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Assimilation of glucose-derived carbon into methanogenic archaea in soil under unflooded condition

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ABSTRACT

Ecology of methanogenic archaea, one of the obligate anaerobes, in soil under unflooded condition has not been well documented. To elucidate whether methanogenic archaea assimilate carbon derived from easily decomposable organic matters in soil even under unflooded condition, we analyzed bacterial and methanogenic archaeal communities in a soil incubated with 0.5% ^{12}C- and ^{13}C-glucose under upland condition (60% of maximum water holding capacity) by DNA-stable isotope probing (DNA-SIP) and denaturing gradient gel electrophoresis (DGGE). The added glucose was rapidly decomposed within 7 days of the incubation and the bacterial community immediately responded to the added glucose. Clone analysis of the ^{13}C-enriched bacterial 16S rRNA genes (16S rDNA) on the days 2 and 7 showed that not only aerobic and facultative anaerobic bacteria closely related to *Bacillus* spp., *Ammoniphilus* spp. (or *Oxalophagus* spp.), *Paenibacillus* spp. and *Azotobacter* spp. but also obligate anaerobes such as *Clostridium* spp. and *Anaerobacter* spp. assimilated glucose-derived carbon. By contrast, DGGE band patterns of methanogenic archaeal 16S rDNA obtained from the incubated soil did not change during the incubation period. However, when DGGE band patterns were compared between the ^{12}C- and ^{13}C-glucose treatments after fractionation by isopycnic centrifugation, some DGGE bands were observed from the heavy DNA fractions in the ^{13}C-glucose treatment on the days 7 and 14. The sequences of these DGGE bands were closely related to *Methanocella* spp., *Methanosaeta* spp., *Methanosarcina* spp., the ZC-I group in *Methanosarcinales* and *Methanobacterium* spp. The present study showed that a part of glucose-derived C flows to methanogenic archaea in the soil during the glucose decomposition even under unflooded condition.

1. Introduction

Methanogenic archaea, which belong to *Euryarchaeota* in the domain Archaea, are unique strict anaerobes that produce methane. They are only able to grow under strict anaerobic and reduced conditions. Since they play an important role as terminal decomposers of organic materials in anaerobic environments, methanogenesis and community structures of methanogenic archaea in various anaerobic environments have been well documented (Chaban et al., 2006; Liu and Whitman, 2008).

Irrigated paddy field is one of the most investigated environments, and many studies have been carried out from viewpoints of environmental science, soil science, biogeochemistry and microbial ecology (Takai and Kamura, 1966; Liesack et al., 2000; Le Mer and Roger, 2001; Conrad, 2005, 2007) for several decades. Considerable findings obtained from these previous studies have contributed to development of mitigation strategy of methane emission from paddy fields (Smith et al., 2007) and better understanding of carbon flow in paddy field ecosystem (Kimura et al., 2004). However, unexplained truth of methanogenic archaeal ecology in soil has remained yet for a long time, that is, their common inhabitation in soil under unflooded condition. Several studies by traditional cultivation methods have shown that methanogenic archaea survive in paddy field soils under unflooded periods (Schütz et al., 1989; Asakawa and Hayano, 1995; Adachi et al., 1996; Asakawa et al., 1998). Molecular analyses also showed that their community structures were relatively constant during unflooded periods (Krüger et al., 2005; Watanabe et al., 2006) and after O_2 exposure (Yuan et al., 2009) and not only rRNA but also transcripts of *mcrA* genes, encoding the key-enzyme for methanogenesis "methyl-coenzyme M reductase (MCR)", survived in the paddy field soils even under such unfavorable conditions (Watanabe et al., 2007, 2009; Yuan et al., 2011). Furthermore, the survival of methanogenic archaea in oxic soils such as air-dried paddy soils (Mayer and Conrad, 1990;

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Asakawa and Hayano, 1995; Miyaki et al., 1996; Ueki et al., 1997; Watanabe et al., 2007) and a desert soil (Peters and Conrad, 1995) has been reported. These previous studies suggested that microhabitats are ubiquitous in not only flooded but also unflooded soils and methanogenic archaea are able to survive under such condition. Although some hypotheses, protection by aggregated cells (Zhilina, 1972; Kiener and Leisinger, 1983; Fetzer et al., 1993), endosymbiosis in cyst of anaerobic ciliates (van Hoek et al., 2000), possession of antioxidant genes (Brioukhanov and Netrusov, 2007) and protection by soil aggregates (Liu et al., 2008), are conceivable reasons of survivability of methanogenic archaea in soil under unflooded condition, no study has shown whether methanogenic archaea participate in carbon cycling during decomposition of organic matters in such unfavorable soils.

Easily decomposable organic matters, such as saccharides, lipids and proteins, which are originated from microorganisms, plant roots and plant residues, are the most common substrates for microorganisms in soil. They are rapidly metabolized and incorporated into microbial cells in soil. During the decomposition processes of easily decomposable substrates in soil, a part of their decomposition products may be supplied to methanogenic archaea in anaerobic microsites developed even under unflooded soil condition. To elucidate whether methanogenic archaea utilize carbon originated from easily decomposable substrates in soil under unflooded condition, in the present study, we incubated a paddy soil with ^{12}C- and ^{13}C-glucose under upland condition, challenging to detect methanogenic archaea assimilating glucose-derived ^{13}C by the DNA-stable isotope probing (DNA-SIP) technique. The SIP technique has been used to link organisms or groups of organisms to a specific function within natural environments (Radajewski et al., 2000; Manefield et al., 2002; Dumont and Murrell, 2005) and involves incorporation of isotope-labeled substrates into nucleic acids that can be used to identify microorganisms assimilating the substrate, that is, active microorganisms. We used the SIP technique to follow carbon flow into methanogenic archaea in a soil and discussed a possible explanation of survivability and common inhabitation of methanogenic archaea in soil under unflooded condition.

2. Materials and methods

2.1. Soil sample

One kilogram of soil (total C content, 12.8 g kg^{-1}; total N content, 1.1 g kg^{-1}; pH [H_2O], 6.3; free iron content, 11.0 g kg^{-1}) was taken from a paddy field in the Anjo Research and Extension Center, Aichi, Central Japan (latitude 34°8′N, longitude 137°5′E) on 1 December 2008 under upland condition, where community structures of soil bacteria and methanogenic archaea have been previously investigated by DGGE analysis (Watanabe et al., 2006; Kikuchi et al., 2007). The soil is classified as the Oxyaquic Dystrudept (Soil Survey Staff, 1999). The soil texture is the Light Clay (Japanese Society of Pedology, 1997), consisting of 122, 312, 281 and 296 g kg^{-1} of coarse sand, fine sand, silt and clay, respectively. The field had been managed under double cropping system and rice or soybean and wheat had been cultivated in summer and winter seasons, respectively. The collected soil was homogenized, passed through a 2-mm mesh sieve and stored at 4 °C until use.

2.2. Experimental design for stable isotope probing (SIP)

The soil sample was preliminary incubated at 25 °C for 7 days to obtain a static condition. In this study, three treatments (control [no-glucose], ^{12}C-glucose and ^{13}C-glucose treatments) with triplicate were established. The homogenized soil was divided into three parts and adjusted to 60% of maximum water holding capacity (251 g H_2O kg^{-1} dry soil) by adding sterilized distilled water (control treatment). In the preparation of ^{12}C- and ^{13}C-glucose treatments, ^{12}C- (Wako, Osaka, Japan) and ^{13}C-D-glucose (99% U-$^{13}C_6$, Cambridge Isotope Laboratories, Andover, MA, USA) was dissolved in sterilized distilled water and added to the soil. The final concentration of glucose was adjusted to 5 g kg^{-1} wet soil. Forty grams of each soil was put into a 200-ml Erlenmeyer flask and capped with an air-permeability silicon plug (C-20, ShinEtsu, Tokyo, Japan). In total, 15 flasks were prepared per treatment. All the preparation of adding glucose was conducted in a cold room (4 °C) to prevent changes in microbial activity that occurs immediately after glucose addition. Then the soils were incubated at 30 °C under aerobic and dark condition.

2.3. Glucose concentration in soil

Five grams of soil was collected from the ^{12}C-glucose treatment at 0, 1, 2, 7, 14, 21 and 28 days after incubation and transferred into a 100-ml polypropylene bottle. Twenty milliliters of sterilized distilled water was added to the bottle. The bottle was horizontally shaken at 210 rpm for 30 min (TS-4 N, TAITEC, Koshigaya, Japan). The suspension was centrifuged under 10,000 × g and the supernatant was transferred into a 50-ml disposable polypropylene tube. The tubes were stored at −20 °C until determination of glucose concentration. Glucose concentration was measured by a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 505 nm using the Glucose C2 (Mutarotase-GOD method) kit (Wako, Osaka, Japan), according to the manufacture's instruction. Serially diluted glucose solution (2.78–27.8 mM) was used as standard reference.

2.4. DNA extraction and quantification

Soil samples collected from the control at 0, 7, and 21 days and the ^{12}C- and ^{13}C-glucose treatments at 0, 2, 7, 14, 21 and 28 days were subjected to molecular analyses. DNA was extracted from 0.5 g of a soil sample by the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA). A Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) was used to disrupt microbial cells in the soil under 5000 rpm for 30 s. A total DNA sample was finally eluted by 100 μl of sterilized TE buffer (10 mM Tris–HCl, 1.0 mM EDTA; pH 8.0). The DNA extracts were quantified using the PicoGreen dsDNA Quantification kit (Molecular Probes, Leiden, The Netherlands) and the Fluoroskan Ascent (Thermo Electron Corporation, Marietta, OH, USA).

2.5. Isopycnic centrifugation for DNA fractionation

The DNA samples extracted from the ^{12}C- and ^{13}C-glucose treatments were subjected to the isopycnic centrifugation as described by Lueders et al. (2004) with small modification (Lee et al., 2011). In brief, a DNA sample with three replications was combined after confirming the variations among the replicates by DGGE analysis. Then 5 μg of DNA was dissolved in 12.5 ml of CsCl gradient solution, which was prepared by mixing 10.34 ml of a 9.90 M CsCl solution (dissolved in gradient buffer [0.1 M Tris–HCl, [pH 8.0], 0.1 M KCl, 1 mM EDTA], buoyant density [BD]; 1.850 g ml^{-1}) with 2.16 ml of gradient buffer. The average BD of all prepared solutions was checked with an AR200 digital refractometer (Reichert, New York, NY, USA) and adjusted to 1.720 g ml^{-1} by adding small volumes of CsCl solution or gradient buffer. The solution was centrifuged under 144,000 × g at 20 °C for 36 h with 12 ml polyallomer seal tubes (Hitachi, Tokyo, Japan) and a RPV45T rotor (Hitachi, Tokyo, Japan). Then, centrifuged gradient solutions in the tubes were fractionated from the bottom upwards into 16 equal fractions (750 μl each) by displacing the gradient medium with sterilized ultrapure