Dynamics of Methanotrophs during in situ Bioremediation

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Changes in diversity of type I and type II methanotrophs during a biostimulation field experiment were investigated for 73 days after the start of treatment by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA fragments. DGGE profiles of the type I and type II methanotrophs yielded simple banding patterns. The type I methanotrophs' profile showed a single intense band at day 10 of biostimulation, and sequence analysis showed 99% similarity with *Methylobacter psychrophilus*. The type II methanotrophs' profile showed a single band appeared at day 31, becoming an intense band after day 40. Sequence analysis of this band pattern showed 99% similarity with *Methylocystis parvus*. These results suggest a possible shift of numerically dominant methanotrophs from type I to type II during the field experiment.

Key words: methanotrophs, DGGE, bioremediation

Methanotrophs are widespread in natural habitats, and have attracted the interest of microbiologists due to their potential for use in bioremediation^{1–5)} and their importance in the global methane cycle⁶⁾. Two categories of methanotrophs, type I and type II, have been identified using a polyphasic approach^{7,8)}. Type I methanotrophs, which contain bundles of intracytoplasmic membranes and assimilate carbon via the ribulose monophosphate pathway, fall within the gamma-*Proteobacteria* category⁹⁾. Type II methanotrophs, which possess intracytoplasmic membranes arranged around the periphery of the cell and assimilate carbon via the serine pathway, fall within the alpha-*Proteobacteria* category⁹⁾. Among the alpha- and gamma-*Proteobacteria*, all close relatives of methanotrophs are methane utilizers¹⁰⁾, with one exception¹¹⁾. Therefore, phylogenetic placement can be a reliable indicator of methanotrophs.

Since methanotrophs are capable of cometabolic oxidation of trichloroethylene (TCE)⁹⁾, which is one of the most common environmental contaminants in soil and groundwater, this bacterial group has received attention for possible use in bioremediation of TCE. Understanding the commu-

nity structure of methanotrophs during bioremediation would facilitate better management of biostimulation with methane injection. However, traditional culture-based approaches are limited for this purpose, because many methanotrophs are difficult to cultivate^{12,13)}. Therefore, culture-independent approaches such as molecular biological techniques must be utilized to examine methanotrophs in the natural environment.

Employing the 16S rRNA gene as a molecular marker, microbiologists have been able to identify the presence of novel, uncultured organisms in nature¹⁴⁾. We exploited the restricted phylogeny of methanotrophs to design type I and type II methanotroph-specific 16S rDNA primers. Using the primers for PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis, changes in the diversity of the methanotrophic bacterial community during a biostimulation field experiment were investigated.

The objectives of this investigation were to better understand the phylogenetic affiliation of the methanotrophs that grew in response to the addition of methane to the on-site biostimulation test site located in Kururi, Kimitsu City, Chiba Prefecture, where the groundwater was contaminated with TCE, and to monitor changes in the diversity of type I and type II methanotrophs during the biostimulation field

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experiment.

Materials and Methods

Sample site, treatment, and sampling

The location of the sampling well, injection well, and extraction well at Kururi experimental site in Chiba prefecture are illustrated in Figure 1. The TCE concentration in groundwater at the experimental site was approximately 200 µg/l and the biostimulation was started on September 25 in 1998. The aquifer characteristics in this field have been described by Hanada *et al.*¹⁵⁾. Details of bacterial abundance and results of TCE remediation studies were reported by Iwamoto *et al.*¹⁶⁾. Biostimulation was conducted by circulating chemically augmented groundwater between the injection well and extraction well at 1.5 l/min. Injected groundwater was constantly augmented under the following conditions; methane addition to a dissolved methane concentration of 10 mg/l for 190 min, no addition of chemical substances for 50 min, addition of oxygen (dissolved oxygen concentration of 30 mg/l) and nutrient salts (KNO₃, 30 mg/l; KH₂PO₄, 15 mg/l) for 190 min and no addition of chemical substances for 50 min. Groundwater samples (500 ml) were collected for microbiological analysis before the start of biostimulation treatment (September 24, 1998), and 10, 24, 31, 40, 45, 52, 60, 66, 73 days after the start of

treatment. The samples were transported to the laboratory on ice and used for DNA extraction within 24 h.

16S rDNA primer design

Type I and type II methanotroph-specific primers were designed by aligning representative 16S rDNA sequences of methanotrophs obtained from DDBJ, GenBank, and RDP with Clustal W (version 1.74). Selected regions unique to each group were tested against GenBank with BLAST (version 2.0.11) and FASTA (version 3.0t74) to check their specificity. The methanotroph-specific primers are listed in Table 1. Chromosomal DNA was extracted from *Methylomonas* sp. KSP III, *Methylomonas* sp. KSW III, *Methylocystis* sp. M, *Methylosinus trichosporium* OB3b, *Aeromonas hydrophila* ATCC 7966, *E. coli* K12 W3110, and *Flavobacterium breve* GIFU 3159, and used for PCR amplification with the methanotroph-specific primer sets to check the specificity.

DNA extraction

Bacterial DNA from the groundwater sample obtained at sampling well S2 was extracted by the method of Teske *et al.*¹⁷⁾, modified as follows. The groundwater sample (200 ml) was filtered with polycarbonate filters (Millipore; pore size 0.2 µm) to collect bacteria. The filter was added to 2 ml of AE buffer (20 mM sodium acetate [pH 5.5],

Fig. 1. Scheme of the location of sampling well S2, injection well, and extraction well at Kururi test site.

Table 1. Methanotroph-specific primers.

Name (sequence)	Target genera	<i>E. coli</i> position
MI-f (5'-GGGTTGTAAAGCACTTCAA-3')	<i>Methylomonas</i> <i>Methylobacter</i> <i>Methylobacterium</i>	423–442
MI-r (5'-CTGGATGTCAAGGGTAGG-3')	<i>Methylomonas</i> <i>Methylobacter</i> <i>Methylobacterium</i>	984–1001
MII-f (5'-TTTCGCCAGGGACGATAAT-3')	<i>Methylocystis</i> <i>Methylosinus</i>	437–455
MII-r (5'-GTCAAAAAGCTGGTAAGGTTCTGC-3')	<i>Methylocystis</i> <i>Methylosinus</i>	973–995

1 mM EDTA) and frozen two times in liquid nitrogen and thawed at room temperature. The filter and buffer were added to 6 ml of Tris-EDTA buffered phenol-chloroform-isoamylalcohol (25 : 24 : 1, pH 8.0) and 60 µl of 25% (w/v) sodium dodecyl sulfate (SDS). After 5 min of incubation at 60°C, the solution was cooled on ice for 3 min and then centrifuged for 5 min at 3,500 g. The supernatant was transferred to a new tube and 250 µl of 2 M sodium acetate, pH 5.2, was added. Nucleic acids were precipitated with 2.5 volumes of 100% ethanol for 3 h at –20°C, and the precipitation was followed by 60 min of centrifugation at 3,500 g. The pellet was washed with 75% ethanol, dried for 10 min under a vacuum, and redissolved in 50 µl of sterile distilled water.

PCR amplification

The DNA extracted from groundwater samples was amplified by PCR with the reagents supplied with AmpliTaq Gold™ (Perkin-Elmer Applied Biosystem) at a magnesium ion concentration of 3 mM and 20 pmol of each primer in a 50 µl reaction mixture. To amplify type I methanotrophic 16S rDNA, the primers MI-f-GC clamp and MI-r were used (Table 1). PCR was carried out under the following conditions; 9 min at 95°C for hot start, followed by 35 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 3 min. The final extension step was at 72°C for 7 min. To amplify type II methanotrophic 16S rDNA, the primers MII-f-GC clamp and MII-r were used (Table 1). PCR was carried out under the following conditions; 9 min at 95°C for hot start, followed by 38 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 3 min. The final extension step was at 72°C for 7 min. The presence and size of amplification products were determined by polyacrylamide gel electrophoresis and ethidium bromide staining.

DGGE analysis

PCR products (30 µl) were loaded onto 6.5% (w/v) polyacrylamide gel in 1X TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0). The 6.5% (w/v) polyacrylamide gel (acrylamide/bisacrylamide [37.5 : 1]) was made with denaturing gradients ranging from 45 to 65% : 100% denaturant contains 7 M urea and 40% formamide. The electrophoresis was run at 55°C, first for 10 min at 20 V and subsequently for 12 h at 100 V. After electrophoresis, the gel was stained for 20 min with SYBR Gold nucleic acid gel stain (Molecular Probes) as specified by the manufacturer. The DGGE gel was scanned with a FluorImager (Molecular Dynamics) using 488 nm argon laser and digital images were obtained by Image QuANT (ver. 4-2-J).

Sequencing of DGGE fragments

The selected DGGE bands were excised with a razor blade. The DNA was retrieved from the acrylamide block with a Centriloter (Amicon) at 150 V for 3 h. The DNA was re-amplified with the same primer set but no GC-clamp. PCR products were purified with a Wizard DNA Clean-up system (Promega) and used for subsequent sequence analysis. The sequence analysis was performed on an ABI 310 automated DNA sequencer (Perkin-Elmer) with the dye-terminator cycle sequencing reaction according to the manufacturer's guidelines.

Nucleotide sequence accession numbers

The sequence data for the selected DGGE bands, i.e. band 1 and band 3 have been deposited in DDBJ, with accession no AB040500 and AB040501, respectively.

Results and Discussion

Methanotroph-specific phylogenetic probes have been developed to detect novel methanotrophs in the natural environment¹⁸). However, most only target one genus. Because of this high specificity, these probes are not suitable for the investigation of the diversity of methanotrophs by DGGE. Therefore, we designed 16S rDNA primers with a broader specificity for type I and type II methanotrophs (Table 1). The number of mismatches between the target region on 16S rRNA and primer is less than 2 in each case. To determine the specificity of the PCR primers, chromosomal DNAs from four methanotrophs and other nonmethanotrophs were used as a template in PCR. Figure 2 (A) shows that only type I methanotrophs, *Methylomonas* sp. KSP III and *Methylomonas* sp. KSW III, gave a PCR product of the expected size with the type I-specific primer set, MI-f-GC clamp and MI-r. Figure 2 (B) shows that only type II methanotrophs, *Methylocystis* sp. M and *Methylosinus trichosporium* OB3b, gave a PCR product of the expected size with the type II specific primer set, MII-f-GC clamp and MII-r. We could separate the bands originating from the four tested strains on the DGGE gel with two primer sets.

Understanding changes in the diversity of the target bacteria during bioremediation will help to make the technology more reliable. However, current knowledge on this subject is insufficient due to limitations associated with traditional culture-based approaches. In the present study, DGGE was used to provide an overview of the diversity of

type I and type II methanotrophs during a biostimulation field experiment. Figure 3 shows the results of DGGE analyses with the type I (A) and with type II methanotroph-specific primer sets (B). The DGGE profiles yielded simple banding patterns. The type I methanotrophic community profile showed a single intense band (band 1) at day 10. This band disappeared at day 24, and faintly re-emerged after day 45. Sequence analysis of this band indicated 99% similarity with *Methylobacter psychrophilus*, which is a type I methanotroph. There is no report that *Methylobacter* possesses soluble methane monooxygenase (sMMO), which has received special attention in the bioremediation of TCE because the enzyme contributes to degradation of TCE. A diffuse band (band 2) was observed throughout the biostimulation treatment. This band was excised from the gel, its DNA retrieved, reamplified with the same type I methanotroph-specific primer set, and analyzed using DGGE. The resulting profile did not show a single band. Therefore, this band may be considered an artifact of the mixed template reaction. The type II methanotrophic community profile showed a single band appearing at day 31, becoming an intense band after day 40. Sequence analysis of this band indicated 99% similarity with *Methylocystis parvus*, which is a type II methanotroph and has sMMO¹²). These results suggest a shift in the methanotrophic community from type I, which has no sMMO, to type II, which has sMMO during the biostimulation treatment.

The amount of methane available has been reported to influence competition between the type I and type II methanotrophs^{19–21}). In growth, type II methanotrophs are

Fig. 2. PCR amplification of methanotroph DNA with type I methanotroph 16S rDNA-specific primers (A) and type II methanotroph 16S rDNA-specific primers (B). Lane 1 contained the pHY size marker. Lanes 2 to 9 contained PCR products obtained after amplification of methanotroph and non-methanotroph DNA template. Lane 9 contained no template DNA. Lane 2, *Methylomonas* sp. KSP III; lane 3, *Methylomonas* sp. KSW III; lane 4, *Methylocystis* sp. M; lane 5, *Methylosinus trichosporium* OB3b; lane 6, *Aeromonas hydrophila*; lane 7, *Escherichia coli*; lane 8, *Fravobacterium breve*.

Fig. 3. DGGE analysis of PCR-amplified 16S rDNA fragments generated with primers specific for type I methanotrophs (A) and for type II methanotrophs (B) from sampling well S2 groundwater. The time course of the experiments is indicated above the lanes in days. B1 day, one day before the start of treatment.

more successful than type I methanotrophs at high methane concentrations, whereas type I methanotrophs are more successful at lower methane concentrations. The change of dominant methanotroph may be dependent on the concentrations of CH_4 and O_2 in groundwater. Continuous methane addition to in situ groundwater appears to favor the growth of type II methanotrophs. It is interesting, and perhaps fortunate, that the methane addition makes type II methanotrophs more competitive, because a number of type II methanotrophs possess sMMO, which is a key enzyme for TCE degradation and has received special attention in the bioremediation of TCE. There seemed to be a correlation between the presence of the type II methanotroph and TCE removal at the experimental site. The removal of TCE stabilized after day 40¹⁶⁾, when a single intense band of type II methanotroph appeared.

The PCR-DGGE approach employed in this investigation

is effective for monitoring changes in the methanotrophic bacterial community during in situ biostimulation. Combining the approach with quantitative molecular techniques like FISH²²⁾, may yield a more detailed picture of the changes in the methanotrophic bacterial community during biostimulation.

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