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# Toward Cloning of the Magnetotactic Metagenome: Identification of Magnetosome Island Gene Clusters in Uncultivated Magnetotactic Bacteria from Different Aquatic Sediments[▽]

Christian Jogler,[1] Wei Lin,[2] Anke Meyerdierks,[3] Michael Kube,[4] Emanuel Katzmann,[1] Christine Flies,[3] Yongxin Pan,[2] Rudolf Amann,[3] Richard Reinhardt,[4] and Dirk Schüler[1]*

*LMU Department Biology I, Microbiology, Großharderner-Str. 2, 82152 Planegg-Martinsried, Germany[1]; Biogeomagnetism Group, Paleomagnetism and Geochronology Laboratory, State Key Laboratory of Lithospheric Evolution, Institute of Geology and Geophysics, Chinese Academy of Sciences, Beijing 100029, China[2]; Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany[3]; and Max Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany[4]*

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**In this report, we describe the selective cloning of large DNA fragments from magnetotactic metagenomes from various aquatic habitats. This was achieved by a two-step magnetic enrichment which allowed the mass collection of environmental magnetotactic bacteria (MTB) virtually free of nonmagnetic contaminants. Four fosmid libraries were constructed and screened by end sequencing and hybridization analysis using heterologous magnetosome gene probes. A total of 14 fosmids were fully sequenced. We identified and characterized two fosmids, most likely originating from two different alphaproteobacterial strains of MTB that contain several putative operons with homology to the magnetosome island (MAI) of cultivated MTB. This is the first evidence that uncultivated MTB exhibit similar yet differing organizations of the MAI, which may account for the diversity in biomineralization and magnetotaxis observed in MTB from various environments.**

Magnetotactic bacteria (MTB) synthesize magnetosomes, which are membrane-enclosed organelles comprising crystals of magnetite ($Fe_3O_4$) or, less commonly, greigite ($Fe_3S_4$) (3) that are aligned in intracellular chains along dedicated cytoskeletal structures (26, 36, 38). Magnetic alignment along the magnetic field lines of the earth facilitates navigation in the stratified environment within freshwater and marine sediments (3, 13). MTB do not form a coherent phylogenetic group, but the trait of magnetotaxis is found in species within different phylogenetic clades, including *Alphaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and the *Nitrospira* phylum (1, 3, 10, 41). Different species produce magnetosome crystals with a multitude of different morphologies displaying a broad variety of intracellular arrangements, including one, two, or multiple chains (3, 14). The perfectly shaped magnetosome crystals and highly ordered chain structures cannot be synthesized by chemical methods as yet. Therefore, an understanding of the genetic mechanisms controlling magnetosome formation is also of great interest for the inorganic production of advanced magnetic nanomaterials (3, 13, 28).

Most genes controlling magnetosome formation and magnetotaxis in *Magnetospirillum gryphiswaldense* and other freshwater magnetospirilla are clustered within four major operons (*mamAB*, *mamGFDC*, *mms6*, and *mamXY*) (18, 34, 37, 49) that are part of a large genomic magnetosome island (MAI) (49). It was recently shown that the MAI is also conserved in marine MTB, including the MV-1 magnetotactic vibrio strain and the MC-1 magnetic coccus strain. The homologous genomic regions display similar gene contents and, to a lesser extent, a conserved gene synteny (23). It has been suggested that the MAI was transferred horizontally between different MTB (37). However, the divergence between the MAI regions of strain MV-1, strain MC-1, and the magnetospirilla suggests that the events of horizontal gene transfer (HGT) did not occur very recently.

Despite continued efforts by many laboratories, the majority of MTB are still not available in pure culture. In particular, the huge diversity of uncultivated species with respect to different morpho- and phylotypes and, in particular, magnetosome crystal shapes is not nearly fully represented by cultivated species. Thus, understanding of the genetic diversity of the magnetotaxis and magnetosome biosynthetic machinery has to rely on culture-independent techniques such as the metagenomic analysis of environmental MTB (24).

It has been demonstrated that single genes and even entire operons can be cloned and functionally expressed from uncultivated soil or marine bacteria by using large insert libraries that provide contiguous sections from single organisms (4, 21, 22). The potential to identify and clone genes for metabolic pathways with relevance for biotechnological applications has already been demonstrated in metagenomic projects, such as the identification of polyketide synthase genes from microbial consortia of marine sponges (25) or other environmental samples (8, 31). The cost of sequencing and the challenges that are associated with the management of vast datasets, however, preclude comprehensive genomic studies of highly complex communities. Consequently, approaches that are based on the analysis of a group of bacteria with reduced species diversity are favored. This requires that the sample material is enriched for the target organisms before DNA preparation, for example, by flow sorting, centrifugation, or other physical enrich-

* Corresponding author. Mailing address: LMU Department Biology I, Microbiology, Großharderner-Str. 2, 82152 Planegg-Martinsried, Germany. Phone: 49 (0)89-2180-74502. Fax: 49 (0)89-2180-74515. E-mail: dschuele@mpi-bremen.de.
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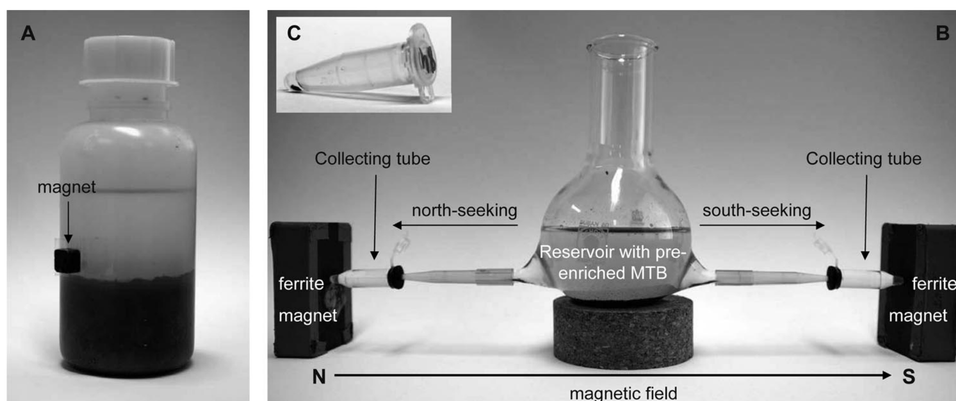


FIG. 1. (A) Direct magnetic collection at a microcosm as previously described (15). (B) "MTB trap" that allows the simultaneous mass collection of north- and south-seeking bacteria (for details, see text). (C) After a collection period of approximately 1 h, a visible cell pellet was found to have resulted from accumulation at the bottom of the collection tube.

ment techniques (32, 42) or by focusing on natural communities with reduced species diversity (48).

Unlike other uncultivated bacteria, MTB exhibit magnetically directed swimming behavior, which enables their selective enrichment from environmental samples without the need of cultivation (16). This approach was utilized in a number of earlier studies uncovering the morphological and phylogenetic diversity of MTB found in environmental populations (10, 43, 45–47). However, these investigations were confined to PCR-based analysis of 16S rRNA genes, ultrastructural studies, and fluorescence in situ hybridization.

In this study we used an improved magnetic collection technique to selectively harvest large numbers of uncultivated MTBs, which allowed the extraction of genomic DNA for the construction of large insert metagenomic libraries from different aquatic habitats. Large parts of the MAI from two uncultivated MTB were identified by hybridization using heterologous magnetosome gene probes and end sequencing. We demonstrate for the first time that uncultivated MTB exhibit a clustered organization of magnetosome genes which resembles that of cultivated species and yet displays variations that may account for the observed diversity in biomineralization and magnetotaxis in MTB from various environments. The levels of similarity between and synteny of magnetosome genes of uncultivated and cultivated MTB provide further evidence for HGT.

## MATERIALS AND METHODS

**Sediment sampling and setup of microcosms.** We sampled four different freshwater sites and one marine habitat in Germany that were previously found to harbor abundant and diverse populations of MTB (14, 44) These were a eutrophic pond near Staßfurt, Saxony-Anhalt (STF) (latitude, 51°50′14.78′′N; longitude, 11°36′19.19′′E), a mesophilic pond in Unterlippach, Bavaria (UL) (latitude, 48°35′15.24′′N; longitude, 12° 4′38.15′′E), a eutrophic pond in Nymphenburg Park in Munich, Bavaria (NYM) (latitude, 48°9′29.10′′N; longitude, 11°29′57.89′′E), Lake Chiemsee (LCH) (latitude, 47°51′24.71′′N; longitude, 12°23′0.21′′E), and an intertidal sandy mudflat from North Sea near Cuxhaven, Lower Saxony (CUX) (latitude, 53°53′34.23′′N; longitude, 8°40′32.61′′E). Sediment samples from the ponds were taken with a shovel from the shore, whereas Lake Chiemsee sediment was collected from the boat at a depth of 10 to 20 m by a bottom sampler. Cuxhaven samples were taken at low tide near the shore. Microcosms were set up in the laboratory by dividing the sediment slurry into aliquots (10 to 75 liters) in 20 to 150 parallels into 500-ml plastic flasks. Each flask contained about 200 ml of sediment and 200 ml of supernatant water. Microcosms were incubated at room temperature (RT) in the dark for at least 1 month prior to magnetic collection.

**Magnetic collection of MTB.** For collection of cells, we used two different methods. Cells from STF were harvested as previously described (16). All other MTB were obtained by a two-step magnetic collection strategy. In the first step, small (1.2 × 1.2 cm) hard ferrite magnets (~400 mT) were placed with the south pole close to a wall of microcosms, immediately above the water-sediment interface (Fig. 1A). After 1 h of collection, a 1-ml water sample was withdrawn from the focus of the attached magnet. Samples were pooled from about 20 microcosms and subsequently transferred to an "MTB trap" (Fig. 1B), which is a modified version of a collection vessel for harvesting uncultured magnetotactic microorganisms (29). This homemade device consists of a reservoir with two opposite funnels pointing toward a collecting tube in parallel to the applied magnetic field of two square ferrite magnets (5 by 7.5 cm) as indicated in Fig. 1B. The collections were performed in up to 10 parallels, with 150 microcosms (equivalent to 30 liters) of sediment in total. Each device was loaded with 100 ml of suspension containing about $10^8$ preenriched cells. The MTB started to swim to the northern or southern collecting tube, and, after 1 h of collection, the liquid was replaced by the next round of primary collections. A total of 5 to 10 subsequent rounds of collection were performed, until a visible MTB pellet was observed (Fig. 1C). After all microcosms of one sample were harvested, both collection tubes were disassembled and cells were pelleted for 10 min at 11,000 × $g$ in a table-top centrifuge. The supernatant was discarded, and cell pellets were frozen at −20°C.

**Extraction of DNA from collected MTB.** The DNA from MTB enrichments was prepared essentially as previously described (52). Briefly, 675 μl of DNA extraction buffer was used to resuspend the individual cell pellets from the magnetic collection. A 2.5-μl volume of proteinase K (20 mg/ml) was added, and samples were incubated at 37°C with agitation (220 cycles/min). Afterward, 75 μl of 20% sodium dodecyl sulfate was added, and samples were heated to 65°C for 2 h. Chloroform and isoamyl alcohol (1:24; 750 μl) were added, and the suspension was incubated at RT for 5 min, with the tube was inverted every minute. After centrifugation at 6,000 × $g$ for 20 min at RT, the aqueous supernatant was transferred into a fresh 1.5-ml reaction tube and the DNA was precipitated with 350 μl of isopropanol at RT for 1 h. The DNA pellet was obtained by centrifugation for 20 min at RT and was washed twice with 70% ethanol at 4°C. Finally, the DNA was resuspended in water by incubation at 55°C for 2 h.

**Fosmid library construction.** Four fosmid libraries were constructed using a CopyControl fosmid library production kit (Epicenter), following the specifications of the manufacturer. The DNA obtained from samples from UL and NYM were pooled prior to library construction, whereas the LCH, CUX, and STF DNAs were used in independent library construction procedures. Ten clones from each library were randomly selected for insertion length determinations by restriction analysis. The LCH library contained an average insert size of 32 kb, the CUX library an average insert size of 36 kb, the NYM and UL libraries an average insert size of 33 kb, and the STF library an average insert size of approximately 30 kb.

Fosmid DNA was prepared by alkaline lysis (35), and contaminating genomic DNA was removed using plasmid-safe DNase (Epicenter) according to the manufacturer's instructions.

**Probe design, Southern blot analysis, and colony filter hybridization.** To facilitate colony filter hybridization and Southern blot analysis, a panel of probes

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TABLE 1. Primers for the amplification of probes from the genome of *M. gryphiswaldense* used for colony filter hybridization and Southern blot analysis

| Primer | Sequence | Target gene | Length of PCR product (bp) |
|---|---|---|---|
| CJ148 | GGATTGGGATTTTGGAGAAC | *mamA* | 699 |
| CJ149 | TGAGACCTTCTACATCGACTGC | | |
| CJ150 | GGGTGCCTGGGAATTATTTT | *mamE* | 2,930 |
| CJ151 | TCAAGCAAGCTAGCCCAAAT | | |
| CJ106 | TTAAGGGGCAGAGAGGGAAT | *mamF* | 1,166 |
| CJ107 | GGCGACGATGAAGCTCTTAC | | |
| CJ152 | GCAGTATCCCACCGTTCAGT | *mamB* | 2,013 |
| CJ153 | CCCATGTTCACCCTCTCTGT | | |

was designed. Regions of highest sequence conservation were selected by multiple sequence alignments of all known magnetosome genes from all sequenced MTB species. Finally, probes were generated by amplifying genomic target sequences from the DNA of *M. gryphiswaldense* strain MSR-1 (DSMZ 6361) via PCR. The primers used are listed in Table 1. After PCR amplification with a PCR Phusion kit (NEB) according to the manufacturer's instructions, DNA fragments were purified through agarose gel electrophoresis, gel extraction, and purification with NucleoSpin Extract III columns (Macherey-Nagel). Probes were generated prior to use with a random hexalabeling kit (Fermentas) with [$\alpha$-$^{32}$P]ATP and with digoxigenin probes (Roche) in the case of the STF library. Southern blot and colony filter hybridization were carried out by essentially standard methods (35).

**Fosmid sequencing.** End sequences from fosmids were determined using pEpiFOS forward and pEpiFOS reverse sequencing primers (Epicenter) with an ABI system according to the instructions of the manufacturer.

Recombinant fosmid DNA was isolated using a Qiagen large-construct kit (Qiagen, Hilden, Germany). For shotgun sequencing, the isolated DNA was fragmented by sonication. The ends of the resulting fragments were repaired with T4 and Klenow polymerase (New England Biolabs, Beverly, MA), and the fragments were size selected, ligated in pUC19 vectors, and transformed into *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA). Two plasmid libraries with 1.3- and 2.5-kb inserts were obtained. Plasmids were isolated by alkaline lysis (BigRobby system; MPI for Molecular Genetics, Berlin, Germany) and used as templates for sequencing with BigDye Terminator chemistry and ABI 3730XL capillary sequencers (Applied Biosystems, Foster City, CA). Sequence quality assessment and assembly were performed using PHRED (11, 12), PHRAP (http://www.phrap.org/phredphrapconsed.html), and Consed (19) software.

**Gene prediction.** Structural rRNAs and tRNAs were determined using Rfam (20) and tRNAscan-SE (30). Protein-coding sequences were predicted by Glimmer3 analysis (9), manually curated using Artemis software (http://www.sanger.ac.uk/Software/Artemis/), and annotated in the High-Throughput Gene Annotation platform (33).

**Phylogenetic analysis.** Alignments of protein sequences were produced with Vector NTI software (Invitrogen). Phylogenetic trees were constructed using neighbor-joining analysis and MEGA 3.1 software. Bootstrap analysis of 100 replicates was performed to validate the positional robustness of the tree topology, and consensus trees are presented.

**Electron microscopy.** Transmission electron microscopy (TEM) was performed with a Philips CM10 (100 kV) or a FEI Tecnai F20 (200 kV) TEM equipped with an Eagle charge-coupled-device camera (4,096 by 4,096 pixels). Cells (either negatively stained by uranyle acetate or left unstained) were adsorbed on carbon-coated copper grids (Plano, Wetzlar, Germany). Images were acquired using EMMenue 4.0 and FEI software.

**Nucleotide sequence accession numbers.** The annotated fosmid sequences have been deposited in GenBank, EMBL, and DDBJ under accession numbers FP312972 to FP312978 and FP312980 to FP312986.

## RESULTS

**Selective collection of uncultivated MTB from different environments.** After incubation for 1 month, the more than 100 microcosms under survey developed diverse and abundant populations of MTB. For example, several marine samples (CUX) contained conspicuous magnetotactic multicellular aggregates (16, 50) in addition to a dominant, homogeneous population of magnetotactic cocci. All microcosms from LCH sediments developed populations of MTB containing several morphotypes, including small spirilla, vibrios, and a large rod with up to 1,000 bullet-shaped magnetosomes that resembled the previously described "*Candidatus* Magnetobacterium bavaricum" (44). A partial 16S rRNA gene with more than 99.7% identity to the sequence of "*Ca*. Magnetobacterium bavaricum" could be amplified from these samples (44). In microcosms from two freshwater ponds (NYM and UL), several coccoid MTB dominated the enrichment, whereas in another eutrophic pond (SFT sample), an apparently monospecific population of magnetococci was predominant, as revealed by TEM and preliminary 16S rRNA gene analysis (14–16).

Of the several techniques tested for magnetic collection of MTB, "magnetic racetrack" sampling (16, 39, 51) was highly specific but yielded only low cell numbers insufficient for library construction, and we failed in attempts to increase the scale using this technique to harvest cells from larger volumes. Direct magnetic collection (16) yielded large numbers of MTB, while the presence of a higher proportion of nonmagnetic contaminants precluded highly selective targeting of MTB. However, a sufficient number of cells was obtained by these techniques to allow extraction of genomic DNA and construction of an STF library. In contrast, by our two-step enrichment procedure, which combines magnetic collection with subsequent enrichment of MTB in a custom-build device, roughly $10^8$ MTB cells per liter of sediment could be collected, while contaminant nonmagnetic organisms were virtually absent (<1%), as determined by light and electron microscopy (Fig. 2) as well as by preliminary 16S rRNA gene diversity analysis (data not shown). Notably, we observed a significant number of cells (up to 90%) that switched swimming polarity after prolonged magnetic enrichment, and the number of "south-seekers" seemed to increase with time and the field strength of the magnet. Frequently, individual cells were observed changing swimming direction. Therefore, an additional funnel extension was added to the vessel which enabled simultaneous collection of both north- and south-seeking subpopulations.

**Construction and screening of four metagenomic fosmid libraries.** Genomic DNA isolated from collected MTB was used for the construction of four fosmid libraries (Table 2) with average insert lengths of about 33 kb. Our initial attempts of screening by PCR using degenerate primers failed due to the poor sequence conservation between magnetosome genes from different species at the DNA level (data not shown). Therefore, two alternative methods were used for the screening of 5,823 clones in total. We first screened the SFT library by filter hybridization using a mixed pool of fragments derived from the *mamA*, *-B*, *-E*, and *-U* genes of *M. gryphiswaldense* (14), which identified a single positive fosmid (0904b6_040730; termed "FOS001" within this study). End sequencing of the other clones yielded a further set of two clones (Magm 9502-ae23 and Magm 9502-ao02) with genes with remote similarity to known magnetosome genes (data not shown). All three clones were subjected to total sequencing (Table 3).

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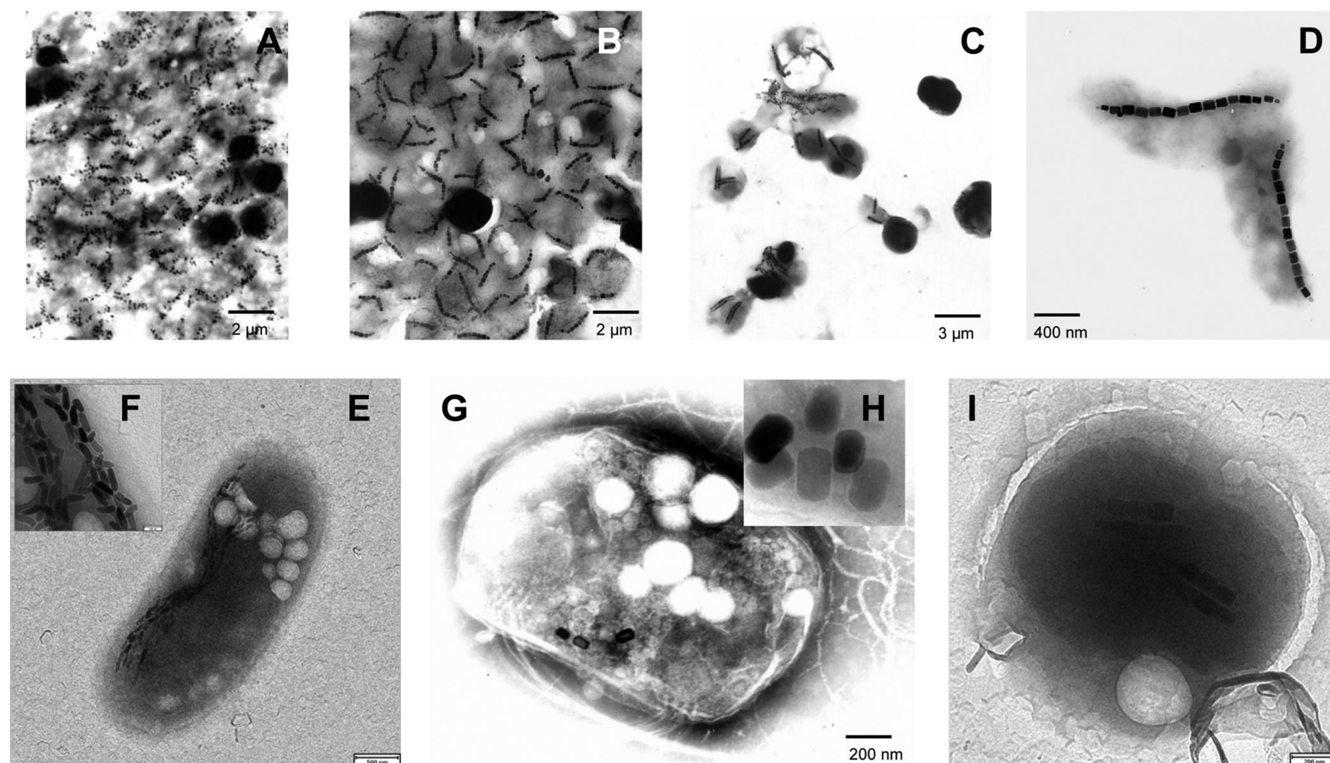


FIG. 2. Examples of different morphotypes present in the sampled habitats. TEM revealed the virtual absence of nonmagnetic contaminants among the highly enriched MTB. The diverse morphotypes included large rod-like "*Ca*. Magnetobacterium bavaricum" cells (C and E). Two different cocci (A, B, C, G, and I) and spirillum-like MTB (D) were observed. The diversity of magnetosome crystals ranged from arrow- or bullet-shaped magnetic particles (F) to cubic and cubo-octahedral morphologies (H). Crystals were arranged either as irregular clusters in cocci of the *Bilophococcus* type (A, G, and H) or in single or multiple chains (B, C, D, F, and I). Scale bars, 500 (E) and 200 (I) mm.

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All other libraries were screened by colony hybridization and subsequent Southern blot analysis with diverse mixtures of probes, including a pool of fragments derived from the *mamA*, *-B*, *-T*, and *-U* genes of *M. gryphiswaldense*, followed by end sequencing. Eleven strongly hybridizing clones (Bb227, Be218, Bg100, Bm116 [termed "Fos002" in this report], Bn096, Bo035, Bs045, Bu093, Bu100, Bt025, and Be094) with end sequences yielding the best hits corresponding to known alphaproteobacterial MTB were selected for total sequencing (Table 3).

**Sequence analysis of metagenomic fosmid clones.** In the case of the STF library, sequencing of three selected candidate clones (Magm 9502-ae23, Magm 9502-ao02, and Fos001) revealed genomic fragments yielding database hits corresponding exclusively to genes of other cultivated MTB. However, except for Fos001, all database hits were virtually unrelated to magnetosome synthesis. The end sequences which initially led to the fosmid selection turned out to represent unclustered genes with weak similarity to magnetosome genes, most likely not related to magnetosome synthesis.

For all other libraries, the sequencing of the fosmid clones Bb227, Be218, Bg100, Bm116 (i.e., Fos002), Bn096, Bo035, Bs045, Bu093, Bu100, Bt025, and Be094 revealed that only Fos002 contained magnetosome genes. All other clones seemed to be of MTB origin, based on database similarities, but lacked clustered magnetosome genes.

The two clones of interest (Fos001 [GC content, 60%] from the STF library and Fos002 [GC content, 61%] from the LCH library) were found to contain several putative operons comprising known magnetosome genes from cultivated MTB (Fig. 3). Clone Fos001 (~36 kb) contains one putative operon with

TABLE 2. Characteristics of the four fosmid libraries constructed in the present study[a]

| Sample | Origin of sample | Characteristic | No of cells collected | Vol of prepared DNA | No. of clones in library | No. of genomes covered[b] |
|---|---|---|---|---|---|---|
| 1 | Chiemsee (LCH) | Mesotrophic | ~$10^8$ | 3.98 μg | 2,100 | ~2.9 |
| 2 | Pond sample[c] | Eutrophic | ~$10^8$ | 7 μg | 2,053 | ~3 |
| 3 | Staβfurt (STF) | Eutrophic | ND[d] | 540 ng | 815 | ~1.1 |
| 4 | Cuxhaven (CUX) | Marine Wadden Sea | ~$10^8$ | 2.36 μg | 820 | ~1.3 |

[a] Sample numbers, numbers of collected cells, amounts of DNA yield, and total numbers of clones in library are shown.
[b] Values are based on a probability of 99% of identification of any DNA fragment in a 5-Mb genome.
[c] Combined sample from UL and pond NYM.
[d] ND, not done.

TABLE 3. Characteristics of fosmids selected for complete sequence analysis

| Sample | Fosmid | Identified by end sequencing[a] | Identified by hybridization[a] | Presence of magnetosome operons[b] | Database accession no. |
|---|---|---|---|---|---|
| 1 | 0904b6_040730/ Fos001 | − | + | + | FP312973 |
| 2 | Magm 9502-ae23 | + | − | − | FP312974 |
| 3 | Magm 9502-ao02 | + | − | − | FP312975 |
| 4 | Bb227 | − | + | − | FP312976 |
| 5 | Be218 | − | + | − | FP312977 |
| 6 | Bg100 | − | + | − | FP312978 |
| 7 | Bm116/Fos002 | − | + | + | FP312985 |
| 8 | Bn096 | − | + | − | FP312980 |
| 9 | Bo035 | − | + | − | FP312981 |
| 10 | Bs045 | − | + | − | FP312982 |
| 11 | Bu093 | − | + | − | FP312986 |
| 12 | Bu100 | − | + | − | FP312983 |
| 13 | Bt025 | − | + | − | FP312984 |
| 14 | Be094 | − | + | − | FP312972 |

[a] +, identified; −, not identified.
[b] +, present; −, not present.

homologues to *mamF*, *-H*, *-I*, *-E*, *-K*, *-M*, *-N*, *-O*, *-P*, *-A*, *-Q*, *-R*, *-B*, and *-S.* Two divergent copies of *mamK*, termed $mamK_I$ and $mamK_{II}$, are present in Fos001. Interestingly, $mamK_I$ encodes a chimeric protein that comprises an N-terminal FtsZ-like domain of 375 amino acids that is fused to a C-terminal domain of 412 amino acids with 47% identity to the actin-like MamK protein from *M. gryphiswaldense* (Fig. 3). The FtsZ domain and the MamK domain appear to represent tubulin- and actin-like proteins, respectively, based on their characteristic lengths, functional domains, and sequence similarities (46% and 25% to *E. coli* FtsZ and MreB, respectively).

In Fos002 (~39 kb), two putative magnetosome gene operons were found. The first operon contains *mamE*, *-K*, *-L*, *-M*, *-N*, *-O*, *-P*, *-A*, *-Q*, *-R*, *-B*, and *-S* and *mamT*. It is separated from the second operon (containing *mamG*, *-F*, and *-D* and *mmsF)* by an open reading frame with strong similarity to the *f*erric *u*ptake *r*egulator (*fur*) gene of *Magnetospirillum* species. In addition, a *mamW* homologue is located downstream of the *mamGFD-mmsF* operon. In both fosmids, the magnetosome clusters seem to extend beyond the ends of the fosmid inserts upstream of *mamE and* downstream of *mamS*, respectively. In addition to the described *mam* and *mms* genes, the regions neighboring the magnetosome operons share further characteristics of known MAIs, such as the presence of three putative transposon-related genes in Fos001 or three hemerythrin-like genes in Fos002 or the presence of genes putatively involved in signaling (two genes in the case of Fos001 and four genes in the case of Fos002). In addition, as with the MAI of previously sequenced MTB, both fosmids contain 10 or 11 hypothetical proteins.

## DISCUSSION

In presenting the findings of our study, we report for the first time the directed cloning of large genomic fragments from uncultivated MTB. High numbers of MTB cells sufficient for library construction could be selectively harvested from different environments by our large-scale enrichment strategy, which utilized the active magnetically directed swimming motility of MTB for mass collection of the bacteria. In disagreement with the long-held belief that MTB from the northern hemisphere predominantly exhibit north-seeking behavior (6, 17), we observed that switching of swimming polarity occurs more frequently than previous observations on uncultivated MTB suggested (40), probably as a consequence of magnetic manipulation, since the switching correlated with the exposure to and strength of the magnetic fields applied for collection (C. Jogler, unpublished data). Whereas this phenomenon clearly requires further investigation, our improved bipolar magnetic trap has the advantage of also simultaneously recovering the substantial number of south-seekers found in several microcosms. Although our initial study was focused on the analysis of highly abundant and oxygen-tolerant MTB species in the population, in future approaches this method could be modified with respect to incubation and collection conditions (e.g., running length, magnetic field configuration, etc.) to target less-abundant or even individual, more oxygen-sensitive species.

All sequenced fosmid inserts and the majority of end sequences yielded database hits corresponding to cultivated MTB species, indicating that, as is consistent with TEM, light microscopic, and 16S rRNA gene analysis, our enrichment process was highly specific and generated only an insignificant background of nonmagnetic contaminants represented in our libraries.

It has been indicated that finding the "needles in the metagenome haystack," i.e., the targeted genes of particular metabolic pathways in complex communities, can be a highly tedious and inefficient procedure unless the diversity of the community under study can be significantly reduced (27). As an extreme example, in a recent study two new glycopeptide gene clusters were identified in a 10,000,000-member DNA megalibrary from desert soil, which contained the equivalent of 100,000 4-Mb bacterial genomes (2). For comparison, the same number of clones (two) harboring large fragments of genomic MAIs was identified by our approach, in which a total of merely 5,823 clones were screened and 400 kb were sequenced from a total insert length of roughly 190 Mb (which is equivalent to about 38 genomes). MTB were shown to represent only below 1% of the total for species in marine and freshwater sediments (16, 41). Thus, for the identification of an MAI-containing clone without preenrichment of MTB the screening of at least 80,000 30-kb clones would have been required by a nonselective high-throughput approach, assuming an average genome size of 5 Mb. This clearly demonstrates the strength and efficiency of our strategy, which is highly selective for both collection and enrichment of MTB as well as for screening and identification of genes relevant to magnetosome formation and thus prevents vast oversampling of th more dominant community members.

Although a complete assessment of species diversity was not within the scope of this study, our preliminary 16S rRNA gene sequence data and previous observations (44) are consistent with the low complexity of the libraries. Since our MTB collections exhibited rather low diversity, we estimated the maximum numbers of species at between 1 (STF), 3 (CUX), and 5 (LCH, UL, and NYM). Assuming an average genome size of 5 Mb for the collected MTB, this would roughly correspond to a number of covered genomes of between one and three (Table 2). Thus, the number of two retrieved magnetosome clusters was within the range of expectations.

We cannot entirely preclude, however, the possibility that we

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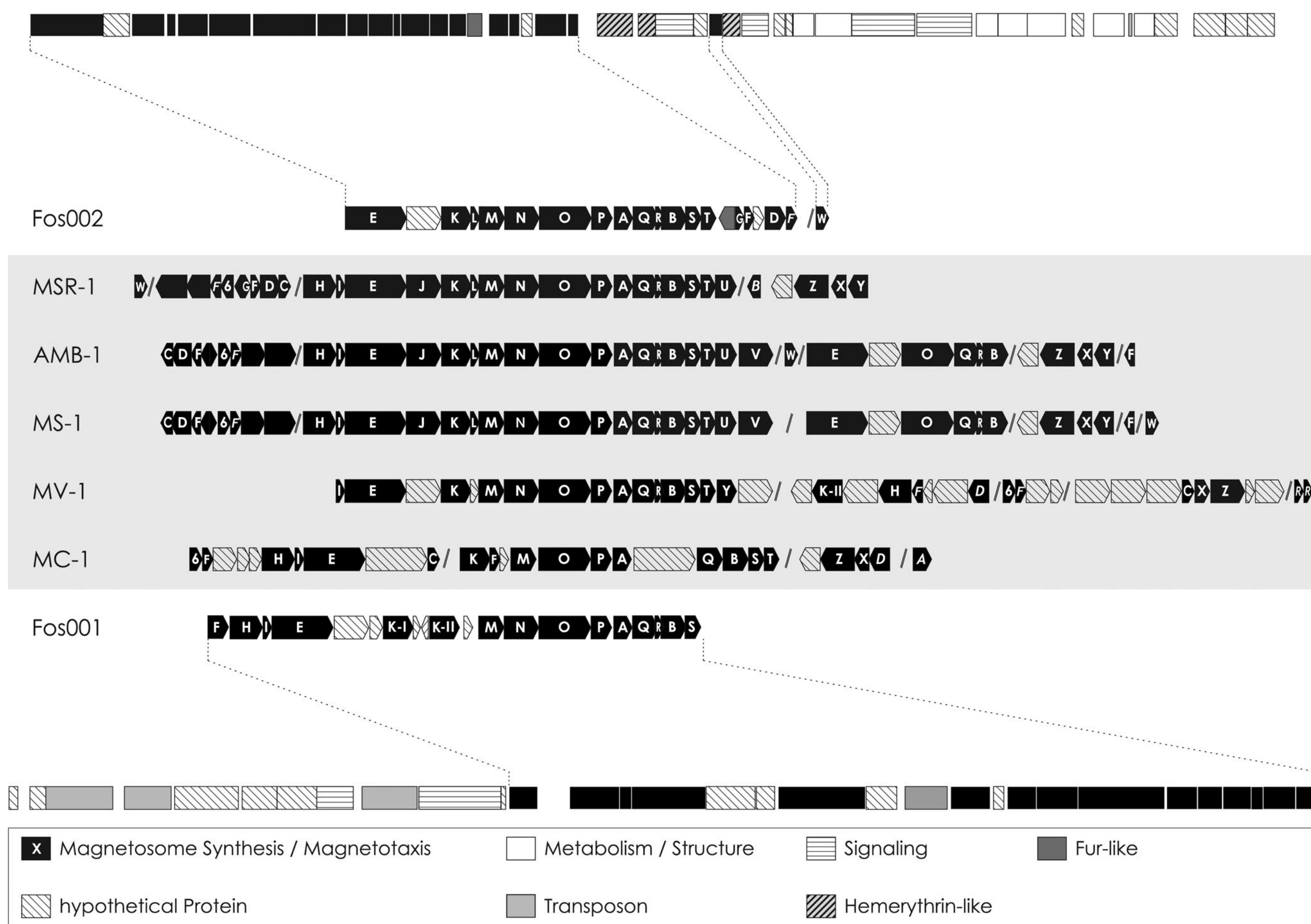


FIG. 3. Molecular organization of two clones harboring magnetosome operons in comparison to the homologous regions from the MAIs of cultivated MTB. In addition to those of magnetosome genes, the entire annotated regions of both metagenomic clones are shown. "Conserved magnetosome proteins" are defined as proteins that occur universally in all magnetospirilla, strain MC-1, and strain MV-1. Magnetosome genes are indicated by capital letters, (e.g., in the case of *mamH*, as "H"), while italic notation indicates homologues with a lower degree of conservation (e value < 10E5); e.g., "*R*" for strain MV-1 stands for "*mamR* like" (exception: "*F*" should be read as *mmsF*). MSR-1, *M. gryphiswaldense*; AMB-1, "*M. magneticum*"; MS-1, *M. magnetotacticum*; MV-1, magnetic vibrio strain MV-1; MC-1, magnetic coccus strain MC-1.

have missed further magnetosome gene clusters present in the initial samples and our libraries. If so, this might have been due to the fact that less-conserved genes from distantly related MTB might have escaped our stringent hybridization conditions, which included using probes deduced from known *mam* genes of alphaproteobacterial MTB. In addition, we observed that several uncultivated MTB species, such as "*Ca.* Magnetobacterium bavaricum," were insensitive to the conventional lysis techniques used for isolation of DNA from environmental samples (C. Jogler and M. Niebler, unpublished data), which might have caused a bias that resulted in their underrepresentation within the libraries (52).

**Characteristics of magnetosome clusters from uncultivated MTB.** Sequence analysis of Fos001 and Fos002 provided evidence that magnetosome genes are also clustered in the genomes of uncultivated MTB within regions strongly resembling putative MAIs. The MAIs of both clones exhibit similarities to but also differences from MAIs in cultivated MTB, which may account for the observed diversity of magnetosome biomineralization and chain organization within several uncultivated MTB. For example, Fos001 contains two *mamK* genes. A peculiarity of $mamK_I$ is that it encodes a chimeric protein comprising an N-terminal FtsZ-like domain fused to a C-terminal MamK-like domain. In magnetospirilla, the actin-like MamK is assumed to form the cytoskeletal magnetosome filament involved in magnetosome chain assembly (26, 38). The computed similarity of only 62.35% between $MamK_{II}$ and the MamK-like domain of $MamK_I$ indicates that they might represent paralogues with slightly distinct functions. FtsZ is a tubulin-like cytoskeletal protein known to assemble into a ring-like structure at the future site of the septum during bacterial cell division (5, 7). However, the fusion of a tubulin-like domain and an actin-like domain within a single polypeptide of Fos001 provides further support for the hypothesis that the two proteins may interact and that, in addition to MamK, FtsZ-like cytoskeletal structures might be involved in magnetosome assembly (34, 38).

The two identified clones share characteristics including rather conserved gene order and content in comparison to the magnetospirilla and MV-1. Interestingly, the *mam* operons of both fosmids lack the *mamJ* gene, which was shown to be involved in

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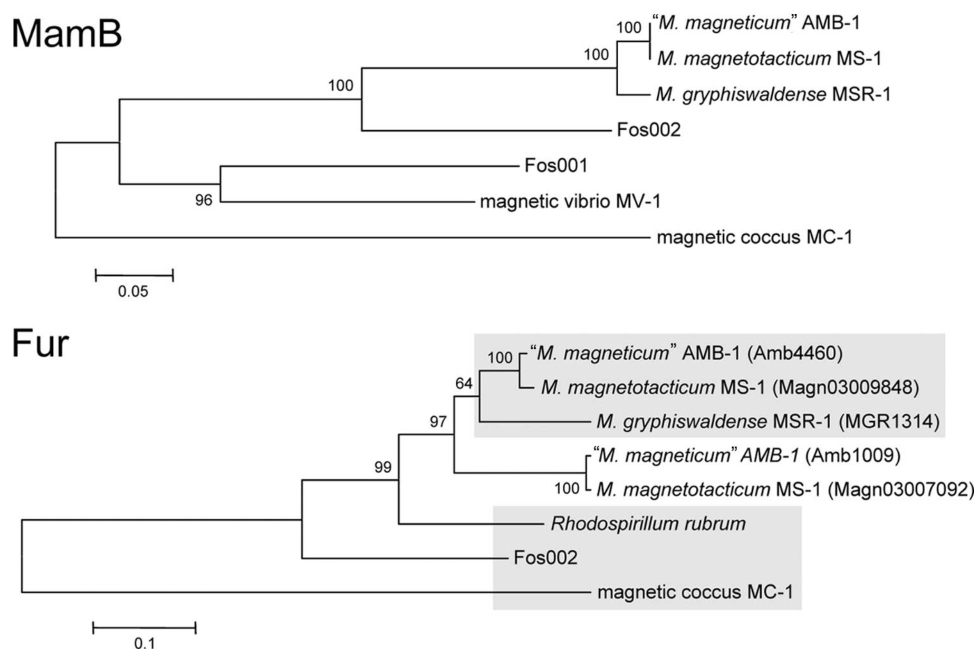


FIG. 4. Similarity trees illustrating the phylogenetic relationships of selected genes. The upper panel shows the phylogenetic affiliations of MamB proteins from all MTB sequenced thus far as an example. Other magnetosome proteins form congruent trees (with some exceptions, as mentioned in the text). The lower panel shows the similarity tree of the Fur (ferric uptake regulator) protein identified in studies of Fos002 that serves as an alternative phylogenetic marker. Shaded boxes indicate Fur proteins from MTB, Fos002, and *Rhodospirillum rubrum*.

magnetosome chain assembly in magnetospirilla (36). Fos002 shows similarity to magnetospirilla with respect to gene content (i.e., the presence of *Magnetospirillum*-specific *mamL* and *mamW* genes) and a conservation of *mamKLMNOPAQRBST* and *mamGFD* organization which is unique within this group (23). The phylogenetic analysis of magnetosome proteins as well as of the ferric uptake regulator protein (Fig. 4) points toward an origination of Fos002 from a *Magnetospirillum* species. The close linkage of this *fur* gene with magnetosome genes might indicate a functional role in magnetosome-related iron metabolism. However, the sequences of, e.g., MmsF and MamP encoded by Fos002 show only 51.4% and 47.3% identity to their homologs in *M. gryphiswaldense*, respectively, and those of "*M. magneticum*" show 96.26% and 78.99% identity to the corresponding *M. gryphiswaldense* sequence.

Fos001 contains homologues to *mamH* and *-I*, which show the same organization as the *mamAB* operon of magnetic spirilla, with the exception of *mamF*, which is part of a different operon in all other sequenced MTB. The duplication of *mamK* in Fos001 was previously also found in the magnetic vibrio MV-1 (23). With some exceptions (*mamQ*, *mamM*, and *mamH*, which branched close to MC-1 and the magnetospirilla), the phylogenetic analysis revealed that the closest similarity is to MV-1 and indicates that Fos001 most likely originates from a bacterium resembling a magnetic vibrio or coccus. However, protein alignments revealed sequence identities in the range from 29 to 100%. For example, MamP of *M. gryphiswaldense* shows only 46.5% sequence identity to that of strain MV-1 and 39.5% to that of strain MC-1.

The detection of homologous magnetosome genes in uncultivated MTB further supports the theory of HGT postulated recently for MTB evolution (37). According to this theory, independent events of HGT led to the appearance of magnetosome genes in individual phyla, while all *Magnetospirillum* species result from one common ancestor, which acquired the MAI before individual species of magnetic spirilla divided (23).

In conclusion, we demonstrated a powerful and economical strategy for metagenomic analysis of MTB from complex environmental samples, thus bypassing the need for their cultivation. Our study provides the first analysis of genes presumably controlling magnetosome formation in uncultivated MTB. As the vast majority of MTB cannot be cultivated, similar studies of different aquatic environments can be expected to provide unprecedented insights into the genetic diversity of these organisms and, in particular, into the genetic basis of magnetotaxis and magnetosome formation. Future approaches might allow functional analysis of the newly discovered magnetosome genes by "surrogate genetics" and heterologous expression of individual genes and entire operons. In addition, the possibility of analysis and reconstruction of entire genomes from individual uncultivated MTB has now come into reach, and realizing that possibility will be just a matter of increased sequencing efforts and improved binning techniques in future studies.

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