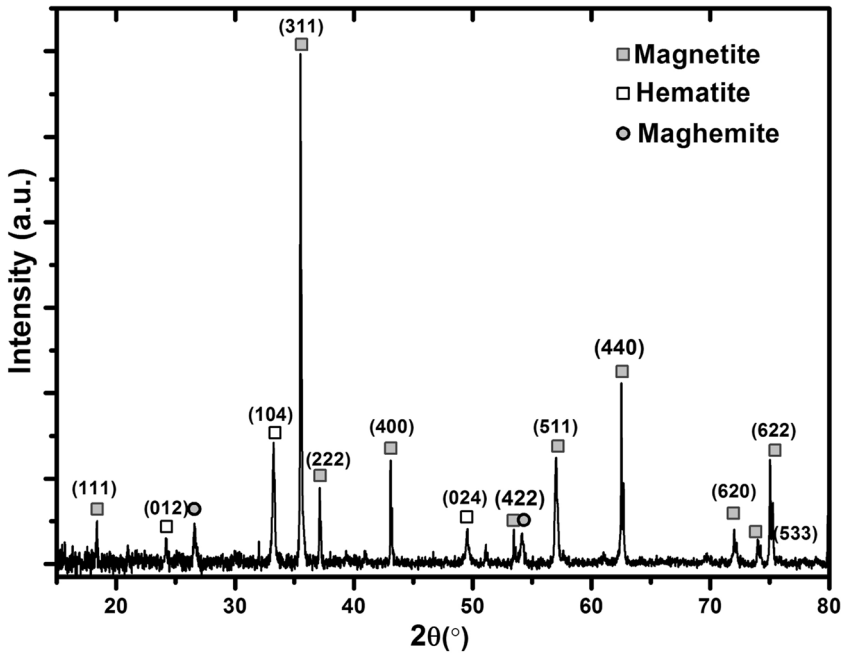

Fig. 1 Typical XRD pattern observed for pond sediment showing the presence of magnetite and hematite phase in pond sediment. A small fraction of oxidized form of magnetite and maghemite is also visible possibly due to surface oxidation of magnetite during the washing of sediment by ethanol

identities of the XRD patterns are attributed to the spinal structures of magnetite (Fe_3O_4-JCPDS-04-005-4551) and maghemite (γ Fe2O3-JCPDS-3901346) and hematite (Fe_2O_3-JCPDS-01-079-1741), a trigonal form of iron oxide. A comparative analysis of maximum intense peaks shows magnetite to be the dominant species (75 %) coexisting with hematite at substantially lower levels (25 %). A small fraction of maghemite can be attributed to the surface oxidation of the magnetite.

Furthermore, the surface microstructure of the material extracted magnetically from the sediment was characterized by scanning ion microscopy (SIM), shown in Fig. 2a. Figure 2a reveals the high occurrence of particles exhibiting spiral growth with triangular morphology and well faceted steps. The

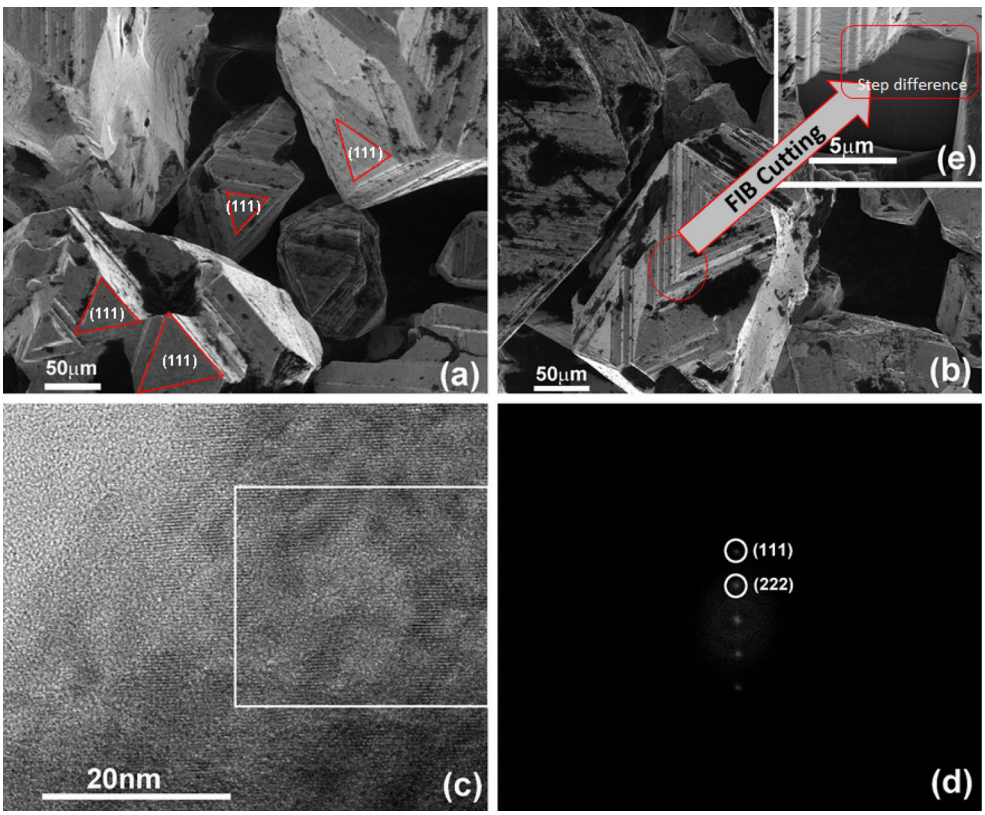

Fig. 2 Scanning ion micrographic image of sediment **a** indicating the spiral step growth along (111) crystallographic direction as suggested by the triangular morphology. **b** The *encircled section* subject to FIB cross-section cutting is shown in the inset Fig(e) indicating the presence of defects between the two steps. **c** The HRTEM micrograph showing the lattice presence typical of a crystal structure. **d** FFT of square section of **c** indicating the stacking of (111) type planes

triangular morphology is indicative of the epitaxial growth of large particles along (111) direction as in the case of magnetite polyhedron [21]. Further, in order to confirm the difference between the two spiral steps, the grain was drilled using a focused ion beam (FIB). The selected section (encircled) shown in Fig. 2b was cut by FIB, and the interior cross-sectional image (inset of Fig. 2b–e) indicates the coexistence of multi-structures between the two steps which might possibly arise from the structural defect occurring during the formation of spiral steps. Furthermore, this observation has also been confirmed by high resolution TEM (HRTEM). The bright field micrograph (Fig. 2c) of the pond sediments shows the presence of lattice fringes. The fast Fourier transform (FFT) in Fig. 2d originates from the square region of Fig. 2b. The FFT shows the presence of (111) types of crystallographic planes of magnetite with lattice parameters a=b=c=8.40 Å. Surface topography along with the crystallographic observation confirms the presence of magnetite in the pond sediment. Given the mineralogical evidence of magnetite in the samples, from this point onwards in the manuscript, we will refer to the magnetically extracted fraction as "magnetite enriched" and the non-magnetic fraction as "magnetite depleted".

Bacterial Community in Magnetite-Enriched and Magnetite-Depleted Fractions

From 8 samples, 4 being the magnetite-depleted fraction and 4 being the magnetite-enriched fraction, a total of 244,947 sequences were obtained. The number for reads ranged from 5227 to 39,587 for each sample. Rarefaction curves with the first 5000 sequences in each sample showed that the magnetite-enriched fraction had higher OTU accumulation rates than magnetite-depleted fraction (Fig. 3). Both the magnetite-depleted and magnetite-enriched fractions were dominated by Proteobacteria, together with a range of other bacterial groups broadly typical of freshwater anaerobic environments (Fig. S1). The bacterial communities of the magnetite-enriched and magnetite-depleted fractions were distinct (ANOSIM, R^2=0.854, p=0.027; MRPP, A=0.035, p=0.027) on NMDS ordination (Fig. 4). This is also evident in a heat map representing the relative abundance of top 50 OTUs (Fig. 5). The most abundant OTU in the magnetite-enriched fraction (OTU5), which belongs to genus Geobacter, accounts for 3.1 % of reads in the magnetite-enriched fraction and 0.2 % in the magnetite-depleted fraction. The next most abundant OTUs in the magnetite-enriched fraction belonged to *Sulfuricella* and to a novel member of the Betaproteobacteria, both over 20 times more abundant in the magnetite-enriched than the magnetite-depleted fraction (Table S1).

In qPCR analysis, the $\log_{10}$-transformed mean (±standard error) of 16S rRNA gene copy number per gram of soil in bulk sediment and magnetite-enriched fraction were 10.04 (±0.16) and 10.15 (±0.04) for each, showing no significant difference in bacterial cell abundances between the bulk and magnetite-enriched samples (p=1, Wilcoxon rank sum test).

Discussion

The selective abundance of three major OTUs—*Geobacteria*, *Sulfuricella*, and the unknown Betaproteobacteria OTU, in association with magnetite grains suggests that they may be dependent upon the magnetite grain environment or may be playing a role in magnetite formation.

Geobacter is well studied and of broad interest because of its potential practical applications. The ability to conduct electron by pili opened up the possibility of its usage as natural battery. Also, the ability to combine oxidation of organic matter and reduction of ferric iron lead *Geobacter* to be used in bioremediation and in synthesis of natural magnetite which

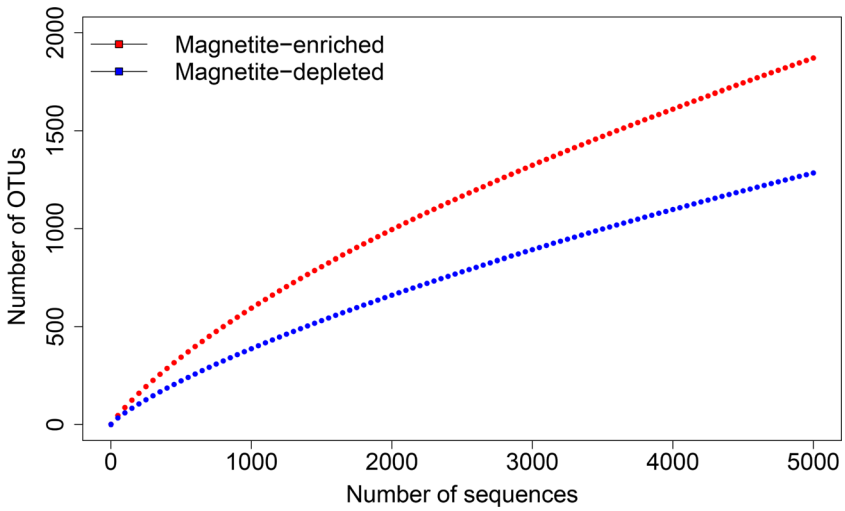

Fig. 3 Rarefaction curve comparing magnetite-enriched and magnetite-depleted samples plotted with first 5000 sequences

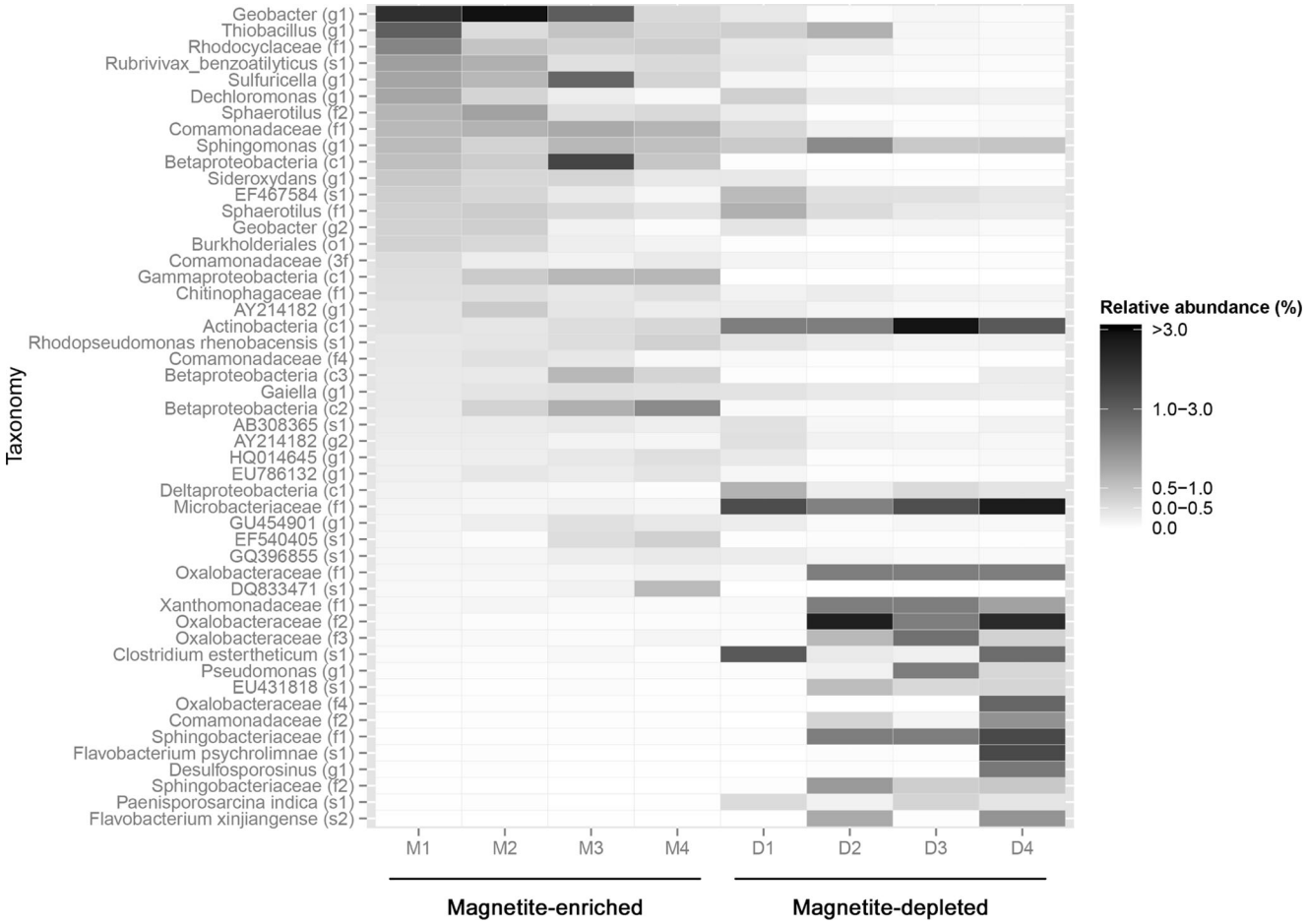

Fig. 4 Heat map showing the relative abundance of the 50 most abundant phylotypes in each sample. The *letter* inside parentheses directly next to each taxon name on Y-axis indicates the lowest classifiable taxonomic level of the sequence ("c" for class, "o" for order, "f" for family, "g" for genus, and "s" for species). The number next to the letter was used to distinguish similar phylotypes (*lower number* indicates higher relative abundance). For example, "(c1)" in "Betaproteobacteria (c1)" denotes it is the most abundant OTU classified up to class Betaproteobacteria

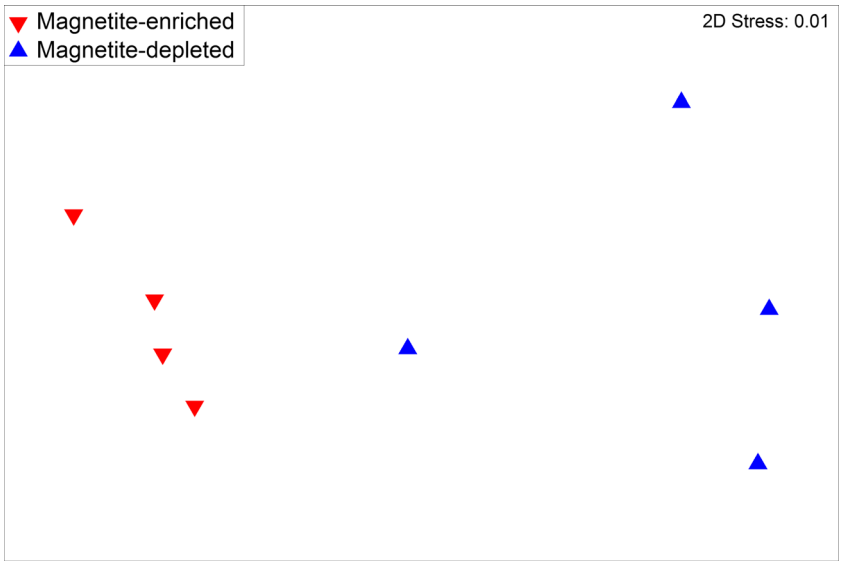

Fig. 5 Unweighted UniFrac ordination of magnetite-enriched fraction vs. magnetite-depleted fraction

is much safer than artificial magnetite for medical purposes. Although various studies have shown that *Geobacter* is able to form magnetite in culture, and other studies have shown the presence of *Geobacter* in natural habitats by using culture-independent methods, being one of the most abundant iron-reducing bacteria, this study provides the strongest evidence so far of an association between *Geobacter* and magnetite formation in natural sediments.

The other two most abundant OTUs, preferentially associated with magnetite grains—*Sulfuricella* and the unknown Betaproteobacteria OTU—may now be regarded as worthy of further investigation as magnetite formers, perhaps involved in similar electron transfers from iron to organic compounds to those carried out by *Geobacter*.

It is also interesting that such distinctively different bacterial communities are associated with the magnetite-enriched grains and magnetite-depleted sediment fractions, respectively. While magnetite is perhaps unusual in being a direct chemical product of bacterial activity and a participant in bacterial activities, it is also possible that distinctive communities partitioned by mineral type are also generally present in soil and sediment. These niche differences may help to explain coexistence of such high bacterial diversity in soils/sediments. Use of other techniques to fractionate minerals within sediments may provide other sets of distinctive niche-partitioned communities.

These findings reported here could be applied to various follow-up studies. The novel approach, partitioning sediment samples using a magnet to separate the magnetic component of the sediment from the non-magnetic, could make it easier and more effective to find new magnetite formers in culture-based studies, with potential practical applications. This could provide a more comprehensive understanding of biotic magnetite formation.

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