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Specific primers for the detection of freshwater alphaproteobacterial magnetotactic cocci

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Summary. Freshwater magnetotactic cocci within Alphaproteobacteria are of ecological interest due to their ubiquitous distribution in aquatic environments as well as their potential roles in iron cycling and the bulk magnetism of sediment. To effectively investigate the diversity and distribution of these cocci, specific primers (FMTCf and FMTCr) were developed. Their specificity, applicability, and effectiveness were then evaluated theoretically and empirically. [**Int Microbiol** 2009; 12(4):237-242]

Keywords: magnetotactic bacteria · freshwater alphaproteobacterial magnetotactic cocci · specific primers ·16S rRNA gene

Introduction

Magnetotactic bacteria (MTB) are able to synthesize membrane-bound [2], nano-sized, and single-domain magnetite or greigite magnetosomes. These particles allow MTB to sense the Earth's magnetic field and to locate the oxic-anoxic interface in chemically stratified environments [1,5,9,24]. MTB are broadly distributed throughout freshwater and marine environments and have been considered to play major roles in iron cycling [9,18,26], and in the bulk magnetism of sediments [22,23,29]. They most commonly include magnetotactic cocci, which are positioned within Alphaproteobacteria [8,10,11,15–17,20,21,25,30–32,35]. Despite the fact that magnetotactic cocci have been studied for more than three decades, their diversity and distribution are not yet fully understood due to difficulties in their culture. So far, only two strains of marine alphaproteobacterial magnetotactic cocci have been successfully grown [14,19], whereas axenic culture of the freshwater variety has not been reported. Therefore, cultivation-independent methods are crucial for investigating the diversity and microbial ecology of freshwater alphaproteobacterial magnetotactic cocci [3]. Classical investigations of these freshwater MTB (and other MTB) exploit their magnetotactic behavior, e.g., through the use of magnetic "capillary racetracks" [37] or magnetic "MTB traps" [13,15], to collect sufficient amounts of MTB, and include the isolation of their 16S rRNA genes through the use of bacterial universal primers. Magnetic approaches are powerful tools for the determination of MTB diversity, and have been successfully used in numerous studies [10,11,13, 15,16,25,27,28,36]. Although magnetic enrichment is a directed and fast approach, its efficiency is highly dependent on the swimming ability of MTB, the magnetic field strength, the swimming distance, and MTB chemotaxis, all of which can result in a biased understanding of the diversity of MTB [17]. Furthermore, in some natural environments, MTB concentrations are too low to allow magnetic enrichment of these bacteria. Instead, environmental samples usually need to be incubated in the laboratory for a certain period of time, during which the MTB community may change [11]

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such that the detected diversity does not reflect the true diversity of MTB in nature.

The use of specific primers allows direct amplification of the partial 16S rRNA genes of freshwater alphaproteobacterial magnetotactic cocci from environmental metagenomic DNA without magnetic enrichment. Theoretically, this approach partly overcomes magnetic enrichment limitations and provides very useful, albeit indirect information about MTB communities [17]. However, to our knowledge, only one set of specific primers (MCF and MCR) for freshwater alphaproteobacterial magnetotactic cocci has been reported [35]. These primers amplify a very short fragment (266 bp) and fail to target newly detected freshwater alphaproteobacterial magnetotactic cocci. The goal of the present study was to design new specific primers that would be more effective in amplifying sequences from freshwater alphaproteobacterial magnetotactic cocci.

Materials and methods

Primers design. After sequences whose sampling site could not be determined were removed, 19 different 16S rRNA gene sequences longer than 1200 bp were identified as corresponding to freshwater alphaproteobacterial magnetotactic cocci (Table 1). These sequences were compared, and specific primers were designed using the Primrose v2.17 program [4] together with visual inspection and manual correction. The specificities of primers FMTCf and FMTCr were theoretically evaluated using the PROBE_MATCH program of the Ribosomal Database Project II (RDP-II, Release 10, Update 11) with default parameters [7].

Extraction of metagenomic DNA from freshwater sediments. Surface sediments (5–10 cm depth) were collected from Lake Miyun (40° 27′ 46.02″ N, 116° 56′ 5.82″ E) and Lake Kunming (39° 59′ 38.52″ N, 116° 15′ 49.14″ E) in Beijing, China. The presence of magnetotactic cocci in Lake Miyun [15,17] and Lake Kunming was confirmed by light and transmission electron microscopy (Fig. 1). Genomic DNA was extracted from 0.5 g (wet weight) of each sediment using the UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

Table 1. In silico analysis of specific primers compared to published 16S rRNA gene sequences of freshwater alphaproteobacterial magnetotactic cocci larger than 1200 bp

			In silico test of primer[a]		
Sequence name	GenBank accession number	Sequence length (bp)	MCF + MCR[b]	FMTCf + FMTCr[c]	Reference
CS103	X61605	1495	+	+	[31]
CS308	X61607	1494	+	+	[31]
CS92	X81182	1498	–	+	[30]
CS81	X81184	1497	–	+	[30]
Magnetic coccus	X80996	1496	+	+	[30]
TB12	X81183	1493	+	+	[30]
TB24	X81185	1494	–	–	[30]
CF2	AJ863135	1427	+	+	[10]
MYG-4	EF370484	1462	+	+	[17]
MYG-5	EF370485	1462	+	+	[17]
MYG-22	EF370486	1462	+	+	[17]
MYG-38	EF370487	1462	+	+	[17]
YDC-1a	FJ667777	1464	–	–	[16]
YDC-1b	FJ667778	1464	–	–	[16]
Clone 7 (OTU B)	EU780674	1466	–	+	[15]
Clone 10 (OTU F)	EU780675	1462	–	+	[15]
Clone 17 (OTU A)	EU780677	1462	+	+	[15]
Clone 29 (OTU G)	EU780680	1464	–	–	[15]
Clone 37 (OTU H)	EU780681	1466	–	+	[15]

[a]In silico test of primer set: + indicates matches to the sequence in target regions; – indicates mismatches to the sequence in target regions.
[b]Primers designed according to reference [35].
[c]Primers designed in this study.

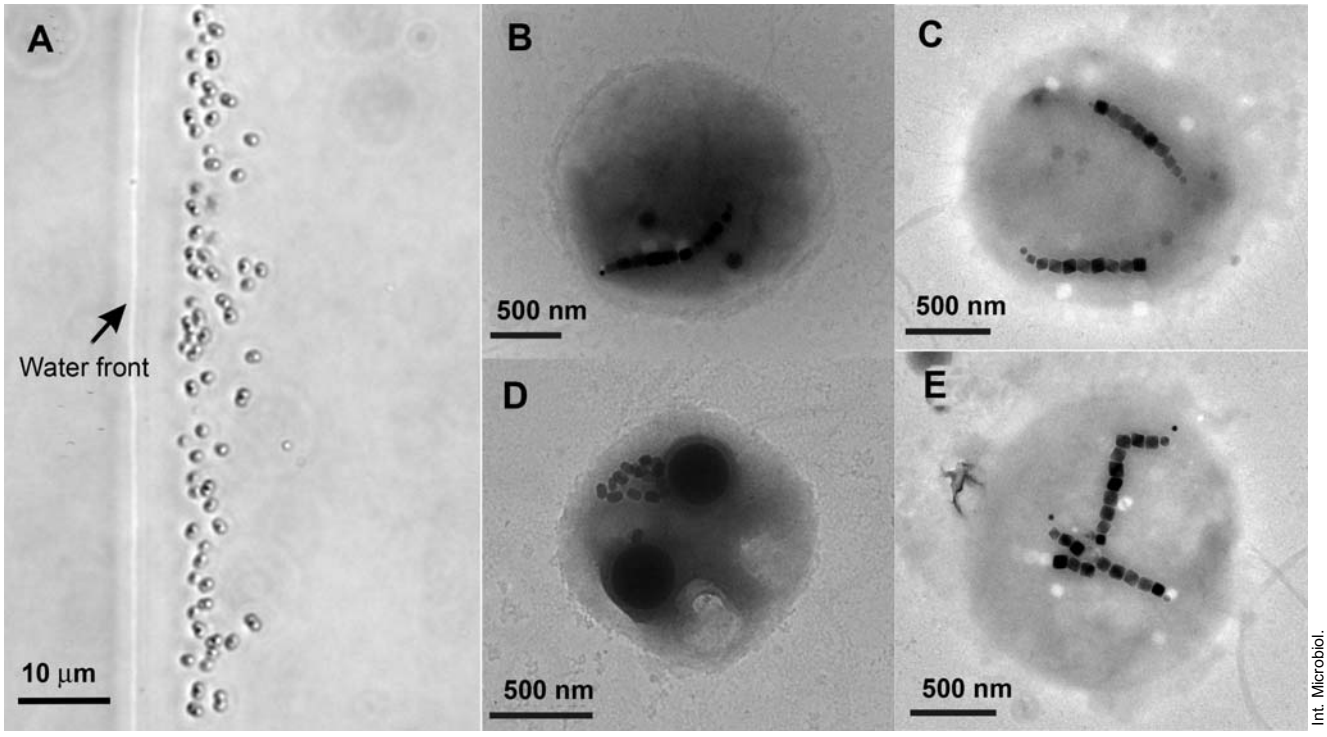


Fig. 1. (**A**) Light and (**B**–**E**) transmission electron microscopy micrographs of magnetotactic cocci from Lake Kunming (Beijing).

PCR amplification using specific primers. To test the applicability of the method and determine the optimal PCR conditions for primers FMTCf and FMTCr, the previously published 16S rRNA gene of uncultured freshwater *Magnetococcus* sp. clone 17 (OTU A) [15] was used as a template. PCR was carried out in a final volume of 20 μl using 0.2 mM of each of the four dNTPs, 2 mM $MaCl_2$, 1.25 U Taq DNA polymerase (Ex Taq, TaKaRa Bio, Shiga, Japan) with 1× Taq buffer, 8 pmol of each primer, and 0.5 μl template DNA. The reactions were run in a T-Gradient thermocycler (Whatman-Biometra, Göttingen, Germany) under the following conditions: 5 min of initial denaturation at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 40–60°C, and 1 min at 72°C, with a final extension at 72°C for 10 min. In addition, environmental metagenomic DNA from Lake Miyun and Lake Kunming was used to examine the robustness of the specific primers. Cycling conditions included an initial 5-min denaturation step at 95°C, 30 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C, and a final 10-min extension at 72°C. All amplification products were analyzed by gel electrophoresis in an agarose gel (0.8%, w/v) stained with ethidium bromide.

DNA cloning, sequencing and phylogenetic analyses. The PCR products of metagenomic DNA were cloned using a commercially available TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Ligation and transformation were carried out according to the manufacturer's instructions. The transformed cells were plated onto Luria-Bertani agar plates containing ampicillin. The 29 randomly selected clones were sequenced using an ABI 3730 genetic analyzer (Beijing Genomics Institute, China), and the resulting sequences checked for chimera formation with the CHECK_CHIMERA software of the RDP-II [7] and the Bellerophon server [12]. The cloned sequences were then compared with existing 16S rRNA genes using GenBank and RDP II, and the 16S rRNA gene sequences aligned using CLUSTAL W [34] (with the following parameters: gap opening penalty = 15; gap extension penalty = 6.66). A phylogenetic tree was subsequently constructed with MEGA v4.0 using the neighbor-joining method (bootstrap phylogeny test: 100 replicates, seed = 64238) [33].

Nucleotide sequence accession numbers. The sequences retrieved in this study were deposited in GenBank under accession numbers GQ338449 to GQ338468.

Results and Discussion

The broad phylogenetic diversity of MTB makes it extremely difficult to design a universal primer set to exclusively amplify all targets. We therefore focused on the most ubiquitous freshwater alphaproteobacterial magnetotactic cocci that formed a coherent phylogenetic lineage [3,32]. A set of specific primers, FMTCf and FMTCr, spanning a region of about 864 bp, was designed. The forward primer, FMTCf (5′-TAAAGCCCTTTYAGTGGGAA-3′), corresponded to positions 431–450 of *Escherichia coli*, and the reverse primer, FMTCr (5′-ACTKCAATCYGAACTGAGACGAGY-3′), to positions 1360–1383 of *E. coli*. The primer FMTCf was a modification of the primer MCF [35], with the C at position 12 replaced by a Y, so that more sequences could be targeted. A theoretical evaluation of primers FMTCf and FMTCr with the program PROBE_MATCH revealed that FMTCf matched with 47 hits, 44 (93.6%) of which belonged to alphaproteobacterial magnetotactic cocci; while the rest were clustered in the Gammaproteobacteria. FMTCr matched with 58 hits, 36 (62.1%) of which were from

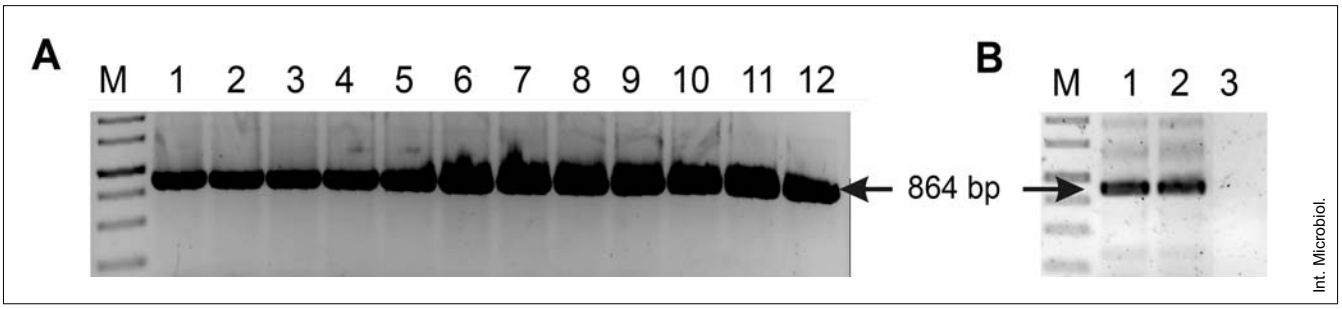
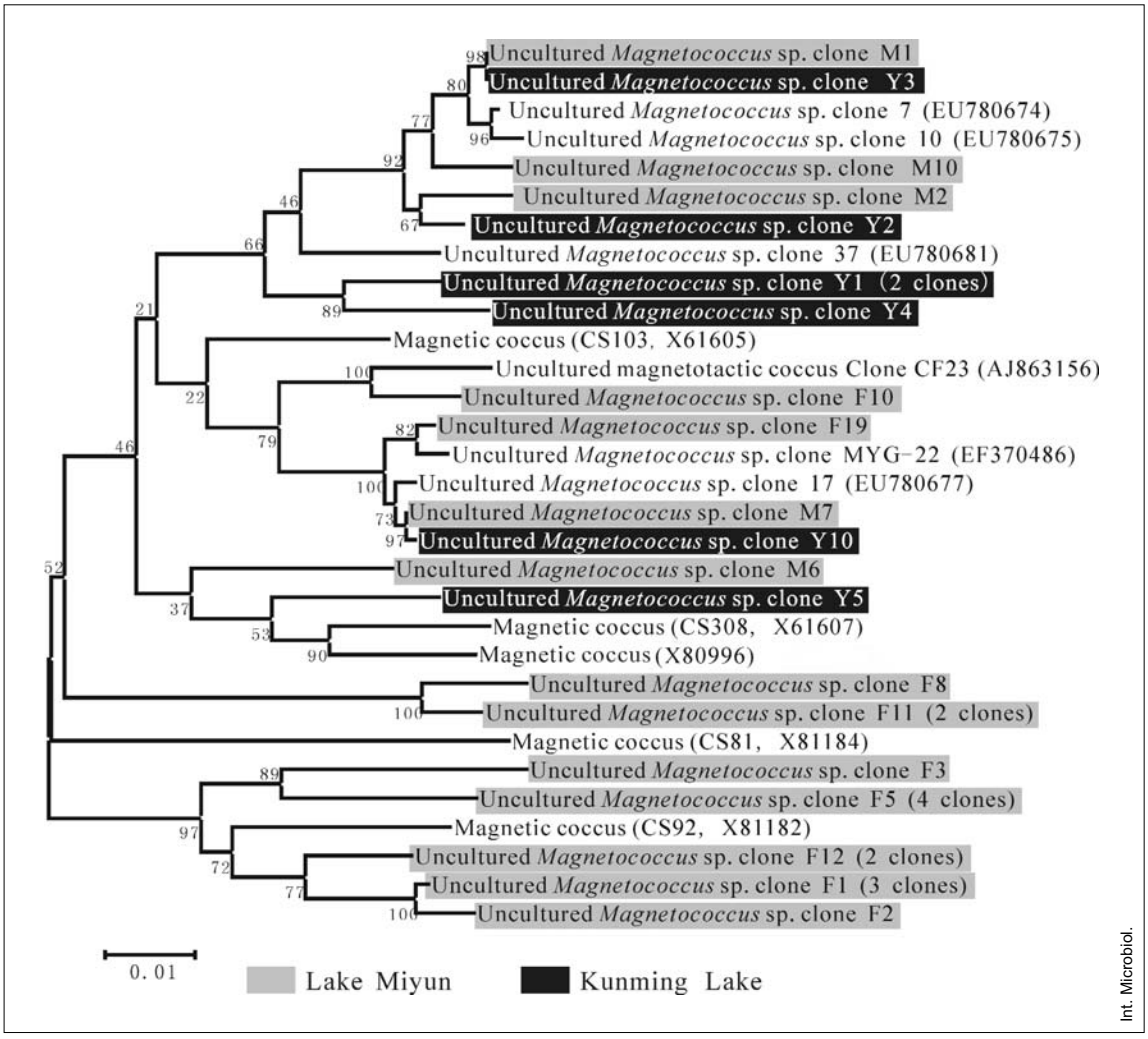


Fig. 2. (**A**) PCR amplification at increasing annealing temperatures using primers FMTCf and FMTCr. The template used in these reactions was the 16S rRNA gene of uncultured freshwater *Magnetococcus* sp. clone 17 (OTU A, EU780677). Lane M, size marker; lane 1, annealing temperature of 40°C; lane 2, 40.6°C; lane 3, 41.9°C; lane 4, 44°C; lane 5, 46.4°C; lane 6, 48.7°C; lane 7, 51.3°C; lane 8, 53.6°C; lane 9, 56°C; lane 10, 58.1°C; lane 11, 59.4°C; and lane 12, 60°C. (**B**) Gel electrophoresis of PCR products from environmental metagenomic DNA with primers FMTCf and FMTCr. Lane M, size marker; lane 1, Lake Kunming (Beijing); lane 2, Lake Miyun (Beijing); and lane 3, PCR negative control.

alphaproteobacterial magnetotactic cocci; the others belonged to non-MTB and were affiliated with the phyla Firmicutes (7 sequences), Acidobacteria (2 sequences), Bacteroidetes (5 sequences), BRC (1 sequence), and unclassified *Bacteria* (3 sequences). Although each primer could potentially bind to bacteria other than MTB, the combined use of the two primers exclusively targeted freshwater alphaproteobacterial magnetotactic cocci.



When the cloned 16S rRNA gene of the freshwater alphaproteobacterial magnetotactic coccus OTU A [15] was used as template, single bands of the expected size (864 bp) were successfully amplified at annealing temperatures of 40–60°C (Fig. 2A), suggesting that the specificities of primers FMTCf and FMTCr were not affected within this temperature range. However, the concentrations of the PCR products declined below 48.7°C (lane 6 in Fig. 1A) and above 59.4°C (lane 11 in Fig. 2A), providing empirical annealing limits for these primers. Therefore, in subsequent experiments, the annealing temperature for primers FMTCf and FMTCr was 50°C.

The robustness of primers FMTCf and FMTCr was experimentally examined using environmental metagenomic DNA extracted from surface sediments of two freshwater lakes, Lake Miyun and Lake Kunming, in Beijing. Although distinct, nonspecific faint bands of various sizes were noted, PCR products generating bands of 864 bp were obtained from both of the tested sediment samples (Fig. 2B). That the development of primers FMTCf and FMTCr had been successful was further confirmed by the fact that all retrieved sequences were affiliated with Alphaproteobacteria and revealed >95% identity with the sequences of published freshwater alphaproteobacterial magnetotactic cocci (Fig. 3).

The overall similarities of the resulting sequences ranged from 90 to 100%, indicating that primers FMTCf and FMTCr were able to amplify a broad spectrum of freshwater alphaproteobacterial magnetotactic cocci (Fig. 3). However, four out of 19 published sequences of freshwater alphaproteobacterial magnetotactic cocci were not fully complementary to primers FMTCf and FMTCr (Table 1), perhaps due to the relatively low similarities of these sequences to those of the other freshwater magnetotactic cocci [15,16,30]. Nevertheless, compared to the previously designed specific primers MCF and MCR [35], the new primers FMTCf and FMTCr extend the current set of MTB group-specific primers [6,35] by amplifying a longer product (about 864 bp) and a greater number of sequences from freshwater alphaproteobacterial magnetotactic cocci (Table 1). These new primers therefore provide a viable method to detect these bacteria. A combination of the specific primers FMTCf and FMTCr with other approaches, e.g., classical magnetic enrichment methods, transmission electron microscopy, and fluorescence in situ hybridization, will improve our understanding of the diversity, distribution, and ecological role of freshwater alphaproteobacterial magnetotactic cocci.

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← **Fig. 3.** Bootstrapped neighbor-joining phylogenetic tree showing the relationships of the16S rRNA genes of freshwater alphaproteobacterial magnetotactic cocci retrieved in this study (shaded sequences) and their close relatives. The percentages of bootstrap replicates are indicated at the nodes.

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