

## Does capillary racetrack-based enrichment reflect the diversity of uncultivated magnetotactic cocci in environmental samples?

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Received 10 August 2007; accepted 13 November 2007.  
First published online 19 December 2007.

DOI:10.1111/j.1574-6968.2007.01029.x

Editor: Elizabeth Baggs

### Keywords

phylogenetic diversity; 16S rRNA gene; capillary racetrack enrichment; metagenome-based PCR; magnetotactic cocci.

### Abstract

The racetrack-based PCR approach is widely used in phylogenetic analysis of magnetotactic bacteria (MTB), which are isolated from environmental samples using the capillary racetrack method. To evaluate whether the capillary racetrack-based enrichment can truly reflect the diversity of MTB in the targeted environmental sample, phylogenetic diversity studies of MTB enriched from the Miyun lake near Beijing were carried out, using both the capillary racetrack-based PCR and a modified metagenome-based PCR approach. Magnetotactic cocci were identified in the studied sample using both approaches. Comparative studies showed that three clusters of magnetotactic cocci were revealed by the modified metagenome-based PCR approach, while only one of them (e.g. MYG-22 sequence) was detected by the racetrack-based PCR approach from the studied sample. This suggests that the result of capillary racetrack-based enrichment might have been biased by the magnetotaxis of magnetotactic bacteria. It appears that the metagenome-based PCR approach better reflects the original diversity of MTB in the environmental sample.

### Introduction

Magnetotactic bacteria (MTB) are a group of mostly aquatic prokaryotes possessing the trait of magnetotaxis, i.e., they can orientate and swim along geomagnetic field lines. This feature is related to intracellular membrane-bound magnetic crystals (magnetite or greigite), named magnetosomes, arranged in one or more chains (Balkwill *et al.*, 1980; Bazylinski *et al.*, 1993). The function of magnetotaxis is assumed to allow MTB to find a favorable position in vertical chemical gradients in stratified environments (Frankel *et al.*, 1997). Despite their ubiquity in aquatic environments (Fassbinder *et al.*, 1990; Bazylinski *et al.*, 1995; Bazylinski & Frankel, 2004), only a few species of MTB are currently available as laboratory pure cultures because most of them are extremely difficult to cultivate. However, MTB can be enriched, relative to other uncultivated bacteria, by taking advantage of their magnetotactic behavior along magnetic field lines, e.g. by the capillary racetrack method (Wolfe *et al.*, 1987). Morphological and phylogenetic diversities of

uncultivated MTB have been uncovered using this approach, as shown in several studies (Meldrum *et al.*, 1993a, b; Schüler *et al.*, 1999; Flies *et al.*, 2005). However, little is known about the effectiveness of this magnetic approach.

The recent development of the 'metagenome' of the environmental sample (Handelsman *et al.*, 1998), which enables the exploration of the phylogenetic and metabolic diversity of microorganisms without cultivation, provides a promising opportunity to investigate MTB from the environmental samples. Thornhill *et al.* (1995) designed a set of PCR primers that are specific to the 16S rRNA genes of magnetotactic cocci and used them to detect cocci in metagenome extracted from aquatic sediment by direct lysis. Recently, another study has examined the diversity of MTB in different microcosms by denaturing gradient gel electrophoresis (DGGE) analysis with metagenomic DNA from a sediment sample (Flies *et al.*, 2005). The data on the phylogenetic diversity of MTB revealed by the metagenome-based approach appear more valuable when

considering the occurrence of mutants that might lose their magnetotactic ability in the environment.

In this study, the 16S rRNA gene sequences of magnetotactic cocci were compared, which were acquired by the capillary racetrack-based PCR and metagenome-based PCR approaches. In order to evaluate whether the capillary racetrack-based enrichment can truly reflect the diversity of MTB in the studied environmental sample, the studied sample was collected from aquatic sediments of the Miyun lake near Beijing.

## Materials and methods

### Natural environmental samples

A sample of surface sediment with water was obtained from the Miyun water reservoir in northeast Beijing, China, in 2005, at a water depth of *c.* 1–2 m. The sample was stored in a plastic bottle and incubated under ambient temperature and low-light conditions in the laboratory. Distilled water was periodically added during the storage. Bacteriodrome (Pan *et al.*, 2005) examinations showed that the fresh sediment sample hosted plenty of live MTB, mainly cocci.

### Extraction and purification of metagenomic DNA from sediments

The metagenomic DNA was extracted from the fresh sediment sample. The procedure was based on the method of Zhou *et al.* (1996) with the following two modifications. First, sediments of 5 g (wet weight) were frozen in liquid nitrogen and ground with a mortar and pestle until thawed. This process was repeated three times until the color of the sample turned white. Second, the resulting crude nucleic acids pellet was resuspended in 100  $\mu$ L double-distilled water instead of 500  $\mu$ L. The quality of nucleic acid was evaluated on a 1% (w/v) agarose gel. The extracted DNA was purified using a Sepharose 4B spin column to minimize humic contamination based on the method of Jackson *et al.* (1997).

### Capillary racetrack-based approach for isolation of MTB

Enrichment of MTB from the same sediment sample was obtained using the capillary racetrack method (Wolfe *et al.*, 1987) modified by Schüler *et al.* (1999), using capillaries of 9 cm length. The wide-mouth end of the capillaries was not plugged. The tip of the capillaries containing MTB was broken off after 30 min collection with the south pole of a permanent magnet next to it. Collections of MTB from a total of 42 capillaries were pooled to contain sufficient cells for the following PCR amplification.

### PCR amplification, cloning and sequencing

For metagenomic DNA, *c.* 270 bp fragments of 16S rRNA gene were amplified from the total DNA using the oligonucleotide primers MCF and MCR (Thornhill *et al.*, 1995). The PCR reaction buffer was set in 50  $\mu$ L volume with the concentration of reagents as follows: 5  $\mu$ L 10  $\times$  Taq Reaction Buffer (Mg<sup>2+</sup> free, TIANGEN, China), 3  $\mu$ L MgCl<sub>2</sub> (TIANGEN), 2  $\mu$ L of each primer, 1  $\mu$ L dNTPs (TIANGEN), 0.5  $\mu$ L Taq DNA polymerase (50 U  $\mu$ L<sup>-1</sup>, TIANGEN), 1  $\mu$ L template DNA and 35.5  $\mu$ L sterile, double-distilled H<sub>2</sub>O. The amplification was carried out for 32 cycles using a Biometra thermal controller (TGRADIENT). The PCR reaction conditions were 3 min of initial denaturation at 95 °C and 30 cycles of 1 min at 95 °C, 2 min at 55 °C, and 2 min at 72 °C. Final extension was carried out for 5 min at 72 °C.

For the MTB enriched by the capillary racetrack method, nearly complete 16S rRNA gene was directly amplified *in vitro* (Saris *et al.*, 1990) with the universal bacterial primers GM3F and GM4R described previously (Muyzer *et al.*, 1995). The PCR reaction buffer was set in 50  $\mu$ L volume with the following concentration of reagents: 5  $\mu$ L 10  $\times$  Taq Reaction Buffer (Mg<sup>2+</sup> free, TIANGEN), 3  $\mu$ L MgCl<sub>2</sub> (TIANGEN), 2  $\mu$ L of each primer, 1  $\mu$ L dNTPs (TIANGEN), 0.5  $\mu$ L Taq DNA polymerase (50 U  $\mu$ L<sup>-1</sup>, TIANGEN) and 36.5  $\mu$ L MTB cell collection enriched by the capillary racetrack method. The amplification was carried out for 35 cycles using the Biometra thermal controller (TGRADIENT). The PCR reaction conditions were as follows: 5 min of initial denaturation at 95 °C and 33 cycles of 1 min at 95 °C, 1 min at 42 °C and 2 min at 72 °C. Final extension was carried out for 10 min at 72 °C.

The PCR products were run on 1% (w/v) agarose gel with 0.5  $\times$  TBE buffer and stained with ethidium bromide. The resulting DNA bands were located under UV illumination, excised from the gel, and purified with the BioSpin Gel Extraction Kit (BioFlux, China). The purified PCR products were then ligated into a pMD18-T TA cloning vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5 $\alpha$ -competent cells. *Escherichia coli* were cultured overnight and the plasmids were extracted from the cloned cells and purified with the Mini-Plasmid Rapid Isolation Kit (BioDev, China). DNA sequencing was performed with an ABI3730 automatic sequencer using the dideoxy chain-termination method (Sanger *et al.*, 1977).

### Phylogenetic analysis

The partial and nearly complete 16S rRNA gene sequences retrieved in this study were compared with sequences stored in GenBank database using the BLAST algorithm. The tool CLUSTAL W version 1.8 (Thompson *et al.*, 1994) was used for automatic sequence alignment. The alignment was further visually inspected and corrected manually. Phylogenetic

analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) based on the neighbor-joining analysis of selected sequences affiliated with known MTB and the sequences retrieved in this study. Bootstrap analysis of 100 replicates was performed to validate the reproducibility of the branching pattern of the tree. The sequences retrieved in this study were deposited in GenBank database under accession numbers EF370484–EF370499.

## Results

### 16S rRNA gene of magnetotactic cocci from metagenome-based PCR approaches

267-bp-long DNA fragments were amplified from metagenomic DNA in this study using the primers MCF, corresponding to nucleotides 385–404 of 16S rRNA gene in strain CS310 and MCR, corresponding to the complement of nucleotides 635–651 of the CS310 sequence, which were completely conserved with the freshwater magnetotactic cocci of strains CS103, CS308 and CS310 (Thornhill *et al.*, 1995). A partial 16S rRNA gene clone library was then constructed. Twelve sequences were acquired and submitted to the GenBank database. All of them were associated with the freshwater magnetotactic cocci. The similarities among them were relatively low, ranging from 88.8% to 99.6% (Table 1).

### 16S rRNA gene of magnetotactic cocci from racetrack-based PCR approaches

Almost complete 16S rRNA genes were directly amplified by PCR using universal bacterial primers GM3F and GM4R from the racetrack-enriched MTB cells without a prior DNA

extraction. Twelve clones were randomly selected and sequenced. In contrast to the results from the metagenome-based approach, only four sequences were found to be similar to magnetotactic cocci; all of these four sequences have very high similarity (>99.8%) and are thus believed to belong to the same species. For detailed analysis, MYG-22 was selected as the representative. According to the comparison of MYG-22 and the sequences obtained using the metagenome-based PCR approach, it was MY-3 obtained using the metagenome-based PCR approach was identical with corresponding fragments of MYG-22 (Table 1).

### Phylogenetic analysis and comparison

According to the phylogenetic tree (Fig. 1), all sequences retrieved in this study were closely related to freshwater magnetotactic cocci affiliated with the *Alphaproteobacteria*, which could be further grouped into three clusters (A–C). Clusters A and B were comprised exclusively of sequences obtained using the metagenome-based PCR approach. In contrast, only one sequence (MYG-22) in cluster C was obtained by both approaches. Clusters A and C comprised very closely related sequences with similarities from 93.6% to 97% and 97.4% to 99.6%, respectively. In contrast, the degree of relatedness in cluster B was low, ranging from 88.4% to 94% sequence identity.

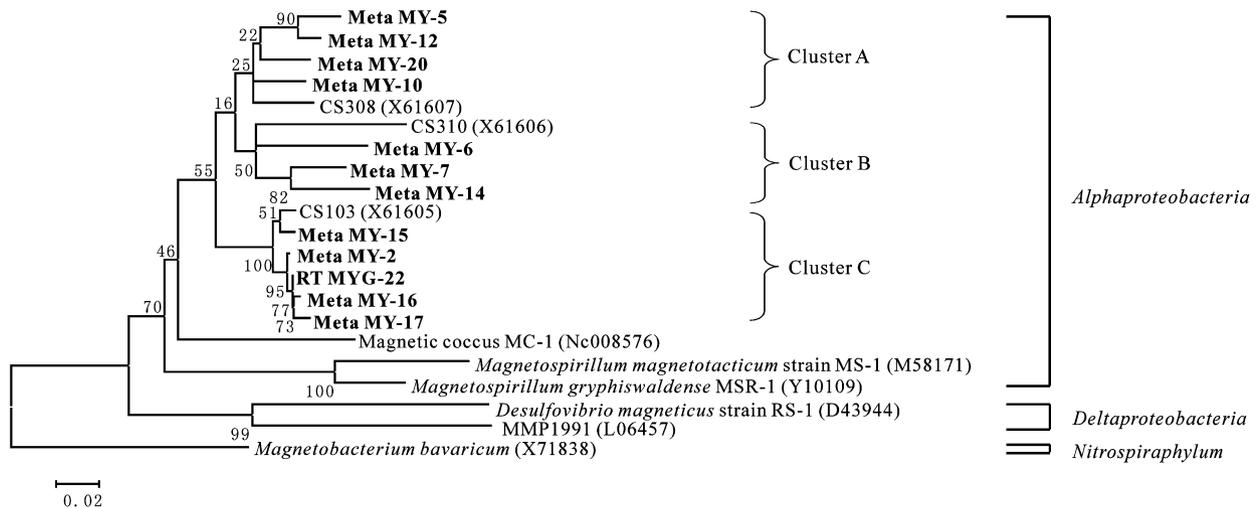
## Discussion

Both the racetrack-based PCR approach and the metagenome-based PCR approach revealed magnetotactic cocci in the studied Miyuan lake sample. Bacteriodrome and light microscopy confirmed the dominance of magnetotactic cocci. The capillary racetrack enrichment is mainly based

**Table 1.** Similarity values of 16S rRNA gene sequences of magnetotactic cocci acquired from this study

Sequence	Approach	Accession no.	% Similarity													
			1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	
1. MY-2	Metagenome-based approach	EF370488	–													
2. MY-3		EF370489	99.6	–												
3. MY-5		EF370490	90.6	90.3	–											
4. MY-6		EF370491	89.9	90.3	90.3	–										
5. MY-7		EF370492	91.0	91.4	92.1	91.0	–									
6. MY-10		EF370493	92.9	92.9	93.6	91.0	91.8	–								
7. MY-12		EF370494	92.1	91.8	97.0	90.3	92.1	94.8	–							
8. MY-14		EF370495	89.5	89.5	91.8	90.3	94.0	90.6	91.8	–						
9. MY-15		EF370496	97.8	98.1	91.0	90.6	92.1	93.3	93.3	91.0	–					
10. MY-16		EF370497	99.3	99.6	89.9	89.9	91.0	92.5	91.4	89.1	97.8	–				
11. MY-17		EF370498	98.9	99.3	89.5	89.5	90.6	92.1	91.0	88.8	97.4	98.9	–			
12. MY-20		EF370499	93.6	93.6	93.6	90.3	91.8	95.1	95.5	91.0	94.4	93.3	92.9	–		
13. MYG-22*	Racetrack-based approach	EF370486	99.6	100	90.3	90.3	91.4	92.9	91.8	89.5	98.1	99.6	99.3	93.6	–	

\*According to the alignment results, only 267 bp were chosen from 1462 nucleotides for this similarity analysis.



**Fig. 1.** Phylogenetic tree based on 16S rRNA gene, showing the relationship between newly found magnetotactic cocci and closely related bacteria. The tree is generated using neighbor-joining analysis. The sequences determined in this study are written in bold. Sequences obtained using the metagenome-based PCR approach are labeled Meta and the sequence obtained using the racetrack-based PCR approach is labeled RT. Labels A, B and C refer to clusters of closely related bacteria that are discussed in the text. Numbers at nodes represent bootstrap values for the nodes (100 times resampling analysis).

on the magnetically directed swimming behaviour of the magnetotactic bacteria. An obvious advantage of the capillary racetrack-based method is that almost complete 16S rRNA gene can be directly amplified from the enriched pure cells by PCR using universal bacterial primers (e.g. GM3F and GM4R). For comparison, the metagenome-based PCR approach (Thornhill *et al.*, 1995; Zhou *et al.*, 1996; Handelsman *et al.*, 1998) was used to study the phylogenetic diversity of cocci in the same sample.

Sequences belonging to magnetotactic cocci acquired from the metagenome-based approach of this study are distributed in three clusters (Fig. 1). As limited by the shortness of amplified DNA fragments, some loss of resolution is to be expected and deeper phylogenetic relationships are not well resolved (e.g. note the low bootstrap value between MY-15 and CS103). Identical sequences (e.g. MY-3 and MYG-22) are revealed from the same sediment sample using both the metagenome-based PCR and racetrack-based PCR approaches, demonstrating the effectiveness of the two different methods. Although the same resulting sequences were found by both methods, the 16S rRNA gene retrieved from the racetrack-based PCR approach had a lower diversity than that obtained using the metagenome-based PCR approach (Table 1).

It is not surprising that the racetrack-based PCR approach revealed fewer cocci species than that of the metagenome-based PCR approach, because the racetrack enrichment sample may have been biased due to the following reasons. The species with lower swimming ability may have been missed in the racetrack enrichment process. The

intensity of the magnetic field, the swimming distance and medium, and chemotaxis may have also negatively affected the efficiency of the racetrack enrichment. In other words, the racetrack enrichment heavily relies on the magnetotaxis of MTB and thus the magnetically extracted species may be dominated by 'good swimmers'. In contrast, the metagenome-based approach does not need the magnetic extraction process. Moreover, it should be noted that possible artifacts could arise from the different quality of templates and the different length of amplified 16S rRNA gene for the two approaches. Almost full-length 16S rRNA gene was directly amplified from cells, which could decrease the yield of the cloned genes in the racetrack-based PCR approach. Thus, it would be worthwhile to compare the two approaches using exactly the same experimental parameters in future investigations.

Based on transmission electron microscopy observations on magnetic extraction samples, one kind of magnetotactic coccus was noted to be dominant in the studied sample, which had a diameter of 1–2  $\mu\text{m}$  and contained one straight magnetosome chain. Therefore, it is tempting to speculate that these sequences (e.g. MY-3 and MYG-22) may come from the observed most abundant magnetotactic coccus. It is also unclear whether magnetotactic cocci in clusters A and B (Fig. 1), which occurred with the metagenome-based PCR approach but not with the racetrack-based PCR approach, are nonmagnetosome mutants or have, for some reason, lost their ability to swim. Additional examination by FISH could help resolve these questions. Studies such as these will further advance one's understanding of the true diversity of magnetotactic cocci in the Miyun lake sediments. In brief,

these findings suggest that the capillary racetrack-based PCR approach may reflect a biased composition of MTB mainly due to the limitation of the enrichment process. To fully understand the diversity of MTB in environmental samples (e.g. sediments), it is thus important to combine metagenome-based and racetrack-based PCR approaches with FISH and transmission electron microscope analyses.

## Acknowledgements

The authors thank the microbiology group at Shandong University for their kind help in the 16S rRNA gene analyses, C. Jogler and C. Lang at Ludwig-Maximilians-University for constructive suggestions and A.J. Biggin at the University of Utrecht for improving the English on an earlier version of manuscript. The authors also thank two anonymous reviewers for their constructive comments. This work was supported by NSFC grants (40325011 and 40221402) and CAS project (KZCX-3-SW-150).

## References

- Balkwill DL, Maratea D & Blakemore RP (1980) Ultrastructure of a magnetic spirillum. *J Bacteriol* **141**: 1399–1408.
- Bazylinski D & Frankel R (2004) Magnetosome formation in prokaryotes. *Nat Rev Microbiol* **2**: 217–230.
- Bazylinski DA, Heywood BR, Mann S & Frankel RB (1993) Fe<sub>3</sub>O<sub>4</sub> and Fe<sub>3</sub>S<sub>4</sub> in a bacterium. *Nature* **366**: 218.
- Bazylinski DA, Frankel RB, Heywood BR, Mann S, King JW, Donaghay PL & Hanson AK (1995) Controlled biomineralization of magnetite (Fe<sub>3</sub>O<sub>4</sub>) and greigite (Fe<sub>3</sub>S<sub>4</sub>) in a magnetotactic bacterium. *Appl Environ Microbiol* **61**: 3232–3239.
- Fassbinder JWE, Stanjek H & Vali H (1990) Occurrence of magnetic bacteria in soil. *Nature* **343**: 161–162.
- Flies CB, Peplies J & Schuler D (2005) Combined approach for characterization of uncultivated magnetotactic bacteria from various aquatic environments. *Appl Environ Microbiol* **71**: 2723–2731.
- Frankel RB, Bazylinski DA, Johnson MS & Taylor BL (1997) Magneto-aerotaxis in marine coccoid bacteria. *Biophys J* **73**: 994–1000.
- Handelsman J, Rondon MR, Brady SE, Clardy J & Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* **5**: 245–249.
- Jackson CR, Harper JP, Willoughby D, Roden EE & Churchill PF (1997) A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Appl Environ Microbiol* **63**: 4993–4995.
- Kumar S, Tamura K & Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.
- Meldrum F, Mann S, Heywood BR, Frankel RB & Bazylinski DA (1993a) Electron microscopy study of magnetosomes in a cultured coccoid magnetotactic bacterium. *Proc R Soc Lond B* **251**: 231–236.
- Meldrum FC, Mann S, Heywood BR, Frankel RB & Bazylinski DA (1993b) Electron microscopy study of magnetosomes in two cultured vibrioid magnetotactic bacteria. *Proc R Soc Lond B* **251**: 237–242.
- Muyzer G, Teske A, Wirsén CO & Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165–172.
- Pan Y, Petersen N, Winkelhofer M, Davila A, Liu Q, Frederichs T, Hanzlik M & Zhu R (2005) Rock magnetic properties of uncultured magnetotactic bacteria. *Earth Planet Sci Lett* **237**: 311–325.
- Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467.
- Saris PE, Paulin LG & Uhlen M (1990) Direct amplification of DNA from colonies of *Bacillus subtilis* and *Escherichia coli* by the polymerase chain reaction. *J Microbiol Methods* **11**: 121–126.
- Schüler D, Spring S & Bazylinski DA (1999) Improved technique for the isolation of magnetotactic spirilla from a freshwater sediment and their phylogenetic characterization. *Syst Appl Microbiol* **22**: 466–471.
- Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.
- Thornhill RH, Burgess JG & Matsunaga T (1995) PCR for direct detection of indigenous uncultured magnetic cocci in sediment and phylogenetic analysis of amplified 16S ribosomal DNA. *Appl Environ Microbiol* **61**: 495–500.
- Wolfe RS, Thauer RK & Pfennig N (1987) A 'capillary racetrack' method for isolation of magnetotactic bacteria. *FEMS Microbiol Ecol* **45**: 31–35.
- Zhou J, Bruns MA & Tiedje JM (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**: 316–322.