Characterizing of novel magnetotactic bacteria using a combination of magnetic column separation (MTB-CoSe) and *mamK*-specific primers

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Running title

Novel approaches for MTB investigation

ABSTRACT

Magnetotactic bacteria (MTB) belong to different taxonomic groups according to 16S rRNA or whole-genome phylogeny. Magnetotactic representatives of the class *Alphaproteobacteria* and the order *Magnetococcales* are the most frequently isolated MTB in environmental samples. This bias is due in part to limitations of currently

available methods to isolate MTB. Here we describe a new approach for isolation of MTB cells that does not depend on cell motility and will allow collecting bacteria both south- and north-seeking movement. We also designed a specific primer system for the gene encoding the MamK protein that effectively detects diverse MTB phylogenetic groups in any sample type. The combination of these two approaches allowed the identification of a novel MTB belonging to the family *Syntrophaceae* of the class *Deltaproteobacteria*. Moreover, we found that *Nitrospirae* bacteria predominated in the MTB fraction of a sample taken from Lake Beloe Bordukovskoe near Moscow, Russia. We describe the novel dominant *Nitrospirae* bacterium '*Candidatus* Magnetomonas plexicatena' and propose its taxonomic name.

IMPORTANCE

Among magnetotactic bacteria (MTB), the members of phyla *Proteobacteria*, *Nitrospirae* and '*Ca*. Omnitrophica' have been studied extensively using the existing approaches. However, in recent years, analyses of the metagenomic databases have revealed the presence of MTB in phylogenetic groups, which had not been previously detected using standard approaches. This finding indicates that the biodiversity of MTB is much broader than is currently known. The difficulty of identifying MTB based on comparative analysis of 16S rRNA genes lies in the existence of closely related species of non-magnetotactic bacteria. Moreover, there is an absence of 16S rRNA MTB sequences from such taxonomic groups as '*Latescibacteria'* and *Planctomycetes*. In addition, the standard methods of separating MTB can benefit bacteria with high motility. Developing novel strategies for investigation offers great

promise towards identifying MTB groups. We have proposed new approach to separate MTB cells from environmental samples and have also proposed a specific primer system for the MTB identification.

INTRODUCTION

Prokaryotes having directed active movement that is guided by geomagnetism are collectively called magnetotactic bacteria (MTB) (1). The term MTB has no taxonomic meaning such that it representatives are physiologically, morphologically and phylogenetically different and share only the ability to synthesize special organelles called magnetosomes. Magnetosomes consist of nanosized magnetite (Fe_3O_4) (2) or greigite (Fe_3S_4) (3–5) crystals surrounded by a lipid bilayer membrane having proteins specific to the organelle (6, 7). Magnetosomes frequently assemble into chains inside the cell (8). MTB evolved the ability to conduct a special type of movement called magnetotaxis, which is based on orientation relative to magnetic field lines (9, 10).

MTB are found among the phyla *Proteobacteria*, *Nitrospirae*, *Planctomycetes*, the candidate phylum *'Omnitrophica'* and the candidate phylum *'Latescibacteria'* (11–15). Despite the high diversity of MTB found in environmental samples, they are difficult to isolate in axenic culture. Therefore, culture-independent techniques are an indispensable approach to study these bacteria.

Unlike other uncultivated bacteria, MTB can be isolated from environmental samples based on their magnetotatic activity. Isolation methods include primary separation using a magnet (16), as well as various magnetic trap techniques, such as 'race-track'

(17) or ‘MTB-trap’ (18). Although such magnetic enrichment approaches are efficient for isolation of environmental MTB, members of the *Alphaproteobacteria* and *‘Etaproteobacteria’* classes tend to predominate when these separation methods are used (14, 18–23). Since they are in higher concentration on the environment, are usually faster (24, 25) and more resistant to higher oxygen concentration than other MTB (26). The time used in standard concentration methods (20–30 minutes) make it hard the separation of MTB that have slow swimming ability (e.g., *Magnetovibrio blakemorei* MV-1) (27). Longer concentration time leads to the emergence of non-magnetic pollutants, which makes the enrichment of slowly swimming cells difficult. The magnetic field intensity, the distance to the magnet and chemotaxis can also negatively affect enrichment efficiency of MTB. Due to these and other characteristics, a narrow group of bacteria is preferentially collected using standard methods, which gives a biased representation of MTB diversity in the microbial community (26).

In some cases, nonmagnetic pollutants may interfere with the phylogenetic definition of MTB. This interference is because the ability to synthesize magnetosomes is not a taxonomic descriptor, and both MTB and non-MTB may belong to the same taxonomic group. For example, the magnetotactic *Desulfovibrio magneticus* RS-1 is closely related to *D. carbinolicus* and *D. burkinensis* (16S rRNA sequence similarity of 99.5% and 99.4%, respectively), which do not form magnetosomes (28). Furthermore, the frequency of MTB in the microbial community is low (~1–3%), as shown in studies using next generation sequencing (NGS) techniques (29, 30). Therefore, identification of MTB using 16S rRNA sequences alone is difficult, since

there are no universal identification markers for MTB. To date, MTB-specific primers are available only for the identification of individuals from the *Magnetospirillum* genus (31) and freshwater coccus from *Magnetococcales* order (32). The absence of MTB reference strains in different taxonomic groups also complicates the description of MTB diversity based on 16S rRNA sequence analysis. For example, putative magnetotosome genes were found in contigs from the draft genome of bacteria belonging to *'Latescibacteria'* and *Planctomycetes* phyla, but no 16S rRNA sequences were associated with these contigs (13, 14). As a result, MTB having high levels of 16S rRNA similarity to non-MTB and MTB that are present with low abundance in the community can remain undetected.

Development of improved techniques for detection and separation of MTB is thus needed for isolation of novel MTB that are less frequent and/or swim slowly.

In this study, we propose a novel approach for MTB isolation called **MTB Co**lumn **Se**paration (MTB-CoSe) that overcomes the problems associated with bias separation. Additionally, we developed a universal primer system for identifying MTB in an environment that addresses the limitations of using 16S rRNA profiling for MTB populations. We chose as an universal marker gene for MTB the gene encoding the actin-like protein MamK, which drives the ordered arrangement of the magnetosome chain in MTB cells (33).

RESULTS

Primer system design

All known MTB possess the genomic magnetosome island (MAI), comprising clusters of genes that regulate magnetosome mineralization (34). MTB share a common set of genes in the *mamAB* operon, which has been recognized as being indispensable for biomineralization. Currently, there are nine highly conserved primary genes in magnetite- and greigite-producing MTB: *mamA*, *mamB*, *mamM*, *mamQ*, *mamO*, *mamI*, *mamP*, *mamK* and *mamE* (12). For primer system design, we analyzed all nine genes across all known MTB (Table S1). The analysis of conserved regions allowed selection of genes that are amenable to the design of universal primer systems. The *mamM* and *mamB* genes, as well as *mamE* and *mamO*, are homologues, and thus are difficult to differentiate. Therefore, they were excluded from the analysis. Furthermore, *mamA*, *mamP* and *mamQ* genes have conserved sites only within a narrow taxonomic group, which precludes their use in a universal identification system. The *mamI* gene was highly conserved, but has a short nucleotide sequence. Thus, the *mamK* gene was chosen as the most optimal molecular marker for MTB identification. Based on analysis of conserved regions of *mamK*, several primer system variations were constructed and tested. The best results were demonstrated for nested PCR using two pairs of degenerate primers (mamK_79F-mamK_577R and mamK_86F-mamK_521R; Fig. S1). Upon testing this primer system on MTB, a product with the desired length of ~440 bp was obtained. Testing of non-MTB *M. aberrantis* and *E. coli* as negative controls yielded no product. Sequencing of the PCR products obtained with the primer set showed that the *mamK* gene fragment had the correct sequence. In testing the primer system on

DNA from the total microbial community, a product of the correct length was also detected (Fig. 1a).

Together these results showed that the method of magnetotactic bacteria identification based on *mamK* as a molecular marker is applicable not only to axenic cultures but also to total DNA from whole communities.

MTB-CoSe approach

Magnetic columns were used as a new method to separate MTB from enrironmental samples. This method minimizes oxygen sensitivity and motility bias seen in classical methods of MTB magnetic separation. In addition, it performs north- and south-seeking MTB isolation simultaneously.

Initially, testing was performed on an axenic culture of *M. magneticum* AMB-1 mixed with *E. coli* DH10B at a 1:3 ratio. The cell suspension was loaded into the column and rinsed with PBS buffer. The presence of *E. coli* cells was checked in the flow-through and after elution of the magnetic fraction. Separation of the cell suspension was successful in that *E. coli* was found in the flow-through and AMB-1 was retained in the column. We then tested this method on a bottom-sediment sample from Lake Beloye Bordukovskoe, which is characterized by low pH levels, a reduced quantity of anions and cations and a high organic carbon content (Table S2). Immediately after MTB-CoSe, a light microscopy examination revealed the presence of $1.12 \pm 0.06 \times 10^6$ MTB in 20 ml of the total sample, indicating that up to 10^8 MTB cells can be obtained from one liter of sediment. Vibrios were the most common MTB morphotype, and ovoid cells, which accounted for about 30% of the magnetic

fraction, were also detected. Nonmagnetic cells were also present in small quantities. The isolated MTB cells were divided into two parts. One part was used to isolate DNA for metagenome sequencing and creating libraries of the *mamK* and 16S rRNA gene sequences. The second part was fixed for FISH/TEM studies.

Diversity analysis by *mamK* and 16S rRNA clone libraries

Clone libraries from the *mamK* and 16S rRNA gene sequences consisted of 346 and 449 sequences, respectively, after the removal of chimeras and low-quality reads. Based on the MamK sequences, a phylogenetic analysis was conducted that yielded six OTUs. The tree topology was compared with the tree based on the 16S rRNA sequence, and identity with the nearest microorganisms was obtained to identify the phylogenetic position of the detected OTUs (Fig. 2). Based on the MamK sequences, the OTUs LBB_01, LBB_02 and LBB_03 were the most well-represented and formed a separate branch on the phylogenetic tree together with the MamK sequences of well-known strains of the Nitrospirae phylum. OTU LBB_01 was dominant (87% of the clone library) and formed a separate clade. The closest to LBB_01, with 61.8% sequence identity, was MamK sequence from *'Candidatus* Magnetobacterium bavaricum*'* TM-1. The dominant group LBB_01 was also present on the phylogenetic tree based on 16S rRNA sequences and forms a separate branch within the phylum *Nitrospirae*. The identity with *'Ca*. Magnetobacterium bavaricum*'* TM-1, *'Ca*. Magnetominusculus xianensis*'* and *'Ca.* Magnetoovum mohavensis*'* 16S rRNA sequences were 92.2%, 89.9% and 89.0%, respectively. The second OTU, LBB_02, clustered with the MamK sequence of *'Ca*. Magnetominusculus xianensis*'* HCH-1

with 85.4% homology. OTU LBB_02 was present on the tree based on 16S rRNA and also clustered with *'Ca*. Magnetominusculus xianensis*'* HCH-1. The sequence similarity was 99.2%, which indicated that they belong to the same species. The third most-frequent OTU (7.2%) was LBB_03. On the phylogenetic tree based on MamK, LBB_03 formed a separate clade with Nitrospirae bacterium MYbin3 with 87.5% sequence similarity. OTU LBB_03 was also present on the tree of 16S rRNA sequences; it formed a branch together with cultivated members of the genus *Thermodesulfovibrio* of the family *Nitrospiraceae* and clustered with C_I49, *'Ca*. Thermomagnetovibrio*'* HSMV-1 and MYbin3 sequences. The sequence identity with these three species was 95.8%, 91.2% and 92.8%, respectively. The level of similarity with 16S rRNA sequence from *'Ca*. Magnetobacterium bavaricum*'* TM-1 was 87.9%, which indicates that they may belong to different families. The sequence similarity of the 16S rRNA sequence of OTUs LBB_01 and LBB_02 was 84–86% with the closest cultivated strain *Thermodesulfovibrio hydrogeniphilus* of the family *Nitrospiraceae*, which indicates that they belong to a separate family. In accordance with the taxonomy proposed by Parks et al. (35), the level of similarity and branching order suggest their affiliation with the family *'Ca*. Magnetobacteriaceae*'*. OTU LBB_03 and *'Ca* Thermomagnetovibrio*'* HSMV-1 belong to the same family as strains of the genus *Thermodesulfovibrio*, but form separate genera.

The minor groups were OTUs LBB_05 and LBB_06, which clustered with the MamK sequences from MTB belonging to *Magnetococcales* and *Rhodospirillales* order; they represented 0.3% and 0.9% of the *mamK* clone library, respectively. OTU LBB_05 clustered with MamK belonging to Alphaproteobacterium WMHbin7 and

clustered with the MamK sequence obtained in the fosmid library Fos002 described by Jogler et al. (18). The level of similarity with MamK of WMHbin7 and MamK from Fos002 was 100%. The level of similarity with MamK of the nearest cultured organism, *Magnetospirillum magneticum*, was 74.6%. OTU LBB_05 was found in the 16S rRNA library and had a high level of similarity with the magnetotactic vibrio LM-1 (98.5%) and the bacterium WMHbin7 (98.3%). Due to the high level of similarity in both the MamK and 16S rRNA sequences, it is possible that OTU LBB_05, bacterium LM-1, WMHbin7 and the bacterium from the fosmid library Fos002 are the same species.

OTU LBB_06 formed a separate branch with the MamK of HA3dbin1, belonging to the order *Magnetococcales*. The level of similarity between sequences was 98.4%. The level of similarity with the MamK of the cultivated coccus *'Magnetofaba australis'* IT-1 was 66.4%. OTU LBB_06 was also present on the 16S rRNA phylogenetic tree and had 98.9% similarity to *Magnetococcus* sp. clone OTU2.

One interesting finding was the presence of OTU LBB_04, which had no MamK that was closely related to any known MTB. The level of similarity to the closest MamK of Nitrospirae bacterium MYbin3 was 69.2%, with coverage of 45%. Thus, the phylogenetic affiliation of OTU LBB_04 required a more thorough analysis. After searching for the nearest homologues for MamK of OTU LBB_04, a protein with a high sequence identity (97.0%) was found in the IMG database. This *mamK* gene was found in metagenome 3300021602, which was obtained by Neufeld et al. (36) from a boreal shield lake in Ontario. After binning of the metagenome, a bin (MAG_21602_syn32) containing the target *mamK* sequence was obtained (Table S3).

The 16S rRNA sequence of the received bin was also identified (acc. № Ga0194060_100555694). Analysis of that sequence on GTDB showed that it belongs to the order '*Syntrophales'* and, according to NCBI, the order *Syntrophobacterales*. MTB of this taxonomic group have not been previously detected. On the 16S rRNA phylogenetic tree, OTU LBB_04 formed a separate branch with representatives of the *Syntrophaceae* family. The 16S rRNA sequence derived from the MAG_21602_syn32 was also included in this cluster and had 96.8% identity with the 16S rRNA gene of OTU LBB_04. The level of similarity with the closest validly described strains, *Syntrophus aciditrophicus* and *Smitella propionica*, was 93.5% and 91.2%, respectively, which indicates that OTU LBB_04 belongs to a novel genus within the family *Syntrophaceae*.

In addition to the findings described above, there were sequences on the 16S rRNA tree belonging to the genera *Sphingomonas*, *Halomonas*, *Rhodococcus*, and *Pseudomonas*, constituting 12.2%, 10.4%, 4.8% and 2.4% of the entire library, respectively. Members of these genera can produce metal particles (37), meaning that they can be attracted by a magnet alongside MTB. Thus, for all OTUs identified on the MamK tree, the corresponding OTU on the 16S rRNA tree was determined. Based on those 16S rRNA gene sequences, probes were designed to identify the morphology of the matching MTB by FISH-TEM.

FISH-TEM evidence for new MTB groups

To correlate the phylogenetic data with the MTB morphotype, FISH-TEM was performed. The LBB_01 probe hybridized only with vibrioid-shaped bacteria (Fig.

3a-c). TEM images of the same area used for FISH analysis (Fig. 3d) revealed that this group of MTB presented a thick chain of magnetosomes organized along the long axis of the bacterial cell body (Fig. 3e). The observed magnetosomes were anisotropic (Fig. 3f) and presented [111] as the elongation axis. In addition, the fast Fourier transform (FFT) pattern of the magnetosome crystalline structure corresponded to the iron oxide magnetite (Fig. 3g). The LBB_03 probe correlated to another vibrioid cell presenting anisotropic magnetosomes in a single chain (Fig. 4a-e), whereas the LBB_02 probe was associated with a small, ovoid MTB with two magnetosome chains of anisotropic crystals (Fig. 4f-j). The probe LBB_04 identified a rod-shaped MTB with disorganized magnetosomes located close to the center of the bacterial cell body (Fig. 4k-o).

Cell morphology of LBB_01

Further analysis of cells compatible with those identified using the LBB_01 probe confirmed that these cells have a vibrioid shape and a 2.0 ± 0.4 μm and 0.5 ± 0.1 μm length and width, respectively (*n*=32). Each cell contained 33 ± 9 anisotropic magnetite magnetosomes (*n*=32) organized as described above (Fig. 3e). The magnetosome tips were not always sequentially oriented and were sometimes pointed in opposite directions (Fig. 3f), as was previously seen for other MTBs with anisotropic magnetosomes (38). Immature crystals were found in different chain segments. A detailed analysis of the magnetosome size showed that they have a mean length and width of 108 ± 21.1 nm and 45 ± 8.1 nm, respectively, and a mean shape factor (width/length) of 0.45 (*n* = 1061; Fig. S2).

General information and phylogeny of the LBB_01 genome

Using metagenomic data, we assembled the genome of the dominant group LBB_01 into 1154 contigs that included 2.3 Mbp with N_{50} equal to 3183. The completeness of the genome assembly was 65.15% with 1.87% contamination. The average G + C content was 42.1 mol%.

To determine the phylogenetic position of the LBB_01 genome we obtained, we built a phylogenomic tree (Fig. 5). *'Ca.* Magnetobacterium bavaricum*'* TM-1 and *'Ca.* Magnetoovum chiemensis*'* CS-04 were not included in the analysis because they failed the quality check due to a high percentage of contamination (34% and 17%, respectively). The genomes quality scores were determined as completeness – 5x contamination and had a threshold equal to 50. Bacterial genomes for which the quality score was less than 50 were not selected for analysis, according to Parks et al. (35). These two species had quality scores of -78.41 and 3.11, respectively, and thus were not analyzed further. As a result, the LBB_01 genome and the genomes of members of the genus *Thermodesulfovibrio* formed a separate branch from *Nitrospira moscoviensis* on the phylogenomic tree. Inside this branch, the LBB_01 genome, together with other MTB, clustered separately from the genus *Thermodesulfovibrio*. In addition, based on the 16S rRNA sequence, LBB_01 formed a branch separate from the genera *'Ca.* Magnetobacterium*'* and *'Ca.* Magnetominusculus*'*. Thus, based on the phylogenomic analysis, the strain LBB_01 represented a novel species and belonged to a novel genus within the family *'Ca.* Magnetobacteriaceae*'*.

Magnetosome genes in the LBB_01 genome

Analysis of the LBB_01 genome revealed genes for 17 magnetosome proteins that were identified in several contigs (Fig. S3a; Table S4). Among them, several proteins coded by the main *mamAB* operon, such as MamA, MamE, MamI, MamK, MamM and MamO-Cter, were found. MamK sequence was identical to OTU LBB 01 sequence retrieved from a clonal library. Homology analyses based on amino acid sequences of those Mam proteins showed that the magnetosome proteins of the strains belonging to the *Nitrospirae* phylum (Table S4) share the highest identity with them. A phylogenetic analysis based on the concatenated amino acid sequences of MamAIKMP proteins showed that LBB_01 clustered together with the Mam proteins of *'Ca* Magnetobacterium*'* and *'Ca* Magnetominusculus'. However, upon analysis of conserved Mam proteins, strain LBB_01 formed a separate branch on the phylogenomic tree (Fig. S3b). A comparison of the topology of the phylogenomic tree of core proteins and the tree of concatenated proteins MamAIKMP indicated a vertical inheritance of strain LBB_01 MAI.

In addition to proteins encoded by the main operon, 10 *mad* related to the magnetotactic *Deltaproteobacteria* and *Nitrospirae* were also found (39). Three of them formed a small genomic cluster comprising *mad24*, *mad25* and *mad26*. *Mad28* was present in two copies. *Man1*, a gene found only in the genome of MTB belonging to the *Nitrospirae* phylum, was also identified in the LBB_01 genome at a site adjacent to *mamK* (40). This organization of magnetosome genes resembled the island of *'Ca* Magnetobacterium casensis*'* MYR-1. The specific operons *mamGFDC*, *mamXY* and *mms6* related to *Alphaproteobacteria* and *Magnetococcales* were not found.

DISCUSSION

The amount of information concerning MTB has been increasing significantly. Among the growing body of genomic data, new taxonomic groups of MTB have been found, which indicates a wider diversity of MTB than was previously known (13, 14). Developing new approaches to study MTB in natural samples will expand our knowledge of the diversity and distribution of MTB in different environments. In this study, we proposed a new approach to study MTB diversity that allows collection of cells without relying on motility or taxis characteristics and uses a universal marker gene. A large number of MTB cells can be collected directly from the bottom sediment, thus bypassing the collection stage that requires a magnet. We demonstrated that a large fraction of the gathered cells can be used to obtain DNA suitable for sequencing the metagenome, whereas the separated cells could be subjected to single-cell sorting for analysis of minority groups. In addition, due to the preservation of viability, the cells can be used to produce enriched cultures.

To identify certain groups of bacteria in microbial communities, one effective method is to identify genes specific to bacterial groups (41). Currently, there are many primer systems used to study bacteria that share some functional trait (42–46). For instance, genes encoding two reaction center proteins, PufLM (47, 48) and the Fenna-Matthews-Olson-protein (FmoA) (49) are used to study bacterial photosynthesis, whereas ammonia monooxygenase (*amoCAB*) genes (50) are mostly applied to investigate ammonia-oxidizing archaea. However, there is no universal primer system for all MTB, even though these bacteria have magnetosome mineralization

genes that could serve as primer targets. Here we developed a MTB-specific primer system and tested it on the magnetic fraction collected from Lake Beloye Bordukovskoe. We found that *mamK* gene sequences can be used to identify and distinguish different taxonomic groups of MTB. Moreover, *mamK* can be used as a marker gene to investigate the diversity of MTB.

By combining novel enrichment and detection approaches, we found many representatives of the phylum *Nitrospirae* in oligotrophic Lake Beloye Bordukovskoe. In previous studies, *Magnetococcales* and *Rhodospirillales* were reported to be the dominant group of MTB in freshwater environments. However, using both 16S rRNA and *mamK* gene analyses in our approach, we showed that these two types of MTB were instead minority groups. Our results are consistent with an earlier study that showed a significant predominance of MTB belonging to the *Nitrospirae* phylum when the DNA was isolated directly from the sediment without prior separation of the MTB. (29). Our finding is also supported by evidence that *Nitrospirae* is the dominant MTB phylum in the freshwater lakes Miyun and Chiemsee in China and Germany, respectively (51). MTB of the orders *Magnetococcales* and *Rhodospirillales* may also be overrepresented due to the high number of reference sequences among the *Alphaproteobacteria* and *'Etaproteobacteria'* classes. As such, studies of MTB diversity typically focused on bacteria in these two classes. Studying the diversity of MTB based on a functional gene will expand our understanding of MTB diversity in various environments.

Using both 16S rRNA and *mamK* gene analyses, we identified *mamK* and corresponding 16S rRNA genes that belonged to a previously unknown taxonomic

group of MTB. The 16S rRNA analysis classified the bin MAG_21602_syn32 related to OTU LBB_04 into the class *Deltaproteobacteria*. Furthermore, FISH/TEM analysis confirmed that the OTU LBB_04 of the *Syntrophaceae* family belongs to a MTB and showed that the *Syntrophaceae* magnetosomes are bullet-shaped.

Using MTB-CoSe, we could collect suitable numbers of cells for DNA extraction and subsequent metagenome sequencing. We obtained the metagenome of the dominant group of the phylum *Nitrospirae*, which currently contains only one family, *Nitrospiraceae*, comprising 3 genera: *Leptospirillum*, *Nitrospira* and *Thermodesulfovibrio*. The branching order of the phylogenomic tree and the 15% difference in 16S rRNA sequences with the closest cultivated species, *Thermodesulfovibrio hydrogeniphilus*, suggest that strain LBB_01 belongs to a separate family. The 8% sequence difference from bacteria of the candidate genus '*Ca.* Magnetobacterium' indicated that strain LBB_01 likely represents a novel genus and species within the candidate family '*Ca.* Magnetobacteriaceae', for which we propose the name '*Ca.* Magnetomonas plexicatena'. The family *'Ca.* Magnetobacteriacea*'* currently includes several candidate genera to which only MTB belong: *'Ca.* Magnetobacterium, '*Ca*. Magnetoovum*', 'Ca*. Magnetominusculus*'* and the proposed genus *'Ca.* Magnetomonas*'*. Members of these genera differ in their morphology: '*Ca.* Magnetobacterium bavaricum', '*Ca.* Mb. Casensis' and the bacteria of Lake Miyun '*Ca.* Mb. bavaricum*'*-like are rod-shaped and synthesize multiple chains of bullet-shaped magnetosomes (51). Meanwhile, '*Ca.* Magnetoovum chiemensis' CS-04 (52) and '*Ca.* Mo. Mohavensis' LO-1 are large ovoid and synthesize only 100–200 magnetosomes per cell (53). For bacteria of the genera '*Ca.*

Magnetominusculus', the morphology of '*Ca.* Mm. xianensis' HCH-1, was previously unknown (54), but our analysis showed that these bacteria are small ovoid and synthesize two bundles of bullet-shaped magnetosomes. The newly described genus '*Ca.* Magnetomonas' has a vibrioid shape and synthesizes one bundle of bullet-shaped magnetosomes.

CONCLUDING REMARKS

This study presents novel approaches to investigating magnetotactic bacteria that allowed us, for the first time, to identify MTB of the family *Syntrophaceae*. Our methods using *mamK* as a marker gene and column separation allowed the identification of low-abundance MTB in different microbial communities. Our approach could be used to characterize magnetotactic representatives from '*Latescibacteria*' and *Planctomycetes* phyla, for which the magnetosome morphology has not yet been determined.

TAXONOMIC PROPOSALS

Phylogenetic analysis of the LBB_01 genome classified it as a novel genus. We propose Latin names for the novel candidate genus and species:

Candidatus Magnetomonas

Magnetomonas (Ma.gne.to.mo'nas. Gr. n. *magnes*, *-etos* a magnet; N.L. pref. *magneto-* pertaining to a magnet; N L. fem. n. *monas* unit, monad; N.L. fem. n. *Magnetomonas* a magnetic monad)

Candidatus Magnetomonas plexicatena

Magnetomonas plexicatena (ple.xi.ca.te'na. L. past part. *plexus* interwoven; L. fem. n. *catena* chain; N.L. fem. n. *plexicatena* an interwoven chain)

MATERIALS AND METHODS

Sampling and physicochemical analysis

Water and sediment samples were taken from the freshwater lake Beloye Bordukovskoe, Shatura District, Russia (55°37'56"N, 39°44'38"E). Samples having 1:2 sediment:water were incubated in the dark at room temperature for several months. Water from the microcosm was analyzed according to 41 parameters at the National Research Nuclear University MEPhI water quality control testing laboratory. The elemental composition of organic carbon (C%) in the sediment was measured using a Flash 1112 elemental analyzer coupled with a Thermo-Finnigan Delta V Plus isotope mass spectrometer (Thermo Fisher Scientific, USA) at the Core Facility of IPEE A.N. Severtsov RAS.

mamK primer system design and selection of amplification conditions

We searched for nucleotide sequences of magnetosome genomic island (MAI) genes in GenBank and IMG databases. Nucleotide sequence alignment procedures were performed using the ClustalW algorithm (55). Primer design was carried out using the program Oligo6 (Molecular Biology Insights, USA).

The selection of PCR conditions and testing of the ability of the designed primers to identify the target product were performed using end-point PCR. The reaction buffer (25 µl) had the following composition: 5 µl Mas^{CFE}TaqMIX-2025 buffer (Dialat

LTD, Russia), 0.5 pmol/µl each primer, 0.04% BSA and 10–50 ng of a DNA template. Amplification was performed on a Mastercycler gradient instrument (Eppendorf, Germany). The temperature-time profile of the reaction was: initiation, 3 min at 95 °C; 4 cycles of 30 s at 95 °C, 40 s at 58 °C, and 1 min at 72 °C; 36 cycles of 30 s at 95 °C, 40 s at 52 °C, 1 min at 72 °C; and a final cycle of 7 min at 72 °C. A fragment of the *mamK* gene was detected using nested PCR using two pairs of degenerate primers: external (mamK_79F and mamK_577R) and internal (mamK_86F and mamK_521R) (Table S5).

The efficiency, specificity and versatility of the primer system was tested on axenic cultures of the genus *Magnetospirillum* (*M. moscoviense* BB-1, *M. kuznetsovii* LBB-42 and *M. caucaseum* SO-1) and on a sample from the River Uda containing the magnetotactic coccus UR-1 of the order *Magnetococcales* only. DNA from the nonmagnetic bacteria *Escherichia coli* DH10B and *M. aberrantis* SpK served as negative controls. *M. aberrantis* is closely related to MTB and cannot form magnetosomes (56). *E. coli* carries the gene *mreB*, which codes for a rod-shape determining protein and has high sequence similarity to *mamK*. Amplified 16S rRNA sequences were used as controls for the DNA template. The resulting PCR products were sequenced to control nonspecific annealing of the primers. Amplification of the *mamK* gene was also verified in DNA isolated from whole microbial communities from the Soda Lake Kiran and Moskva River, which contain different taxonomic groups of MTB (23).

Magnetotactic bacteria column separation (MTB-CoSe)

The proposed method involves use of MACS® columns (Miltenyi Biotec, Germany) (Fig. 6). Since large sediment particles can occupy space in the column and in turn increase the washing time, we introduced additional steps. To desorb cells from large clay particles, PBS buffer (final concentration 1.25X) and glass microbeads (2 g; 150–200 µm diameter; Sigma-Aldrich, USA) were added to a 50 ml tube containing 20 ml of the samples (sediment + water). Additional filtration and centrifugation steps allowed removal of large soil particles and concentrated the bacterial cells. The mixture was then placed on a ThermoShakerTS-100 (Biosan, Latvia) for 15 min at 100 rpm before the homogenate was transferred to a Bunsen flask and filtered using a paper filter under vacuum pressure to remove large soil particles. The filtrate was transferred to 50 ml tubes and centrifuged (Eppendorf Centrifuge 5804R) for 10–15 minutes at 8,000 rpm. The majority of the supernatant was discarded, and 2 ml was retained for cell resuspension. Next, a miniMACS column was washed with 1.25X PBS buffer and placed on a magnet, and the cell mixture was applied to the column. MTB cells that adsorbed onto the column were washed 4–5 times with 2 ml 1.25X PBS buffer until there was a complete absence of cells in the washing liquid. Next, the column was removed from the magnet, and the MTB cells were eluted twice with 100 µl 1.25X PBS buffer into a clean 1.5 ml tube. For samples having high cell concentrations, the eluate was reapplied onto the column, which was rinsed to completely remove nonmagnetic cells. The presence of MTB cells and nonmagnetic bacteria was assessed by microscopy of 5 µl aliquots of eluted liquid.

This method is suitable for separating MTB from a large sample volume in a relatively short time. After completing all the manipulations, the magnetic fraction had low amounts of nonmagnetic pollutants.

Light and electron microscopy

The bacterial morphology after MTB-CoSe was examined using an Eclipse E200 (Nikon, Japan) light microscope. For conventional transmission electron microscopy (TEM), magnetically enriched cells were deposited on Formvar-coated 300-mesh copper grids, washed with distilled water and imaged using a Morgagni (FEI, USA) TEM operated at 80 kV. For high-resolution TEM (HRTEM), the same grids prepared for conventional TEM were imaged using a Tecnai G2 F20 FEG (FEI, USA) operated at 200 kV and equipped with a 4k×4k Gatan UltraScan 1000 CCD camera. Measurements and fast Fourier transform from the HRTEM images were obtained using Digital Micrograph software (Gatan, USA).

Amplification, cloning and sequencing fragments of genes encoding 16S rRNA and *mamK*

Genomic DNA was extracted using a DNeasy PowerSoil kit (Qiagen, Netherlands) according to the manufacturer's instructions. Amplification of the 16S rRNA sequences was performed using the universal primers 27F and 1492R (57). The PCR products were purified from a 0.7% agarose gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega, United States) according to the manufacturer's recommendations. Cloning was carried out with the pGEM-T Easy Vector System I

(Promega, USA). *E. coli* DH10B competent cells were transformed using a multiporator (Eppendorf, Germany). The target insert of the *mamK* gene was sequenced using the primer M13F, and 16S rRNA was sequenced using the primers 341F, 530F, 1114F and 519R (58). Sequencing was performed using the Sanger method on an ABI3730 DNA Analyzer sequencer (Applied Biosystems, USA) with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer.

Phylogenetic analysis

Nucleotide and amino acid sequences were aligned using MAFFT (59). The GTDB-Tk v. 0.1.3 toolkit was used to find 120 single-copy bacterial marker genes and construct multiple alignments of concatenated single-copy gene sequences (35). Concatenated *mamAIKMP* sequences were obtained using Gblocks (60). Obtained sequences of the MamK and 16S rRNA were grouped in OTUs using identity threshold 97%. Phylogenetic analysis was performed using the IQ-TREE program (61) with selection of an evolutionary model using ModelFinder (62) and estimation of branch supports using UFBoot2 (63). The MamK and 16S rRNA sequences for the identified OTUs were deposited in GenBank under the accession numbers MK636828-33 and MK63285-90, respectively.

Fluorescence in situ hybridization/transmission electron microscopy (FISH/TEM)

For fluorescence *in situ* hybridization (FISH), collected MTB cells were fixed in 3% paraformaldehyde for 1.5 hours. For the association of the retrieved *rrs* gene sequences with different MTB morphotypes, a drop of sample was added to center-marked or index copper grids covered with Formvar for electron microscopy. A thin layer of carbon was sputtered onto the grids (Balzers CED-030/Baltec) atop each sample to provide stabilization. FISH was performed on each grid using conditions and buffers described in Pernthaler et al. (2001) (64) and 30% formamide as the hybridization buffer. After this procedure, the grids were stained with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 10 minutes, placed between a glass slide and cover glass, and observed with a Zeiss AxioImager microscope equipped with an AxioCam Mrc (Zeiss, Germany).

Probe sets used for FISH were designed based on the *rrs* sequences retrieved from sequencing of the magnetically enriched samples (Table S5). In addition, a mix of EUB388I, EUB388II and EUB388III bacterial universal probes labelled with Alexa 488 was used as a control (65).

After performing FISH, the same grids were placed on a Morgagni (FEI, USA) transmission electron microscope (TEM) operated at 80 kV, and images were taken in the same region where hybridization with the specific probe occurred.

Genome sequencing

Libraries were constructed with NEBNext DNA library prep reagent set for Illumina, per the kit protocol. Sequencing was performed using the Illumina HiSeq 1500 platform with single-end 220-bp reads. The dried DNA was dissolved in 50 µl MQ.

Raw reads were quality-checked with FastQC v. 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and low-quality reads were trimmed using Trimmomatic v. 0.36 (66). The quality-filtered reads were assembled *de novo* with SPAdes v. 3.12.0 using the default settings (67). Annotations of the scaffolds were carried out using the NCBI Prokaryotic Genome Annotation Pipeline (68). Results for this whole-genome shotgun project were deposited in DDBJ/ENA/GenBank under the accession number SNNQ00000000. To evaluate genome statistics, an automatic assembly quality evaluation tool (QUAST) was used (69).

Metagenome binning and analysis

The assembled metagenome of Lake Beloe Bordukovskoe and the metagenome 3300021602 obtained in the IMG database were binned using three different tools (MaxBin2 (70), MyCC (71), and Busy Bee Web (72)) prior to dereplication and refinement with the DAS Tool (73), which performs a consensus binning to produce the final bin set. Genome contamination was removed using an approach based on taxonomic assignments in RefineM v. 0.0.24 (74). Completeness and contamination rates were assessed using CheckM v. 1.0.12 (75) with the 'lineage wf' command and default settings. Magnetosome island genes were found using local BLAST compared with reference sequences of magnetotactic bacteria.

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Figure legends

Figure 1. Evaluation of nested PCR for MTB analyses. **a)** PCR amplicons obtained with the primers mamK_86F-mamK_521R after nested PCR on MTB and non-MTB isolates and the entire microbial community; **b)** PCR amplicons with16S rRNA used

as a control DNA template. Lane 1: control sample without DNA; Lane 2: *E. coli* DH10B; Lane 3: *Magnetospirillum aberranis* SpK; Lane 4: *M. moscoviense* BB-1*;* Lane 5: *M. kuznetsovii* LBB-42; Lane 6: *M. caucaseum* SO-1; Lane 7: Magnetococcales bacterium UR-1; Lane 8: Moskva River; Lane 9: Soda Lake Kiran; Lane M: DNA ladder GeneRuler 100 bp Plus.

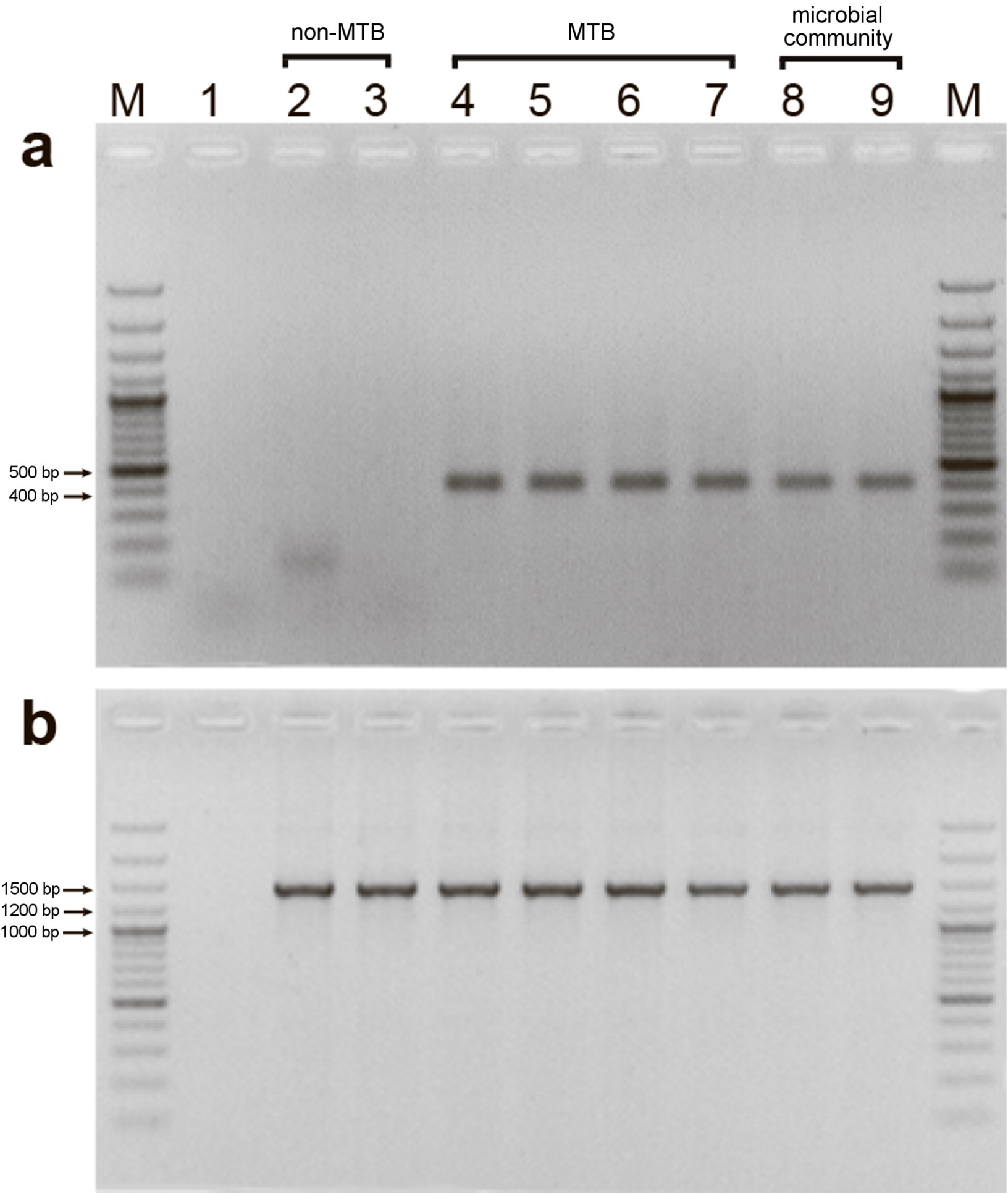
Figure 2. Maximum-likelihood phylogenetic trees based on **a)** magnetosome associated protein MamK (285 amino acid sites) reconstructed with evolutionary model LG+I+G4; and **b)** 16S rRNA sequences (1,399 nucleotide sites) reconstructed using the evolutionary model GTR+F+I+G4.

Figure 3. FISH-TEM of the MTB '*Ca*. Magnetomonas plexicatena' LBB_01 and HRTEM of its magnetosomes. **a)** Phase contrast image of magnetically enriched environmental sample deposited on Formvar- coated TEM grids; **b)** Bacteria observed following hybridization with a EUB probe; **c)** Image after hybridization with a probe specific for *Ca*. Magnetomonas plexicatena species. The arrow highlights a small coccus that was not hybridized with the specific probe; **d)** TEM image of the same region marked as a box in panel **a**; **e)** Higher magnification of the highlighted area in **d** showing a chain of anisotropic magnetosomes organized along the long axis of the cell; **f)** Higher magnetic activity of the area inside the square in figure **e**. Magnetosomes in this cell had occasional rough edges and a bullet shape; **g)** HRTEM of the magnetosome indicated by an asterisk in **f**. FFT of the magnetosome crystalline structure support the possibility that these magnetite magnetosomes align along an [111] elongation axis.

Figure 4. FISH-TEM image of MTB from environmental samples. The circles and arrows indicate cells that did and did not, respectively, hybridize with the specific probe tested. **a**), **f)** and **k)** Phase contrast image of magnetically enriched environmental samples on Formvar-coated TEM grids; **b)**, **g)** and **l)** Bacteria present in the same region as imaged in **a**, **f** and **k**, respectively, stained with DAPI; **c**), **h)** and **m)** Image of the same area as shown in **b**, **g** and **l**, respectively, after hybridization of bacterial cells with the EUB probe; **d)** Image of the same area captured in image **c** after hybridization with the LBB_03 probe; **e)** TEM image of the same cell that showed hybridization with the LBB_03 probe in **d**. The cell has a rod shape and anisotropic magnetosomes; **i)** Image of the same area captured in **h** after cell hybridization with probe LBB_02; **j)** TEM image of the MTB that showed hybridization with probe LBB_02 in image **i**. The MTB is a small coccus having two chains of anisotropic magnetosomes; **n)** TEM image of the same area represented in image **m** after hybridization with probe LBB_04. This cell had many disorganized anisotropic crystals in the cytoplasm.

Figure 5. Maximum-likelihood phylogenetic tree derived from concatenated core protein sequences. Phylogenetic analysis was performed with a LG+F+I+G4 model based on 34,747 amino acid positions. The scale bar represents amino acid substitutions per site.

Figure 6. MTB-CoSe separation procedure that allowed collection of magnetotactic components directly from sediment samples.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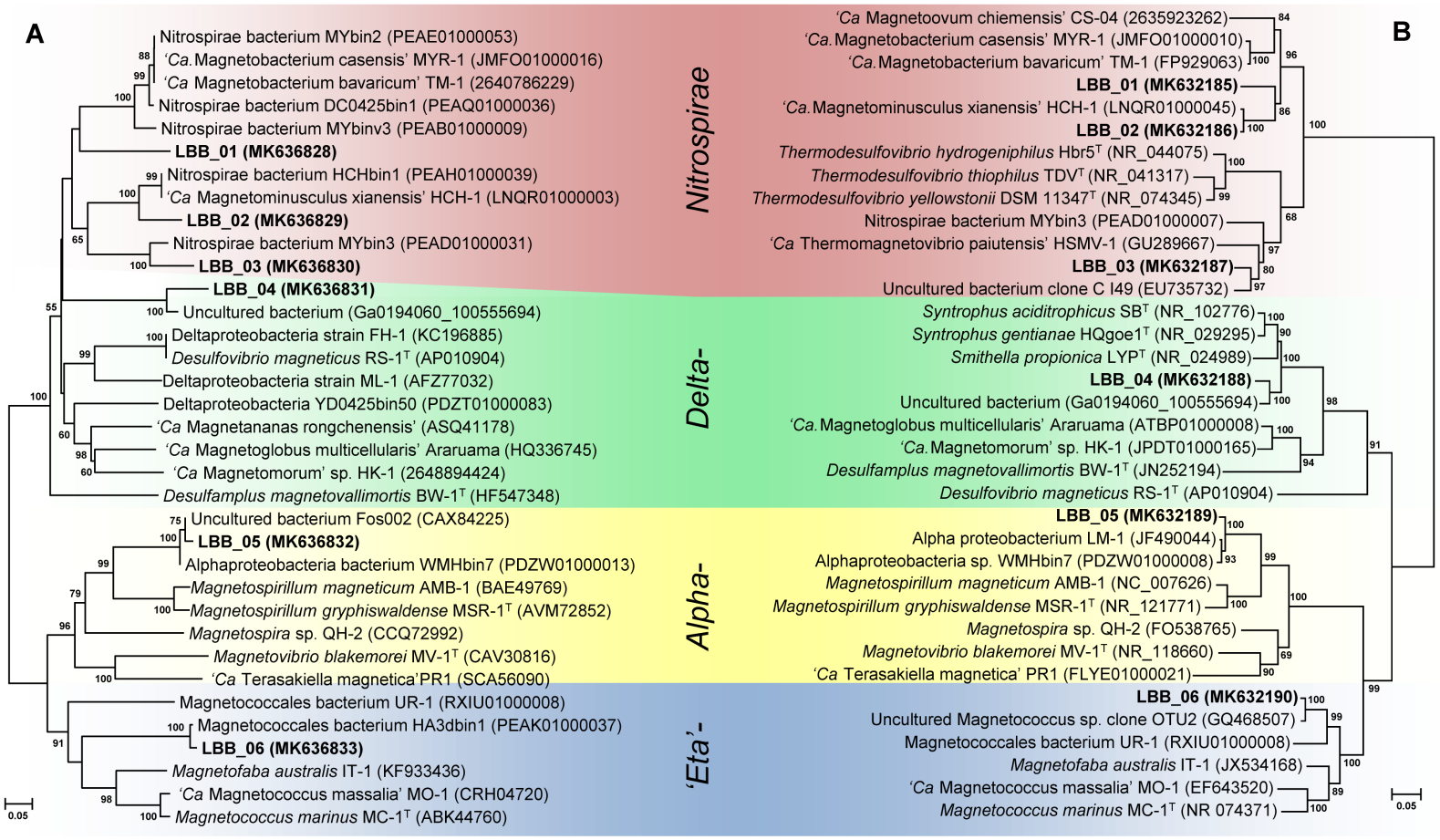
1200 bp
1000 bp


A
B
Nitrospirae
Delta-
Alpha-
'Eta'-
Nitrospirae bacterium MYbin2 (PEAE01000053)
'Ca. Magnetobacterium casensis' MYR-1 (JMFO01000016)
'Ca Magnetobacterium bavaricum' TM-1 (2640786229)
Nitrospirae bacterium DC0425bin1 (PEAQ01000036)
Nitrospirae bacterium MYbinv3 (PEAB01000009)
LBB_01 (MK636828)
Nitrospirae bacterium HCHbin1 (PEAH01000039)
'Ca Magnetominusculus xianensis' HCH-1 (LNQR01000003)
LBB_02 (MK636829)
Nitrospirae bacterium MYbin3 (PEAD01000031)
LBB_03 (MK636830)
LBB_04 (MK636831)
Uncultured bacterium (Ga0194060_100555694)
Deltaproteobacteria strain FH-1 (KC196885)
Desulfovibrio magneticus RS-1T (AP010904)
Deltaproteobacteria strain ML-1 (AFZ77032)
Deltaproteobacteria YD0425bin50 (PDZT01000083)
'Ca Magnetananas rongchenensis' (ASQ41178)
'Ca Magnetoglobus multicellularis' Araruama (HQ336745)
'Ca Magnetomorum' sp. HK-1 (2648894424)
Desulfamplus magnetovallimortis BW-1T (HF547348)
Uncultured bacterium Fos002 (CAX84225)
LBB_05 (MK636832)
Alphaproteobacteria bacterium WMHbin7 (PDZW01000013)
Magnetospirillum magneticum AMB-1 (BAE49769)
Magnetospirillum gryphiswaldense MSR-1T (AVM72852)
Magnetospira sp. QH-2 (CCQ72992)
Magnetovibrio blakemorei MV-1T (CAV30816)
'Ca Terasakiella magnetica'PR1 (SCA56090)
Magnetococcales bacterium UR-1 (RXIU01000008)
Magnetococcales bacterium HA3dbin1 (PEAK01000037)
LBB_06 (MK636833)
Magnetofaba australis IT-1 (KF933436)
'Ca Magnetococcus massalia' MO-1 (CRH04720)
Magnetococcus marinus MC-1T (ABK44760)
0.05
'Ca Magnetoovum chiemensis' CS-04 (2635923262)
'Ca. Magnetobacterium casensis' MYR-1 (JMFO01000010)
'Ca. Magnetobacterium bavaricum' TM-1 (FP929063)
LBB_01 (MK632185)
'Ca. Magnetominusculus xianensis' HCH-1 (LNQR01000045)
LBB_02 (MK632186)
Thermodesulfovibrio hydrogeniphilus Hbr5T (NR_044075)
Thermodesulfovibrio thiophilus TDVT (NR_041317)
Thermodesulfovibrio yellowstonii DSM 11347T (NR_074345)
Nitrospirae bacterium MYbin3 (PEAD01000007)
'Ca Thermomagnetovibrio paiutensis' HSMV-1 (GU289667)
LBB_03 (MK632187)
Uncultured bacterium clone C I49 (EU735732)
Syntrophus aciditrophicus SBT (NR_102776)
Syntrophus gentianae HQgoe1T (NR_029295)
Smithella propionica LYPT (NR_024989)
LBB_04 (MK632188)
Uncultured bacterium (Ga0194060_100555694)
'Ca. Magnetoglobus multicellularis' Araruama (ATBP01000008)
'Ca. Magnetomorum' sp. HK-1 (JPDT01000165)
Desulfamplus magnetovallimortis BW-1T (JN252194)
Desulfovibrio magneticus RS-1T (AP010904)
LBB_05 (MK632189)
Alpha proteobacterium LM-1 (JF490044)
Alphaproteobacteria sp. WMHbin7 (PDZW01000008)
Magnetospirillum magneticum AMB-1 (NC_007626)
Magnetospirillum gryphiswaldense MSR-1T (NR_121771)
Magnetospira sp. QH-2 (FO538765)
Magnetovibrio blakemorei MV-1T (NR_118660)
'Ca Terasakiella magnetica' PR1 (FLYE01000021)
LBB_06 (MK632190)
Uncultured Magnetococcus sp. clone OTU2 (GQ468507)
Magnetococcales bacterium UR-1 (RXIU01000008)
Magnetofaba australis IT-1 (JX534168)
'Ca Magnetococcus massalia' MO-1 (EF643520)
Magnetococcus marinus MC-1T (NR 074371)
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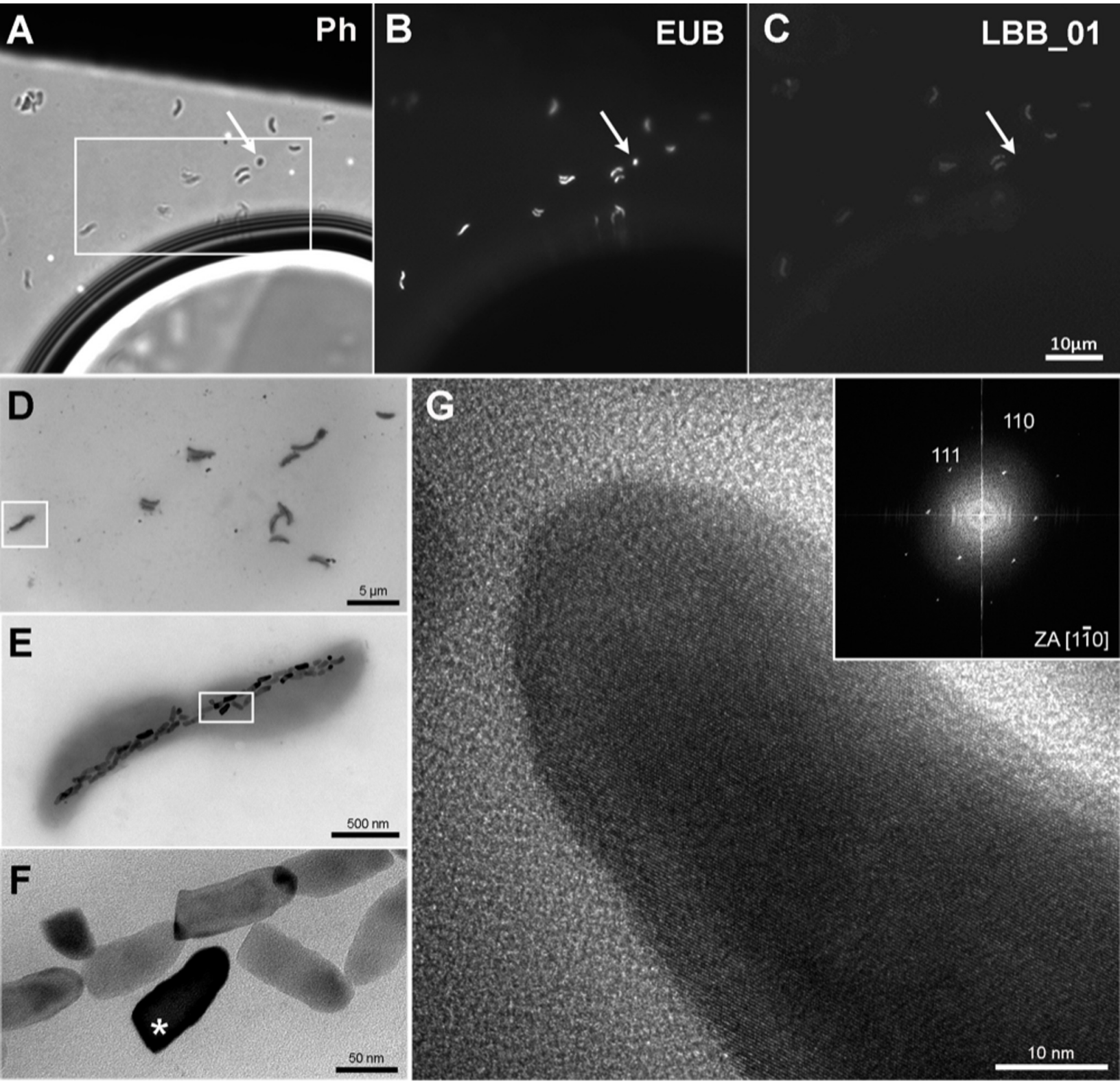
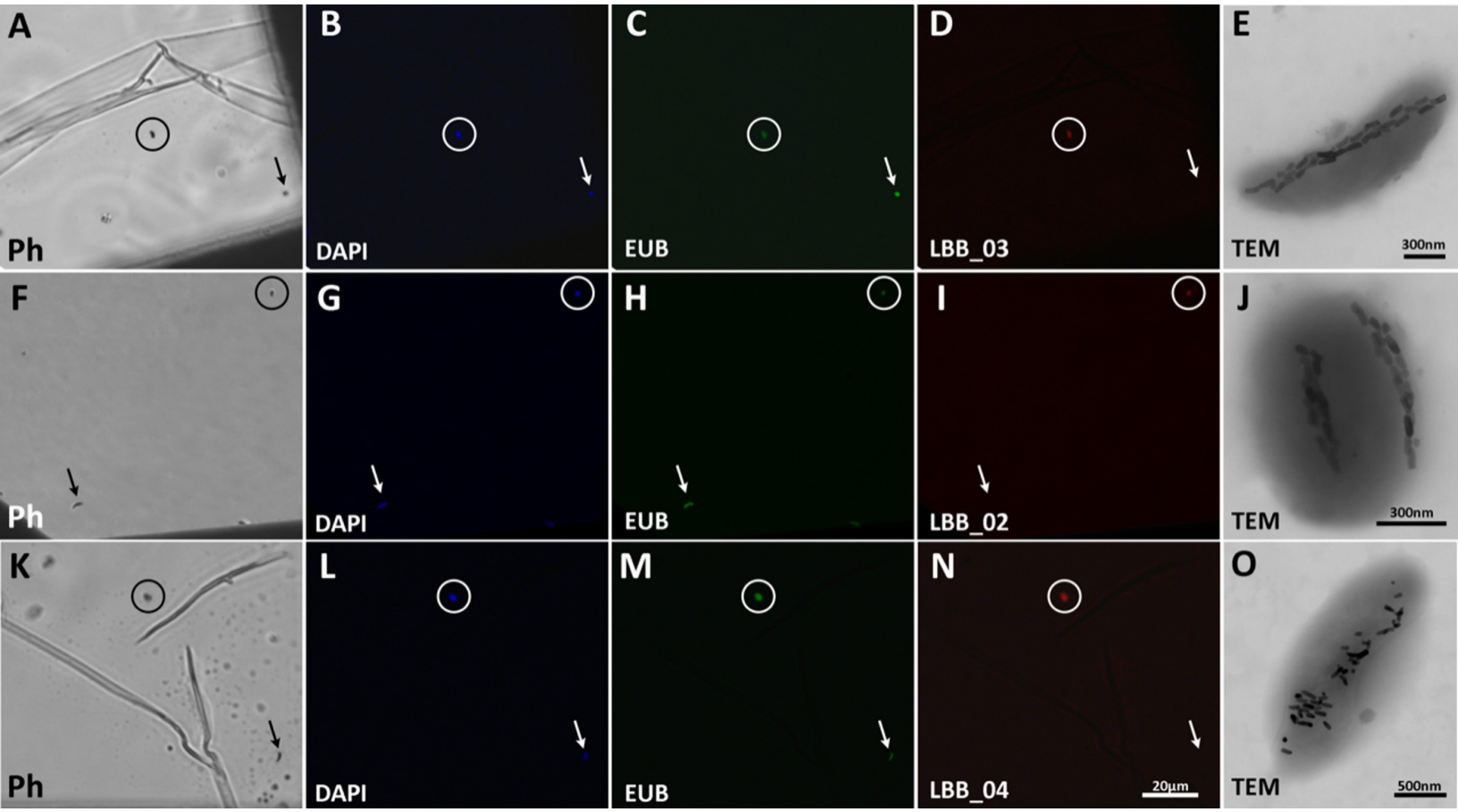
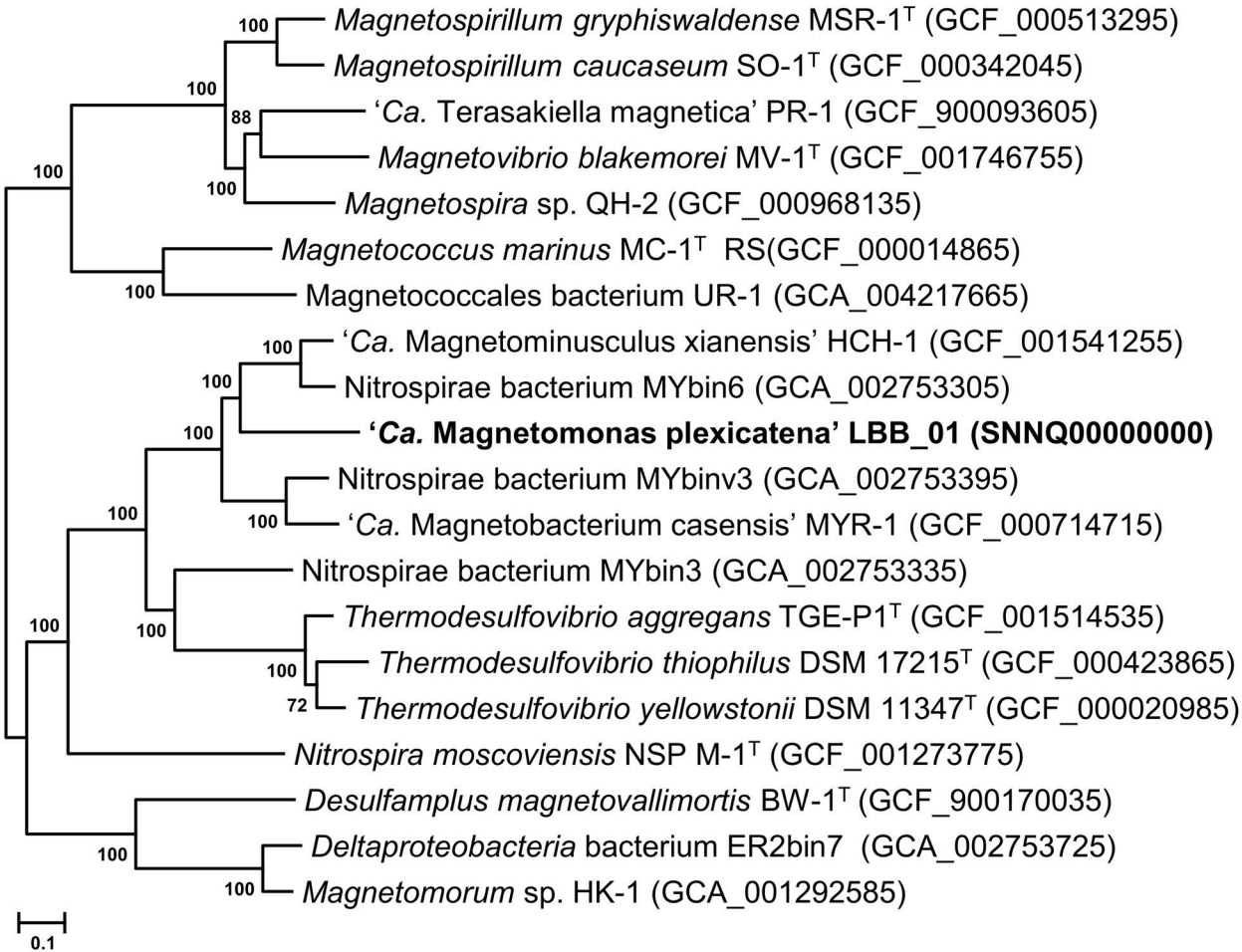

A
Ph
B
EUB
C
LBB_01
10µm
D
5 µm
E
500 nm
F
*
50 nm
G
110
111
ZA [1$\bar{1}$0]
10 nm


A
Ph
B
DAPI
C
EUB
D
LBB_03
E
TEM
300nm
F
Ph
G
DAPI
H
EUB
I
LBB_02
J
TEM
300nm
K
Ph
L
DAPI
M
EUB
N
LBB_04
20µm
O
TEM
500nm


Magnetospirillum gryphiswaldense MSR-1T (GCF_000513295)
Magnetospirillum caucaseum SO-1T (GCF_000342045)
'Ca. Terasakiella magnetica' PR-1 (GCF_900093605)
Magnetovibrio blakemorei MV-1T (GCF_001746755)
Magnetospira sp. QH-2 (GCF_000968135)
Magnetococcus marinus MC-1T RS(GCF_000014865)
Magnetococcales bacterium UR-1 (GCA_004217665)
'Ca. Magnetominusculus xianensis' HCH-1 (GCF_001541255)
Nitrospirae bacterium MYbin6 (GCA_002753305)
'Ca. Magnetomonas plexicatena' LBB_01 (SNNQ00000000)
Nitrospirae bacterium MYbinv3 (GCA_002753395)
'Ca. Magnetobacterium casensis' MYR-1 (GCF_000714715)
Nitrospirae bacterium MYbin3 (GCA_002753335)
Thermodesulfovibrio aggregans TGE-P1T (GCF_001514535)
Thermodesulfovibrio thiophilus DSM 17215T (GCF_000423865)
Thermodesulfovibrio yellowstonii DSM 11347T (GCF_000020985)
Nitrospira moscoviensis NSP M-1T (GCF_001273775)
Desulfamplus magnetovallimortis BW-1T (GCF_900170035)
Deltaproteobacteria bacterium ER2bin7 (GCA_002753725)
Magnetomorum sp. HK-1 (GCA_001292585)
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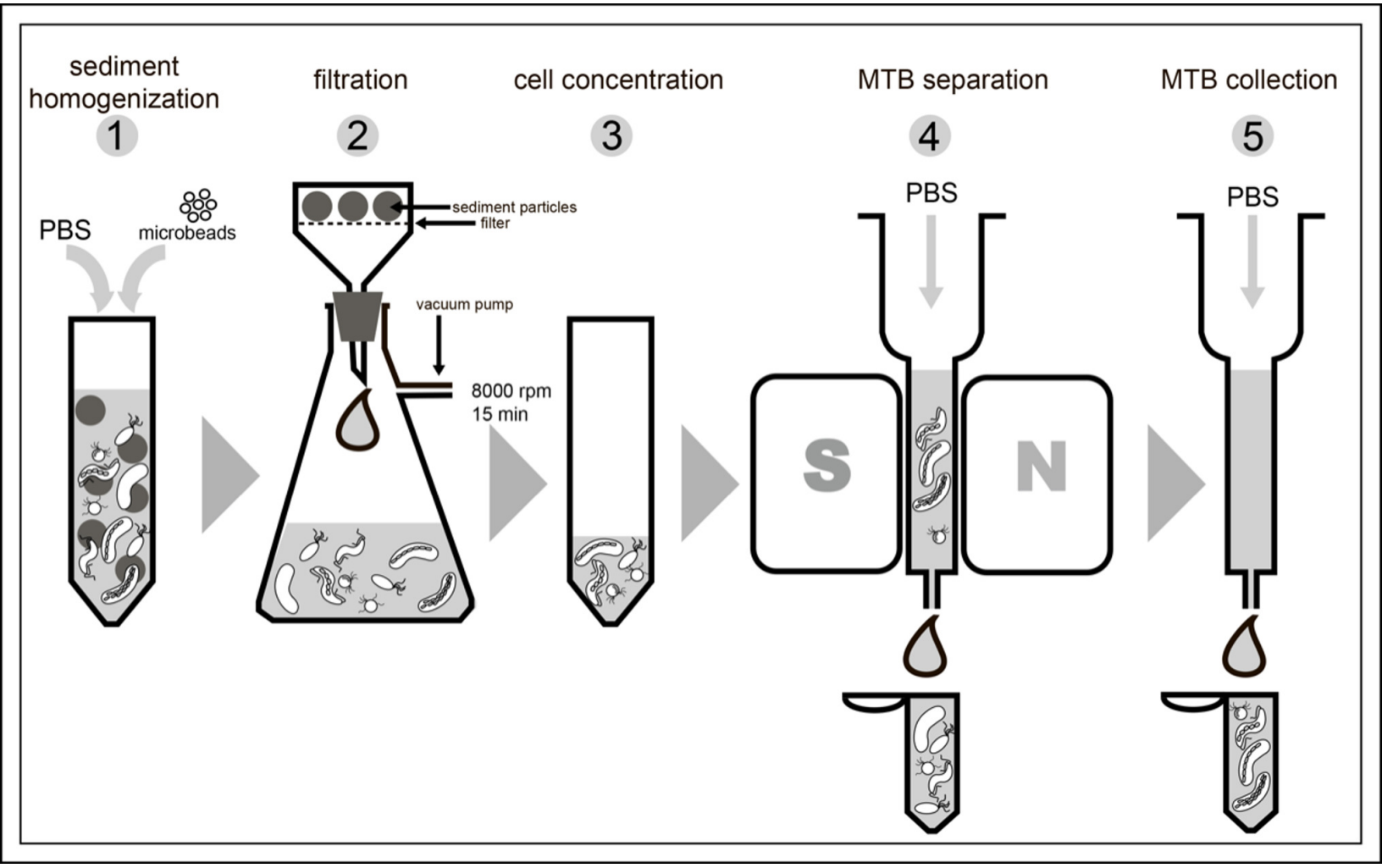
72
100
100
0.1


sediment homogenization
1
filtration
2
cell concentration
3
MTB separation
4
MTB collection
5
PBS
microbeads
sediment particles
filter
vacuum pump
8000 rpm
15 min
PBS
S
N
PBS