

# Chapter 2

## Enrichment, Purification and Characterization of Environmental Magnetotactic Bacteria

### 2.1 Introduction

Although magnetotactic bacteria (MTB) are ubiquitous in various aquatic or sedimentary environments, the majority of them are still unculturable. One of the most intriguing properties of MTB, in contrast to other bacteria, is their ability to swim along the magnetic field lines, which provides an opportunity to enrich and purify uncultivated MTB cells directly from environmental samples. The principal steps of the proposed procedure for MTB diversity analysis are (1) magnetic enrichment and purification of MTB cells from samples, (2) PCR amplification of 16S rRNA genes or other functional marker genes, (3) construction of clone libraries, (4) sequences sequencing, (5) phylogenetic analysis, and (6) other cultivation-independent analyses, such as fluorescence *in situ* hybridization or single-cell analysis.

For step (1), i.e. magnetic enrichment and purification of MTB, ‘capillary racetrack’ method (Wolfe et al. 1987), which uses a capillary tube and magnet to enrich MTB cells from sediments, has been successfully applied in a number of previous studies (e.g. Flies et al. 2005a). Although ‘capillary racetrack’ is an efficient approach, it has the following two drawbacks. First, only a small number of MTB cells (normally  $10^3$ – $10^5$  cells, occasionally up to  $10^7$  cells) could be enriched, which is not enough for further metagenomic characterizations and magnetic measurements. Second, this approach only enriches MTB cells swimming in one direction, but cannot simultaneously recover both north- and south-seeking subpopulations. To overcome the first drawback, Lins et al. (2003) have developed a homemade large-volume apparatus for harvesting much more MTB cells than using the ‘capillary racetrack’ method. However, this apparatus only has one end and thus cannot simultaneously recover both north- and south-seeking cells either.

Another way of investigating MTB diversity is to directly amplify 16S rRNA genes of MTB cells from environmental metagenomic DNA without requirement of magnetic collection. In this way, PCR primers that specifically target particular MTB populations are necessary. Since MTB are polyphyletic, it is difficult to

design one set of primers to target all MTB communities scattered among the *Proteobacteria*, *Nitrospirae*, and the candidate division OP3. So far only one set of specific primers (MCF and MCR) for freshwater *Alphaproteobacteria* magnetotactic cocci has been reported (Thornhill et al. 1995). These primers amplify a very short fragment ( $\sim 266$  bp) and fail to target newly detected MTB populations in this group.

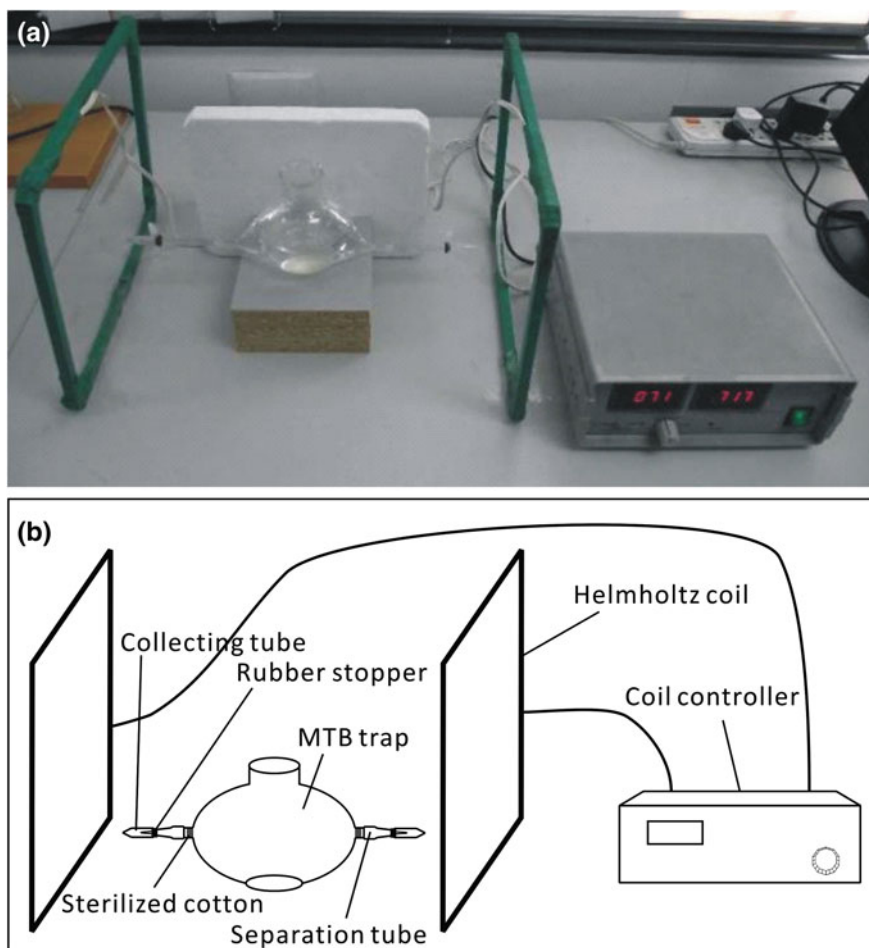
Restriction fragment length polymorphism (RFLP) analysis is a popular method used to survey the phylogenetic diversity of microorganisms (Nocker et al. 2007). This approach is performed to investigate MTB diversity in several studies (e.g. Flies et al. 2005a; Pan et al. 2008). Restriction endonucleases are used in this approach to produce different RFLP patterns, which are defined as operational taxonomic units (OTUs) inferred as single populations within a community. Clearly, selection of restriction endonucleases plays a critical role in RFLP analysis results. Therefore, it is necessary to evaluate the power of different sets of restriction endonucleases in RFLP for identifying the actual populations of MTB.

The aim of this chapter is to develop efficient approaches for MTB enrichment, purification, and community characterization. A double-ended open magnetic separation apparatus and a two-step magnetic enrichment strategy are developed, which allow mass collection of pure environmental MTB for further molecular, genomic and magnetic analyses (Jogler et al. 2009). In addition, a new set of specific primers for freshwater *Alphaproteobacteria* magnetotactic cocci is designed, which could amplify longer product (about 864 bp) and target more populations compared to the old primers (Lin and Pan 2009a). Finally, the resolving powers of different restriction endonucleases are assessed on selected MTB 16S rRNA gene sequences by *in silico* experiments (Lin et al. 2009).

## 2.2 Novel Apparatus and Strategy for MTB Enrichment

For magnetic enrichment of large amount of both north- and south-seeking MTB from environment, a specifically double-ended open separation apparatus (named as MTB trap) is designed (Fig. 2.1) (Jogler et al. 2009). This glass device consists of a reservoir with two opposite funnels connecting with 1.5-ml tubes through rubber stoppers. A small piece of sterilized cotton is loosely packed into the funnel, which will yield much more purified MTB samples. A homemade Helmholtz coil was designed to generate homogenous magnetic field with the strength of about 5–8 times greater than the Earth's magnetic field (approximately 0.25–0.4 mT), by which both north- and south-seeking MTB in the trap can simultaneously swim to the collecting tubes.

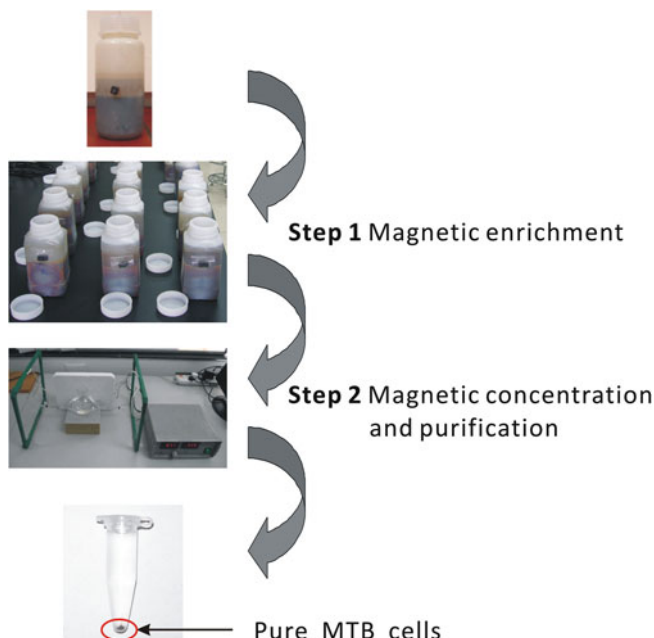
Sediments that contain MTB were divided into 600-ml plastic flasks (microcosms), which were covered with about 100 ml of water. A two-step magnetic enrichment strategy is developed for the MTB trap to harvest large amount of pure MTB cells from microcosms (Fig. 2.2). Step 1 magnetic enrichment: MTB cells were magnetically collected by situating one south magnetic pole and one north



**Fig. 2.1** **a** The photo and **b** The schematic diagram of the double-ended open separation apparatus 'MTB trap'

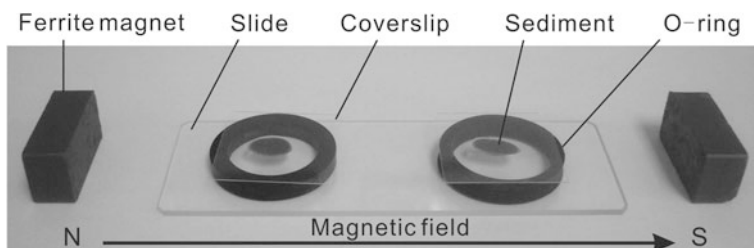
magnetic pole of two ferrite magnets, respectively, on opposite sides of the wall of a microcosm, just above the sediment-water interface. Step 2 magnetic concentration and purification: after 1–2 h of collection, 5 ml of water with accumulated MTB cells near each magnets were removed by a pipette and transferred to the MTB trap. In this step, 20–50 microcosms could be simultaneously collected and pooled. MTB cells in the trap swim along the applied homogeneous magnetic field to the northern or southern collecting tubes. After 2–5 h collection, visible pellets of accumulated pure MTB could be seen by the naked eye at the tips of both collecting tubes (Fig. 2.2).

After MTB collection, carefully disassemble both collecting tubes and centrifuge the tubes in a micro centrifuge at the maximum speed (14,500 rpm) for 3 min

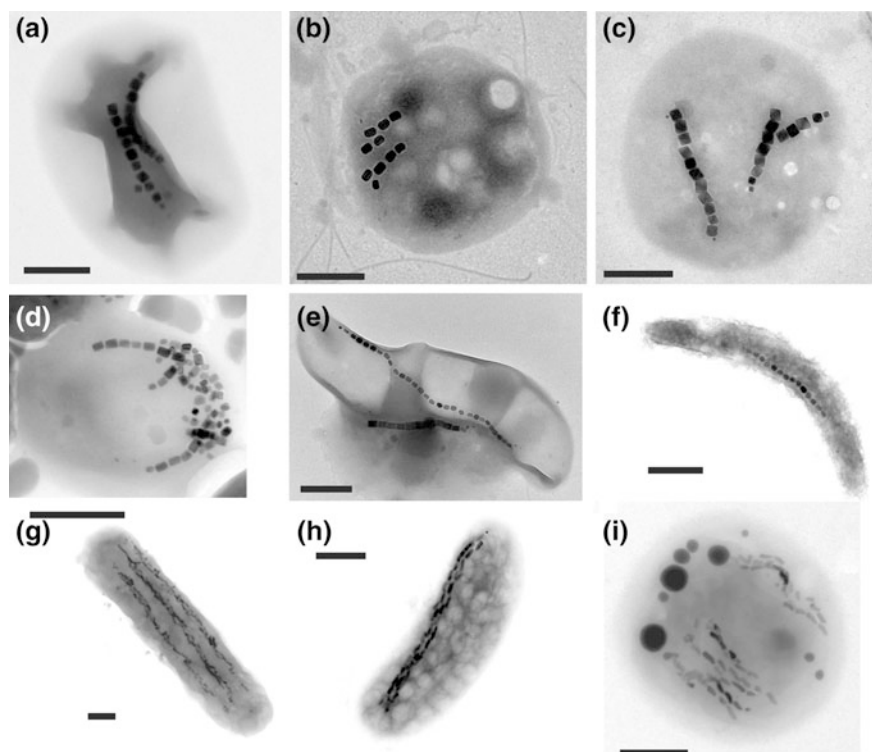


**Fig. 2.2** Procedures of magnetic collection and purification of environmental MTB cells from sediment samples

to pellet MTB cells. Carefully remove the supernatant, add 100  $\mu\text{l}$  of sterilized double-distilled water, gently resuspend the MTB pellet, and mix samples from two tubes. The enriched MTB cells were then observed under light and transmission electron microscope (TEM). A drop of enrichment (20  $\mu\text{l}$ ) was placed on a glass coverslip to check the presence of MTB using the hanging-drop method (Greenberg et al. 2005) under a Olympus BX51 research light microscope (Olympus Optical, Tokyo, Japan) at magnifications of  $\times 400$  and  $\times 1,000$  (Fig. 2.3). For TEM observation, a 20- $\mu\text{l}$  drop of magnetic enrichment was deposited on a formvar-carbon coated grid and allowed to air dry. The grid was



**Fig. 2.3** Schematic diagram of the hanging-drop method



**Fig. 2.4** Representative transmission electron micrographs of MTB cells in Beijing, China. Bars = 500 nm

rinsed with sterilized double-distilled water twice and then observed on a JEM-2100 transmission electron microscope with an accelerating voltage of 200 kV.

Enrichment of enough pure cells is a prerequisite for further diversity, phylogenetic, genomic and magnetic analyses of uncultivated MTB. The MTB trap apparatus and the two-step collection method have the advantages that not only enrich large amount of MTB cells but also collect both north- and south-seeking MTB at the same time. Hanging drop observation and TEM analysis have revealed that contaminant nonmagnetic organisms were virtually absent from the enrichment (<1 %) (Fig. 2.4a–i). The apparatus and the approach developed here are efficient and easy to manipulate, and thus can be used for various MTB studies. The MTB trap is also applicable for collecting and purifying uncultivated magnetotactic protists (e.g., Bazylinski et al. 2012).

## 2.3 Specific Primers for Freshwater *Alphaproteobacteria* Magnetotactic Cocci

In the present study, we focused on the *Alphaproteobacteria* magnetotactic cocci in freshwater environments that form a coherent phylogenetic lineage. These bacteria are the most common MTB group encountered in nature (Moench and Konetzka 1978; Spring et al. 1992; Spring et al. 1994; Thornhill et al. 1995; Spring et al. 1998; Cox et al. 2002; Simmons et al. 2004; Flies et al. 2005b; Flies et al. 2005a; Simmons et al. 2007; Lin et al. 2008; Pan et al. 2008; Lin et al. 2009; Lin and Pan 2009b). To develop a set of specific primers, nineteen 16S rRNA gene sequences longer than 1,200 bp were compared (Table 2.1). The specific primers were designed using the Primrose v2.17 program (available at <http://www.bioinformatics-toolkit.org/>). The forward primer, FMTCf (5'-TAAAGCCCTTTY AGTGGGAA-3'), corresponds to positions 431–450 of *Escherichia coli*, and the reverse primer, FMTCr (5'-ACTKCAATCYGAAGTGAAGAGAGY-3'), corresponds to positions 1360–1383 of *E. coli*. The primer FMTCf was a modification of the primer MCF (Thornhill et al. 1995), which substituted the C at position 12 for Y, thus facilitating targeting more sequences of freshwater magnetotactic cocci. This primer set could amplify PCR product of approximately 864 bp.

Theoretical evaluation of these primers was tested using the PROBE\_MATCH program of the Ribosomal Database Project II (RDP-II, Release 10, Update 11) with default parameters (Cole et al. 2003). FMTCf matched with 47 hits, 44 of which belonged to magnetotactic cocci, the rest were clustered into the *Gamma-proteobacteria*. While, FMTCr matched 58 hits, 36 of which were from magnetotactic cocci. The others belonged to non-MTB bacteria, such as phyla *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, BRC and unclassified Bacteria. Although each of primers potentially binds to bacteria other than MTB, the combined use of both primers exclusively targets the freshwater magnetotactic cocci.

16S rRNA gene of uncultured freshwater *Magnetococcus* sp. clone 17 (OTU A) (Lin et al. 2009) was used as template to test the applicability of primers FMTCf and FMTCr, and to find their optimal PCR condition. PCR reactions in a final volume of 20  $\mu$ l were carried out, using 0.2 mM of each of the four dNTPs, 2 mM  $\text{MgCl}_2$ , 1.25 U *Taq* DNA polymerase (TaKaRa Bio., Shiga, Japan) with  $1 \times$  *Taq* buffer, 8 pmol of each primer and 0.5  $\mu$ l of template DNA. The PCR processes were optimized using a T-Gradient thermocycler (Whatman-Biometra, Göttingen, Germany) using the following conditions: 5 min of initial denaturation at 95  $^{\circ}\text{C}$ , followed by 30 cycles of 30 s at 94  $^{\circ}\text{C}$ , 30 s at 40–60  $^{\circ}\text{C}$ , and 1 min at 72  $^{\circ}\text{C}$ . Final extension was carried out at 72  $^{\circ}\text{C}$  for 10 min. All amplification products were analyzed by gel electrophoresis (0.8 %, w/v). Single bands with expected size ( $\sim$ 864 bp) were successfully amplified at annealing temperatures from 40 to 60  $^{\circ}\text{C}$  (Fig. 2.5a), suggesting that the specificity of primers FMTCf and FMTCr was not affected in this temperature range. However, the concentration of the PCR products declined below 48.7  $^{\circ}\text{C}$  (lane 6 in Fig. 2.5a) and above 59.4  $^{\circ}\text{C}$  (lane 11

**Table 2.1** *In silico* analysis of specific primers compared to published 16S rRNA gene sequences of freshwater *Alphaproteobacteria* magnetotactic cocci larger than 1,200 bp

Sequence name	GenBank accession number	Sequence length (bp)	<i>In silico</i> test of primer <sup>a</sup>		Reference
			MCF + MCR <sup>b</sup>	FMTcF + FMTcR	
CS103	X61605	1,495	+	+	(Spring et al. 1992)
CS308	X61607	1,494	+	+	
CS92	X81182	1,498	—	+	(Spring et al. 1994)
CS81	X81184	1,497	—	+	
<i>M. coccus</i>	X80996	1,496	+	+	
TB12	X81183	1,493	+	+	
TB24	X81185	1,494	—	—	
CF2	AJ863135	1,427	+	+	
MYG-4	EF370484	1,462	+	+	(Flies et al. 2005b)
MYG-5	EF370485	1,462	+	+	
MYG-22	EF370486	1,462	+	+	(Lin et al. 2008)
MYG-38	EF370487	1,462	+	+	
YDC-1a	FJ667777	1,464	—	—	(Lin and Pan 2009b)
YDC-1b	FJ667778	1,464	—	—	
Clone 7	EU780674	1,466	—	+	(Lin et al. 2009)
Clone 10	EU780675	1,462	—	+	
Clone 17	EU780677	1,462	+	+	
Clone 29	EU780680	1,464	—	—	
Clone 37	EU780681	1,466	—	+	

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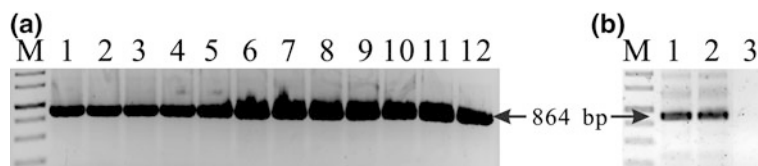
<sup>a</sup> *In silico* test of primer set: + indicates matches to the sequence in target regions; — indicates mismatches to the sequence in target regions

<sup>b</sup> Primers according to reference (Thornhill et al. 1995)

in Fig. 2.5a), providing empirical annealing limits for these primers. We therefore chose 50 °C as the annealing temperature for primers FMTcF and FMTcR in the following experiments.

We further examined the robustness of primers FMTcF and FMTcR using environmental genomic DNA. Surface sediments (5–10 cm) 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. Genomic DNA was extracted from 0.5 g (wet weight) of each sediment using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, USA) according to the manufacturer's instruction. Although some nonspecific faint bands of various sizes were noted, PCR products of expected bands of about 864 bp were obtained from both tested sediment samples (Fig. 2.5b). The target bands were then cloned and transformed using a commercially available TOPO TA cloning kit (Invitrogen, San Diego, USA). In total 29 clones were randomly selected and sequenced using an ABI 3730 genetic analyzer (Beijing Genomics Institute, Beijing, China). The resulting sequences were checked for chimera formation with the CHECK\_CHIMERA software of the



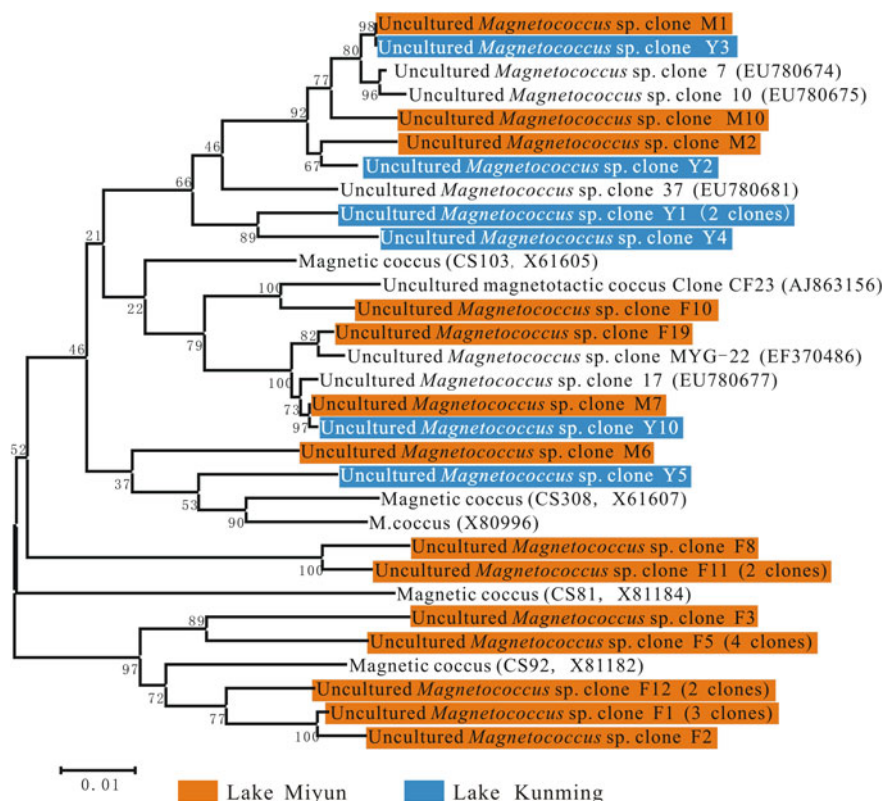


**Fig. 2.5** **a** PCR amplification at increasing annealing temperatures using primers FMTCf and FMTCr. The template used in these reactions was the 16S rRNA gene of uncultured freshwater *Magnetococcus* sp. clone 17 (OTU A, EU780677). Lane M, size marker; lane 1, annealing temperature of 40 °C; lane 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, China); lane 2, Lake Miyun (Beijing, China); and lane 3, PCR negative control. Reprinted with the permission from Lin and Pan (2009a). Copyright 2009 by Spanish Society for Microbiology

RDP-II (Cole et al. 2003) and the Bellerophon server (Huber et al. 2004). Cloned sequences were then compared with existing 16S rRNA genes using the GenBank and RDP-II. The sequences retrieved in this study were deposited in the GenBank database under accession numbers GQ338449 to GQ338468. It is apparent that the development of primers FMTCf and FMTCr is successful since 100 % of retrieved sequences were most related to known freshwater magnetotactic cocci at >95 % similarity (Fig. 2.6). The sequences and their close neighbors were subsequently aligned with ClustalW (Thompson et al. 1994), and the phylogenetic tree was constructed by MEGA 4.0 using a neighbor-joining method with 100 bootstrapping (Tamura et al. 2007). All resulting sequences affiliated with the *Alphaproteobacteria* and the overall similarities of them ranged from 90 to 100 %, indicating that the primers FMTCf and FMTCr are able to amplify a broader coverage of freshwater magnetotactic cocci (Fig. 2.6).

The novel specific primers FMTCf and FMTCr developed here provide a cultivation-independent, reliable and efficient tool to screen environmental samples for the presence of freshwater magnetotactic cocci. Combined with other approaches (e.g., magnetic enrichment, transmission electron microscope, fluorescence *in situ* hybridization, or single-cell analysis), this new primer set will shed new light on the diversity, distribution, and ecological function of freshwater magnetotactic cocci. More sequences retrieved by these primers in future may also be helpful in directing cultivation or identification of novel magnetotactic cocci from freshwater ecosystems. Furthermore, this new primer set could be used to detect fossil 16S rRNA gene sequences of freshwater magnetotactic cocci and therefore may help us better understand paleoenvironmental changes.





**Fig. 2.6** Bootstrapped neighbor-joining phylogenetic tree showing the relationships of the 16S rRNA genes of freshwater *Alphaproteobacteria* magnetotactic cocci retrieved in this study and their close relatives. Modified after the permission from Lin and Pan (2009a). Copyright 2009 by Spanish Society for Microbiology

## 2.4 *In silico* Evaluation of Restriction Endonucleases for RFLP Analysis of MTB

RFLP analysis enables a rapid investigation of microorganism community diversity. The selected restriction endonucleases play a critical role for the analysis results. Moyer et al. (1996) first systematically evaluated the rationale for selecting restriction endonucleases and found that the choice of different restriction enzymes could enhance the information gained from the same bacterial community. In this study, we focused on the evaluation of appropriate restriction endonucleases that are efficient in detecting different MTB populations. Twenty-three nearly complete 16S rRNA gene sequences of MTB were selected for *in silico* analysis, which were from different types of MTB, including cocci, spirilla, vibrioid and rod-shaped bacteria (Table 2.2). Multiple sequence alignments were performed by using

**Table 2.2** Similarity values of selected MTB 16S rRNA sequences from the Genbank database. Two sequences with the similarity above 98 % belong to the same OTU

OTU	Strain	Accession no.	1	2	3	4	5	6	7	8	9	10	11
I	1. CF2	AJ863135	100										
II	2. CS103	X61605	94.8	100									
III	3. CS308	X61607	96.7	94.9	100								
IV	4. MRT-81	EF371482	90.6	91.7	90.1	100							
IV	5. MRT-82	EF371483	90.7	91.9	90.3	99.9	100						
V	6. M-52	EF371485	90.5	90.4	90.7	91.9	92.0	100					
VI	7. M-40	EF371486	90.5	90.4	90.4	92.2	92.3	97.9	100				
VII	8. M-67	EF371491	91.2	92.2	91.3	90.6	90.8	90.9	91.1	100			
VIII	9. MRT-97	EF371493	90.6	91.0	91.0	91.1	91.2	91.0	91.2	91.5	100		
IX	10. Mabrij12	Y13215	91.7	92.1	91.5	90.6	90.8	90.9	90.9	96.8	91.5	100	
X	11. Mabrij58	Y13211	90.3	90.5	90.8	91.7	91.9	96.3	96.0	91.0	91.6	89.9	100
XI	12. MC-1	NC008576	89.2	89.4	90.0	89.3	89.4	89.7	89.3	90.5	91.2	90.3	89.6
XII	13. MYG-22	EF370486	94.1	96.9	92.9	91.7	91.8	90.0	90.0	92.0	90.8	91.8	90.5
XII	14. MYG-38	EF370487	93.9	96.7	92.7	91.4	91.6	89.8	89.7	91.7	90.6	91.6	90.2
XIII	15. MV-1	L06455	83.5	84.6	84.1	83.4	83.6	82.7	83.3	83.8	84.2	84.2	83.0
XIV	16. RS-1	D43944	82.6	83.0	82.7	82.3	82.4	81.8	81.5	82.3	84.0	82.0	81.3
XV	17. AMB-1	D17514	84.5	84.8	83.8	84.5	84.6	83.5	83.9	83.4	84.6	83.6	83.5
XV	18. CC-26	AB033746	84.7	84.5	83.9	84.3	84.5	83.3	83.7	83.3	84.7	83.6	83.5
XV	19. MGT-1	D17515	84.1	84.4	83.4	84.1	84.3	83.1	83.6	83.0	84.2	83.3	83.1
XVI	20. MS-1	M58171	84.1	84.1	83.5	84.0	84.1	82.8	83.2	82.9	84.0	83.1	82.9

(continued)

Table 2.2 (continued)

OTU	Strain	Accession no.	1	2	3	4	5	6	7	8	9	10	11	
XV	21. MSM-6	Y17391	84.8	85.1	84.2	84.8	84.9	83.5	83.9	83.7	84.9	83.9	83.5	
XXVII	22. MSR-1	Y10109	84.9	85.5	84.2	84.9	85.0	83.9	84.5	84.3	84.6	84.4	83.9	
XXVIII	23. WM-1	DQ899734	82.8	83.1	82.3	82.8	83.0	81.8	82.2	81.9	82.9	82.2	81.9	
OTU	Strain	Accession no.	12	13	14	15	16	17	18	19	20	21	22	23
I	1. CF2	AJ863135												
II	2. CS103	X61605												
III	3. CS308	X61607												
IV	4. MRT-81	EF371482												
IV	5. MRT-82	EF371483												
V	6. M-52	EF371485												
VI	7. M-40	EF371486												
VII	8. M-67	EF371491												
VIII	9. MRT-97	EF371493												
IX	10. Mabrij12	Y13215												
X	11. Mabrij58	Y13211												
XI	12. MC-1	NC008576	100											
XII	13. MYG-22	EF370486	89.7	100										
XII	14. MYG-38	EF370487	89.5	99.8	100									
XIII	15. MV-1	L06455	83.1	84.2	84.1	100								
XIV	16. RS-1	D43944	82.2	82.5	82.5	80.5	100							
XV	17. AMB-1	D17514	82.9	84.8	84.6	87.3	80.1	100						
XV	18. CC-26	AB033746	82.8	84.6	84.4	87.3	80.3	98.7	100					
XV	19. MGT-1	D17515	82.5	84.4	84.2	86.9	79.9	99.6	98.4	100				
XXVI	20. MS-1	M58171	82.5	84.1	84.0	87.2	80.0	97.7	97.7	97.4	100			
XV	21. MSM-6	Y17391	83.3	85.0	84.8	87.2	80.5	99.3	99.0	99.0	100	100		
XXVII	22. MSR-1	Y10109	83.2	85.3	85.1	87.5	80.9	95.7	95.2	95.3	94.7	95.8	100	
XXVIII	23. WM-1	DQ899734	81.3	83.1	82.8	86.0	78.8	97.6	97.2	97.3	96.2	98.0	94.3	100

Vector NTI Advance 10.1.1 (Invitrogen, Carlsbad, USA) with default parameters. The exterior ends of sequences were manually trimmed, and the lengths of size were  $\sim 1,350$  bp. Since the sequence similarity between two well-studied model organisms, *Magnetospirillum magnetotacticum* strain MS-1 and *M. magneticum* strain AMB-1, was 97.7 %, here we arbitrarily defined that the identities between two populations  $>98$  % belonged to the same OTU (Sait et al. 2002). According to this criterion, 23 selected MTB were affiliated with 18 OTUs (Table 2.2).

Twelve commonly used sets of restriction endonucleases were evaluated here: *RsaI*; *MspI*; *AluI* plus *RsaI*; *MspI* plus *HaeIII*; *MspI* plus *RsaI*; *HhaI* plus *HaeIII*; *HinPII* plus *MspI*; *MspI* plus *HhaI*; *RsaI* plus *HindIII*; *HhaI*, *RsaI* plus *HaeIII*; *HhaI*, *RsaI* plus *BstUI*; and *AluI*, *DdeI* plus *MspI*. The restriction site determination, the size of RFLP fragments and the expected results of analysis via gel electrophoresis were simulated *in silico* by using Vector NTI Advance 10.1.1 (Invitrogen, Carlsbad, USA). Restriction fragments shorter than 99 bp were not considered and fragments that differ about 7 bp or less in sizes were treated as identical here (Urakawa et al. 1999; Stakenborg et al. 2005). Two parameters were defined in this study: OTUmin, representing the minimal identity of nucleic acid sequences between two strains in the same OUT, and OTUmax, reflecting the maximal identity of nucleic acid sequences between two strains in different OTUs. Both parameters were utilized to evaluate the resolution of the selected restriction endonucleases. The ideal values of OTUmin and OTUmax should be identical with the similarity threshold for different MTB species (i.e. 98 % in this study).

For all selected MTB sequences, the theoretical restriction patterns of different sets of restriction endonucleases were calculated and performed *in silico*. Initial observation revealed that different restriction endonucleases had different power in the ability to differentiate OTUs of MTB (Table 2.3). For example, *MspI* plus *HaeIII*, *AluI*, *DdeI* plus *MspI*, and *MspI* plus *RsaI* were able to detect a large number of different OTUs (18, 17 and 16 OTUs, respectively), while *RsaI*, *RsaI* plus *HindIII*, and *HhaI*, *RsaI* plus *BstUI* could only distinguish 12 OTUs. The average number of restriction fragments per strain for each set of restriction endonucleases was estimated (Table 2.3). The results showed a range of 4–6 fragments per strain. Additionally, the percentages of successfully identified OTUs divided by the real number of OTUs (18 OTUs) were presented (Table 2.3). Of 12 sets of restriction endonucleases, *AluI*, *DdeI* plus *MspI*, *MspI* plus *RsaI* and *MspI* plus *HaeIII* were most efficacious for identifying corrected OTUs (Table 2.3).

We further compared the identities of nucleic acid sequences among same and different OTU(s) acquired by the three sets of restriction endonucleases mentioned above (Table 2.4). The higher OTUmax of *MspI* plus *HaeIII* and *AluI*, *DdeI* plus *MspI*, 99.6 and 99.8 % respectively, meant that using these two sets of restriction endonucleases might overestimate the number of OTUs, i.e., the retrieved number of OTUs by these enzymes was more than the real number of OTUs for some MTB. Correspondingly, lower OTUmin of *MspI* plus *HaeIII* (89.4 %) showed that different strains of MTB might share the same RFLP patterns, which lead to underestimate of community diversity. However, *MspI* plus *RsaI* presented appropriate values of OTUmin and OTUmax (96.7 and 97.7 %, respectively,

**Table 2.3** Summary of *in silico* RFLP analysis of 23 selected MTB 16S rRNA gene sequences

Restriction endonuclease	Number of detected OTUs	Mean number of restriction fragments per strain	%Successful affiliations <sup>a</sup>	OTUs which cannot be differentiated by this set of enzymes <sup>b</sup>
<i>MspI</i> plus <i>HaeIII</i>	18	5	77.8	OTU II, XI and XII
<i>AluI</i> , <i>DdeI</i> plus <i>MspI</i>	17	5	83.3	OTU II and XII; OTU V and VI
<i>MspI</i> plus <i>RsaI</i>	16	5	77.8	OTU II and XII; OTU V and VI
<i>AluI</i> plus <i>RsaI</i>	15	6	66.7	OTU II and XII; OTU VII, IX and X; OTU V and VI
<i>RsaI</i> , <i>HhaI</i> plus <i>HaeIII</i>	15	5	66.7	OTU VII and IX; OTU V and X; OTU XV and XVIII
<i>MspI</i>	15	4	66.7	OTU II and XII; OTU V and VI; OTU X and XI
<i>HinPII</i> plus <i>MspI</i>	14	4	66.7	OTU II and XII; OTU V, VI and VIII
<i>MspI</i> plus <i>HhaI</i>	14	4	66.7	OTU II and XII; OTU V, VI and VIII
<i>HaeIII</i> plus <i>HhaI</i>	13	6	55.6	OTU I and III; OTU V, VII, IX and X; OTU XV and XVIII
<i>RsaI</i>	12	4	50.0	OTU II, V, VI, X and XII; OTU VII and IX;
<i>RsaI</i> Plus <i>HindIII</i>	12	4	50.0	OTU II, V, VI, X and XII; OTU VII and IX; OTU XV and XVI
<i>HhaI</i> , <i>RsaI</i> plus <i>BstUI</i>	12	5	38.9	OTU II and XII; OTU VII and IX; OTU V, VI and X; OTU VIII and XI; OTU XV and XVI

<sup>a</sup> Percentage of successfully identified OTUs divided by the real number of OTUs (18 OTUs, Table 2.2)

<sup>b</sup> Detailed information of OTUs are shown in Table 2.2

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Table 2.4), which were close to the ideal value of 98 %. Thus, *MspI* plus *RsaI* outperformed the others and could better reflect the diversity of selected MTB sequences.

These results clearly indicate that utilization of more restriction endonucleases does not increase the selective power for differentiating MTB. For example, *MspI* alone is able to detect more OTUs than several sets of two or three restriction endonucleases (Table 2.3). Although, set of *MspI* plus *HaeIII* and set of *AluI*, *DdeI* plus *MspI* detect more OTUs in this study, partial of them are false OTUs because the OTUmax of them are very high (99.60 and 99.80 %, Table 2.4). Furthermore, 89.4 % of OTUmin with set of *MspI* plus *HaeIII* suggests that this set of enzymes cannot effectively differentiate selected sequences. Our findings demonstrate that

**Table 2.4** Comparison of OTU<sub>min</sub> and OTU<sub>max</sub> among three sets of restriction endonucleases

Restriction endonucleases	OTU <sub>min</sub> <sup>a</sup> (%)	OTU <sub>max</sub> <sup>b</sup> (%)
<i>MspI</i> plus <i>HaeIII</i>	89.40	99.60
<i>AluI</i> , <i>DdeI</i> plus <i>MspI</i>	96.90	99.80
<i>MspI</i> plus <i>RsaI</i>	96.70	97.70

<sup>a</sup> Minimal identity of nucleic acid sequences between two strains in the same OTU

<sup>b</sup> Maximal identity of nucleic acid sequences between two strains in different OTUs

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exclusive use of RFLP method alone to analyze the community structure of MTB should be treated with care, since this method might bias the diversity of communities by using inappropriate set of enzymes. *RsaI* plus *HindIII* and *MspI* were previously used to investigate the diversity of MTB in microcosms and marine samples (Flies et al. 2005a; Pan et al. 2008). However, both of them only detect 50 and 66.7 % corrected OTUs (Table 2.3), which suggests that these two sets of restriction endonucleases are not appropriate to unravel the real community structure of uncultivated MTB. According to our *in silico* analysis, the *MspI* plus *RsaI* RFLP analysis should have a better resolution power to screen the diversity of MTB. It should be noted that given the faster advances and cheaper price in DNA sequencing technology, MTB clone libraries might be screened by direct sequencing in future rather than using traditionally fingerprint methods, such as RFLP. However, compare to SSU rRNA gene sequencing RFLP could be directly applied with rRNA PCR products to characterize the changes in MTB communities across time and space (so-called community RFLP), and further link these changes with environmental factors.

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Diversity, Biomineralization and Rock Magnetism of  
Magnetotactic Bacteria

Lin, W.

2013, XV, 92 p. 27 illus., 18 illus. in color., Hardcover

ISBN: 978-3-642-38261-1