Preparation of Genomic DNA from a Single Species of Uncultured Magnetotactic Bacterium by Multiple-Displacement Amplification

Atsushi Arakaki, Mie Shibusawa, Masahito Hosokawa, and Tadashi Matsunaga*

Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan

Received 2 September 2009/Accepted 5 January 2010

Magnetotactic bacteria comprise a phylogenetically diverse group that is capable of synthesizing intracellular magnetic particles. Although various morphotypes of magnetotactic bacteria have been observed in the environment, bacterial strains available in pure culture are currently limited to a few genera due to difficulties in their enrichment and cultivation. In order to obtain genetic information from uncultured magnetotactic bacteria, a genome preparation method that involves magnetic separation of cells, flow cytometry, and multiple displacement amplification (MDA) using 629 polymerase was used in this study. The conditions for the MDA reaction using samples containing 1 to 100 cells were evaluated using a pure-culture magnetotactic bacterium, “Magnetospirillum magneticum AMB-1,” whose complete genome sequence is available. Uniform gene amplification was confirmed by quantitative PCR (Q-PCR) when 100 cells were used as a template. This method was then applied for genome preparation of uncultured magnetotactic bacteria from complex bacterial communities in an aquatic environment. A sample containing 100 cells of the uncultured magnetotactic coccus was prepared by magnetic cell separation and flow cytometry and used as an MDA template. 16S rRNA sequence analysis of the MDA product from these 100 cells revealed that the amplified genomic DNA was from a single species of magnetotactic bacterium that was phylogenetically affiliated with magnetotactic cocci in the Alphaproteobacteria. The combined use of magnetic separation, flow cytometry, and MDA provides a new strategy to access individual genetic information from magnetotactic bacteria in environmental samples.

Magnetotactic bacteria synthesize nanosized intracellular magnetic particles, also referred to as magnetosomes, by accumulating iron ions from the environment. Since the first report on the identification of magnetotactic bacteria (2), the morphological and phylogenetic diversity of these organisms has been observed in various aquatic environments (12, 25, 27, 30). However, bacterial strains available in pure culture are currently limited to a few genera. Desulfovibrio magneticus strain RS-1 is the only isolate of magnetotactic bacteria that is classified among the Deltaproteobacteria (13, 23), while Magnetospirillum spp., marine magnetic vibrio strain MV-1, and “Magnetococcus strain MC-1” are phylogenetically affiliated within the Alphaproteobacteria group (24, 27). This limitation is mainly because not much is known about their metabolic requirements, culturing conditions, and obligate coculture requirements.

Isolation and enrichment of magnetotactic bacteria are generally conducted by applying a magnetic field to a container containing a sediment sample from the environment. The capillary racetrack method is a highly selective enrichment technique that separates magnetotactic bacteria from other contaminants (31). The magnetic separation method that involves the use of a large glass apparatus is efficient and suitable for analyzing samples containing more than 100 ml of sediment and water (12, 16). These techniques have been applied to investigate community structure and phylogenetic diversity of uncultured magnetotactic bacteria in the environment based on 16S rRNA analyses (3, 7, 26, 29). In a recent study, DNA isolation enabling gene cloning was examined by magnetically collecting a large number of magnetotactic cells from environmental samples, and two gene fragments, probably containing parts of magnetosome islands (MAIs) derived from magnetotactic bacteria of the Alphaproteobacteria, were identified (12). However, this approach allows only for sequence gene information to be obtained from a heterogeneous bacterial community in the sample.

Multiple displacement amplification (MDA) can generate microgram quantities of high-quality DNA sample from a few femtograms of DNA template (5, 6). We previously revealed that MDA is a powerful tool for whole-genome amplification from the metagenome of an uncultured bacterial community (32). Studies have been conducted to determine the efficacy of MDA for analyzing genomic DNA preparations from a limited number of bacterial cells (14, 17, 21, 22, 28). Complete genomic sequencing of an uncultured gut symbiont in termites has been achieved using MDA products amplified from approximately 1,000 cells (9). Partial genome sequencing using MDA products from a single uncultured cell has also been reported (17, 22). Such targeted genome analyses using MDA products from a single cell or genetically identical microorganisms is advantageous because it allows the assignment of individual genes to the corresponding microorganisms.

In this study, an improved genome preparation method involving racetrack purification and flow cytometry followed by MDA was investigated by using a small number of uncultured magnetotactic bacteria. This method can be used for the identification of...
new genes from rare magnetotactic bacteria in environmental samples.

MATERIALS AND METHODS

Culture conditions and DNA extraction. “Magnetospirillum magneticonum AMB-1” (ATCC 700264) was cultured as described previously (20). D. magnetitritus RS-1 (ATCC 700980 = DSM13731T) was grown with pyruvate and fumarate as described previously (23). The cells were harvested by centrifugation (8,000 × g, 4°C, 5 min) and suspended in 360 ml of lysis buffer (5 mg/ml lysozyme, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After lysis, the samples were mixed with 120 μl of 10% sodium dodecyl sulfate (SDS) and 50 μl of 10 mg/ml proteinase K and incubated at 37°C for 1 h. Then, 50 μl of 5 M NaCl and 100 μl of e7ytrimethyl ammonium bromide were added, and each mixture was incubated at 65°C for 20 min. The lysates were extracted twice with phenol-chloroform (50:50 [vol/vol]) and once with phenol-isooamyl alcohol (24:1 [vol/vol]). The extracts were mixed with 100 μl of 0.3 M sodium acetate, and the nucleic acids were precipitated with absolute ethanol. Each precipitate was resuspended in sterile water. Environmental DNA was prepared using the same procedure.

Sample collection. In May 2007 and July 2008, sediment samples from the upper sediment layer (approximately 5 cm from the sediment surface) and surface water were collected from well water in Kururi, Chiba, Japan. The samples, which were roughly 200 cm² and contained 200 ml of surface water, were collected in a plastic container and stored at 28°C for a few days.

Racetrack purification of magnetotactic bacteria from environmental samples. A neodymium-boron magnet (cylindrical shape; Φ = 2.0 cm; thickness [h] = 1.0 cm) was attached to the side of a plastic bottle at the sediment-water interface for a few hours to collect the magnetotactic bacteria. Approximately 2 to 3 cm³ of the sediment and water near the magnet were then sampled with an interface for a few hours to collect the magnetotactic bacteria. Approximately 0.3 cm³ of the sample was used for a single racetrack experiment. The sealed end of a Pasteur pipette glass capillary was filled with filtered-sterilized and degassed habitat water (31). A piece of wet cotton was placed in the middle of the capillary. A magnet was attached to the side wall of the end of the pipette for 1 h. Finally, the magnetically separated bacteria were recovered by breaking off the pipette end. The solution containing the magnetotactic bacteria was transferred into a 1.5-ml tube with a sterile hypodermic needle.

Electron microscopy. Cells collected by racetrack purification were observed by transmission electron microscopy. One microliter of the sample, containing approximately 10⁵ to 10⁶ cells, was placed on the surface of a carbon-coated collodion-covered copper mesh grid. The cells were then negatively stained with 1% phosphotungstic acid and washed three times with distilled water. After the sample was dried, the cells were observed under a transmission electron microscope (H-700H; Hitachi, Tokyo, Japan).

Flow cytometric analysis and cell sorting. Flow cytometric analysis and cell sorting were performed using an Epics-Altra Coulter flow cytometer (Beckman Coulter, Hialeah, FL) equipped with a 488-nm argon laser and a 100-μm nozzle orifice. After analysis, the cells were stained with the nucleic acid stain SYBR green (Molecular Probes Inc., Eugene, OR). The forward and side scatter intensities and the green fluorescence were measured at 488 nm. Fluorescent beads of known size (flow cytometry size calibration kit; Invitrogen Corp., Eugene, OR) were used as an internal standard for scatter and fluorescence. Only the cells within gate limits were sorted by the AccuSort mode and collected into 96-well plates containing 10 μl of sterile water per well (Roche Diagnostics K.K.) at 1, 10, and 100 cells per well.

Genome amplification by MDA. Solutions containing cells were heated at 97°C for 3 min and placed on ice; this process was repeated three times. The cell lysate was used for an MDA reaction using REPLI-g Mini or REPLI-g Midi kits (Qiagen, Chatsworth, CA). The REPLI-g Midi kit was used for genome amplification from the uncultured magnetotactic cells collected from the environmental sample. The reaction was performed for 12 h at 30°C according to the manufacturer’s instructions. After the reaction, the samples were heat inactivated at 65°C for 5 min. The DNA concentration of the MDA products was spectrophotometrically measured using a Qubit fluorometer (Invitrogen Corp., Eugene, OR) after PicoGreen reagent staining according to the manufacturer’s instructions (Molecular Probes Inc., Eugene, OR).

Quantitative PCR analysis. Specific forward and reverse primers and 6-carboxyfluorescein (FAM)/BHQ fluorescent/quencher probes for 10 single-copy genes of M. magneticonum AMB-1 were designed (Table 1). Quantitative PCR (Q-PCR) was carried out in a 25-μl reaction mixture containing 1 ng of MDA product, 12.5 μl of premixed ExTaq DNA polymerase (TaKaRa BIO, Shiga, Japan), 200 nM forward and reverse primers, and 500 nM TaqMan probe. The

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>Primers</th>
<th>TaqMan probe</th>
<th>Sequence position (gene ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibdF</td>
<td>Putative GTase</td>
<td>5'-GGCCGACATCAGGCGCTGTT-3'</td>
<td>5'-CGACGGGAGCGCTCTA</td>
<td>239-1336 (amb0001)</td>
</tr>
<tr>
<td>mms16</td>
<td>Magnetosome-specific GTase</td>
<td>5'-TCCCGGCGTTGAGTGTC-3'</td>
<td>5'-CAAAGAATCAGGCGCTCTG-3'</td>
<td>593217-593654 (amb0546)</td>
</tr>
<tr>
<td>mms24 (mms4)</td>
<td>Magnetosome protein Mms24 (MamA)</td>
<td>5'-GCCGCAGACCGCTGTT-3'</td>
<td>5'-CGACCGTTCTCGGCC</td>
<td>1033406-1034071 (amb0971)</td>
</tr>
<tr>
<td>irk-10</td>
<td>Putative potassium channel</td>
<td>5'-ACGGACATTACCCACCTTCTGCT-3'</td>
<td>5'-CTCGTGGGCCGGTTG</td>
<td>1795933-1798571 (amb1660)</td>
</tr>
<tr>
<td>adhP</td>
<td>Putative Zn-dependent alcohol dehydrogenases</td>
<td>5'-GGCAAGATCAGGCGCATTAG-3'</td>
<td>5'-TCGTCGACGCTGCG</td>
<td>2320631-2325015 (amb2144)</td>
</tr>
<tr>
<td>pfkA</td>
<td>Putative 6-phosphofructokinase</td>
<td>5'-TGGCAGCGGCGAAGT-3'</td>
<td>5'-ATCGTGGCAGCCTCA</td>
<td>2583328-2584047 (amb2301)</td>
</tr>
<tr>
<td>qor</td>
<td>Putative NADPH:quinine reductase</td>
<td>5'-CGGAAAAACGGCGAATG-3'</td>
<td>5'-CAAAGCCCATGGCTG</td>
<td>4963992-4963015 (amb4555)</td>
</tr>
<tr>
<td>bfr</td>
<td>Putative ferredoxin</td>
<td>5'-AACCGCGACGCTGATTTGTTG-3'</td>
<td>5'-ACGGCGCTTCACGC</td>
<td>3304453-3329681 (amb5080)</td>
</tr>
<tr>
<td>tonB</td>
<td>Putative periplasmic protein TonB</td>
<td>5'-AGCTGACGCTGCTCTA-3'</td>
<td>5'-CTGAAGTCCACCGGAG</td>
<td>3480878-3479937 (amb3122)</td>
</tr>
<tr>
<td>magA</td>
<td>Ferrous transporter</td>
<td>5'-TGTTCGCGACGGGATGAA-3'</td>
<td>5'-TGACAGATCATCCTGCT</td>
<td>4401335-4400031 (amb3990)</td>
</tr>
</tbody>
</table>
Q-PCR protocol was as follows: 10 s at 95°C and then 50 cycles of 5 s at 95°C and 30 s at 60°C. Reactions were carried out by using a Thermal Cycler Dice real-time system (TaKaRa BIO). For determination of the copy number of each gene in the MDA products, fluorescent signals from diluted samples were detected and compared with a standard curve generated with genomic DNA extracted from *M. magneticum* AMB-1. The standard curve was created using a dilution series of solutions containing $1 \times 10^2$ to $1 \times 10^7$ copies of genomic DNA. No-template control (NTC) reactions for both template-free MDA product and template-free water were conducted to determine nonspecific amplification and contamination of the MDA reaction and Q-PCR assays, respectively. Results from three replicates of TaqMan assays for each gene in the MDA product were averaged.

**Sequencing analysis of 16S rRNA.** Bacterial 16S rRNA was selectively amplified from MDA products and environmental DNA by PCR using PrimeSTAR Max Premix (TaKaRa BIO) with the following amplification profile: 98°C for 10 s and 35 cycles at 98°C for 10 s, 55°C for 5 s, and 72°C for 7 s. The primers 27F (5'-AGGTTTGYATYMGGCTAG-3') and 1492R (5'-GGTTACCTTGTTA CGACTT-3') were used (1). The amplified 16S rRNA was cloned into a pCR4-TOPO vector and transformed in TOP 10 Escherichia coli according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA). All 16S rRNA sequences were analyzed with M13 forward and reverse primers using an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA). For the analysis of bacterial communities in sediment sample, the partial 16S rRNA sequences obtained using the forward primer (approximately 600 bp) were compared with those deposited in the DNA Data Bank of Japan (DDBJ) by using the basic local alignment search tool (BLAST) network service. BLAST analysis of the 16S rRNA genes obtained from MDA products of environmental magnetotactic bacteria was performed using a 1,423-bp region. Multiple-sequence alignment and generation of a phylogenetic tree from 16S rRNA sequences were performed with the ClustalW program at DDBJ (http://www.ddbj.nig.ac.jp/top-j.html) and TreeView software, respectively.

**Nucleotide sequence accession numbers.** The 16S rRNA sequences obtained from environmental DNA have been deposited in GenBank, EMBL, and DDBJ under accession numbers AB518579 to AB518668. A representative 16S rRNA sequence obtained from MDA-amplified genomic DNA of 100 magnetotactic bacterial cells has been deposited under accession number AB537162.

**RESULTS**

**Analysis of bacterial communities in sediment samples obtained from the environment.** A sediment sample containing coccoid magnetotactic cells at an approximate density of $1 \times 10^5$ cells/cm$^3$ was used in this study. In order to determine the microbial diversity of this sediment sample, bacterial 16S rRNA sequences (approximately 600 bp of a partial region) were analyzed. A total of 91 clones were sequenced, and 61 clones exhibited more than 87% similarity to the closest matches of 16S rRNA sequences in the DDBJ database (see Table S1 in the supplemental material). The obtained 91 clones were classified into diverse groups of 17 phyla. The predominant bacterial group found in the environment sample was the Betaproteobacteria, which constituted approximately 29.7% of the obtained clones. The remaining sequences were affiliated with the Alphaproteobacteria (14.3%), Gammaproteobacteria (9.9%), Deltaproteobacteria (2.2%), Acidobacteria (1.1%), Cyanobacteria (2.2%), Bacteroides (18.7%), Fibrobacteres (1.1%), Fusobacteria (1.1%), Planctomycetes (1.1%), Lentisphaerae (3.3%), Thermotogae (1.1%), Verrucomicrobia (1.1%), Firmicutes (2.2%), Actinobacteria (1.1%), candidate division OP11 (4.4%), candidate division OP3 (1.1%), and unknown (4.4%). Both aerobic and anaerobic Gram-positive and -negative bacteria were identified in the sediment sample. One of the predominant bacterial groups consisted of the putative iron-reducing and -oxidizing bacteria. The percentage of 16S rRNA clones phylogenetically affiliated with ferrous iron oxidizers, including *Leptothrix, Rhodoferax,* and "Sideroxoaxidans," was approximately 9%, whereas that of the 16S rRNA clones affiliated with iron reducers, including *Shewanella* and *Geobacter,* was 6%. In the 91 sequenced clones, 10 sequences exhibited high similarity with sequences of two uncultured magnetotactic bacteria. Eight clones were closely related to the uncultured magnetic coccus strain MP17. Two clones were related to the uncultured magnetic coccus strain CS81 (27).

**Preparation of cell samples using capillary racetrack method and flow cytometry.** Magnetotactic bacteria were magnetically separated from the sediment sample by the capillary racetrack method (31). Approximately 0.3 cm$^3$ of the sediment was placed in a single capillary. The average cell number obtained by a single racetrack operation was of the order of $10^5$ to $10^6$ cells. Approximately $10^5$ cells were collected from 20 capillaries. The cells were 1.5 to 2.5 μm in size with coccoid morphology, as observed under a transmission electron microscopy (Fig. 1). Magnetosomes in the cells exhibited elongated rectangular shape. The average magnetosome number per cell was...
approximately 40, and they were mainly located as a large cluster within the cell. Aligned magnetoosomes forming a chain-like structure were also observed beside the large cluster. The cells contained large electron-dense granules (approximately 800 nm in diameter) that were probably polyphosphate, as reported previously (7, 25).

Flow cytometric cell sorting was conducted as follows. In bivariate plots, a region of forward scatter (FS) versus side scatter (SS) was set to separate cells from the background (see Fig. S1A in the supplemental material). Gate 1 (G1) in the histogram of SYBR green fluorescence was set to distinguish bacterial cells from other contaminants (see Fig. S1B). Finally, the Gate 2 (G2) histogram of FS was set to distinguish cells from agglomerations (see Fig. S1C). In the FS histogram, the main peak of uncultured magnetic cocci appeared near the position of 1-μm standard fluorescent beads. FS histograms of M. magneticum AMB-1, D. magneticus RS-1, and E. coli indicated slightly smaller distribution profiles (see Fig. S2). Main peaks, in decreasing order, were uncultured magnetic cocci (see Fig. S1), E. coli (see Fig. S2A), strain RS-1 (see Fig. S2B), and strain AMB-1 (see Fig. S2C). The differences were presumably attributed to cell size, cell structure, or chemical composition, as reported previously (4). Cells in G2 were then sorted to prepare samples containing 1, 10, and 100 cells in a 96-well plate. These cell samples were used for further examination.

**Evaluation of MDA product from M. magneticum AMB-1 by TaqMan Q-PCR assay.** In order to investigate bias in genomic DNA amplification by MDA, the amplified products were evaluated by a TaqMan Q-PCR assay. M. magneticum AMB-1, for which the complete genome sequence is available (19), was used as a model sample to investigate amplification efficiency and accuracy of the MDA reaction. Sorted samples containing 1, 10, or 100 cells were directly utilized for MDA reactions using a REPLI-g Mini kit. After a 12-h reaction, the samples were diluted appropriately and used for the Q-PCR assay. Specific primer sets and TaqMan probes were designed for 10 single-copy genomic loci by the racetrack method. In order to overcome this problem, the genomes were amplified by MDA from small quantities of cells. DNA amounts obtained using the REPLI-g Mini kit were 8 to 10 μg, which is sufficient for genome sequencing.

**DISCUSSION**

The magnetotactic bacterium is one of the most common microorganisms in aquatic environments. Although magnetotactic cells are usually found at cell densities of approximately $10^4$ to $10^5$ cells/cm$^3$, these values vary greatly depending on the sampling site (7, 26, 30). In this study, we examined a sediment sample that contained approximately $1 \times 10^5$ magnetotactic bacteria per cm$^3$, and approximately $10^5$ cells were obtained after purification by the racetrack method. However, bacterial genome sequencing currently requires microgram amounts of purified DNA, which corresponds to approximately $10^6$ to $10^8$ cells (11). It is difficult to collect such a large single bacterial population by the racetrack method. In order to overcome this problem, the genomes were amplified by MDA from small quantities of cells. DNA amounts obtained using the REPLI-g Mini kit were 8 to 10 μg, which is sufficient for genome sequencing.
“Nitrospira mosconiensis”

“Candidatus Magnetobacterium bavaricum”

*Desulfovibrio magneticus* RS-1

*Multicellular magnetotactic prokaryotes*

*Escherichia coli*

*Salmonella* serovar Typhimurium LT2

*Magnetospirillum gryphiswaldense* MSR-1

*Magnetospirillum magneticum* AMB-1

*Magnetospirillum magneticum* MGT-1

*Magnetococcus* sp. MC-1

Uncultured magnetic coccus CS81

Uncultured magnetic coccus CS103

Uncultured magnetic coccus TB24

Uncultured magnetic coccus MP17

Uncultured magnetic coccus

FIG. 3. Phylogenetic tree based on 16S rRNA genes of uncultured magnetotactic bacterium identified from 100-cell MDA product. Magnetotactic bacterial strains are indicated by boldface type. A bacterial cell identified from an MDA product is highlighted. *Salmonella* strain is *Salmonella enterica* Typhimurium LT2.

Amplification bias in the MDA reaction increased remarkably when 1 cell and 10 cells were used as templates. Our results indicated that copy numbers of 10 loci in the genome from the 1-cell and 10-cell MDA samples were much lower than those from a 100-cell sample. Difficulties in genomic amplification using small quantities of cells were reported in previous MDA studies. In quantitative PCR analysis of MDA products from single *E. coli* cells, only about 30% of the amplified DNA was estimated to be specific to the *E. coli* genome (22). The remaining 70% was considered to be synthesis artifacts. Genomic DNA libraries of single uncultured marine bacterioplankton were constructed from a single cell and from 100 cells (28); although several metabolic genes were identified from the 100-cell MDA product, none were detected in any of the single-cell MDA products. A complete genomic sequence from uncharacterized termite group 1 bacteria was obtained using the MDA product from approximately 1,000 bacterial cells (9). These results suggested that approximately 100 to 1,000 bacterial cells are required to attain uniform genome amplification by the general procedure. On the other hand, Marcy et al. proposed use of a 60-nl reaction chamber integrated into a microfluidic device, which reduces nonspecific synthesis from contaminant DNA templates and unfavorable interactions between primers, enabling subsequent genome sequence analysis from a single bacterial cell (17, 18). Further investigation will establish a genome amplification method that enables whole-genome sequencing using a single cell.

In this study, genomic-DNA preparation from uncultured magnetotactic bacteria was demonstrated using an environmental sample containing two phylogenetically distinct magnetotactic cocci. Their proportions in the original sample were estimated to be approximately 9% (8/91 clones) and 2% (2/91 clones).
Genomic DNA from an Uncultured Magnetotactic Bacterium

Vol. 76, 2010