Co-Culture of a Methanogenic Archaeon and a Methanotrophic Bacterium on Sterilized Soil in Large Test Tubes: Design for Soil-Mediated Co-Culture

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In a design utilizing a sterilized soil-mediated co-culture of methanogenic archaeon and methanotrophic bacterium, the activities of both the methanogen and methanotroph were examined. It was confirmed that CH₄ was produced by the methanogen after methanotrophic inoculation and suggested that this CH₄ was oxidized by the methanotroph as the concentration of O₂ increased, although emission of CH₄ itself was indirect proof of CH₄-oxidizing activity. On the other hand, in the control treatment, methanogenic inoculation without methanotrophic inoculation at different levels of O₂, CH₄ was not emitted (there was no activity to produce CH₄). These results suggest a symbiotic relation in this co-culture system, because the methanogen provided CH₄ to the methanotroph, while inoculation of the methanotroph was necessary for subsequent production of CH₄ by the methanogen. The development of co-culture systems is essential for understanding the close relationship and interaction between methanogens and the methanotrophs in natural and agricultural environments.

Key words: co-culture, methanogenic archaeon, methanotrophic bacterium, paddy soil

Methane is an important warming gas whose atmospheric concentration has been increasing for decades^{9,21,34}. Methane emission is the net effect of CH₄ production (methanogenesis) and oxidation (methanotrophy). Methanotrophic bacteria (methanotrophs) are obligately aerobic respiratory bacteria that utilize methane as a sole source of carbon and energy for growth^{8,17,29} and hence, are considered important regulators of atmospheric methane fluxes in nature^{10,23}. Methanotrophs have been isolated from a variety of environments including freshwater lakes, wetlands, and the open ocean^{8,17–19,23,27,30,35}. In contrast, methanogenic archaea (methanogens) are strictly anaerobic archaea commonly isolated from natural anaerobic environments, including freshwater and marine sediments, wet and waterlogged soils, the

rumen of animals, and the gut of insects^{7,15,22}. Methanogens produce methane and provide it to methanotrophs, which utilize it as a source of energy. Consequently, there is considerable interest in the interaction between methanogens and methanotrophs with respect to CH₄ production and oxidation in the interfaces of anaerobic and aerobic environments. Gerritse and Gottschal¹⁶ established two-membered mixed cultures of a methanogenic archaeon and an aerobic bacterium in O₂-limited conditions but mentioned that growth of *Methanobacterium formicicum* was markedly inhibited in the co-culture with *Methylocystis* species. Shen *et al.*³² reported methanotroph and methanogen coupling in a granular biofilm under O₂-limited conditions, although the coupling system included other aerobic and anaerobic bacteria. Hence the two-membered co-culture of a methanogen and a methanotroph is not well developed yet, although the two organisms are thought to interact closely with each oth-

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er in natural environments. The development of a model ecosystem with which to study production and oxidation of CH_4 is essential for understanding the mechanism of CH_4 emission from wetland paddy fields^{12,26}. Further, such a model should prove helpful in establishing techniques for the biological control of CH_4 emissions from paddy fields, one of the major anthropogenic sources of CH_4 emission to the atmosphere^{20,24,31,34}. This short report describes a soil-mediated co-culture of methanogen and methanotroph using sterilized paddy soil.

Materials and Methods

Methanogenic and methanotrophic strains and preparation of inoculants

A methanotrophic strain, namely methanotroph isolate R16 (*Methylosinus* sp.) described by Dianou and Adachi¹¹, and a methanogenic strain, *Methanobrevibacter arboriphilicus* strain SA (=DSM7056, =JCM9315) isolated from paddy soil by Asakawa *et al.*⁴, were used in this study. Inoculant solutions for co-culture of the methanotroph and methanogen were prepared in test tubes.

The methanotrophic strain R16 was grown in the nitrate-mineral-salts (NMS) medium described by Whittenbury *et al.*^{11,35}, in a shaking culture at 30°C. The liquid NMS medium (5 ml) was dispensed into 34-ml tubes then autoclaved. The tops of the tubes were sealed with W-shaped butyl rubber stoppers. Then, 6 ml of pure CH_4 was passed through a 0.2 μm pore filter syringe and injected into each tube to give about 18% CH_4 and about 16% O_2 in the head gas-phase. After inoculation, the tubes were incubated at 30°C with shaking. Growth was monitored by measuring optical density at 620 nm with a spectrophotometer (Hitachi U-2000), and CH_4 oxidation was monitored by measuring the concentration of CH_4 in the tube headspace with a gas chromatograph (Hewlett-Packard 5890A/II) equipped with a flame ionization detector and a glass column (1.8 m, 2-mm inner diameter) packed with Porapak R (50/80).

The methanogenic strain SA was grown in modified LPBM medium⁵ which consisted of KH_2PO_4 (0.75 g), K_2HPO_4 (0.75 g), NH_4Cl (1.0 g), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.36 g), trace mineral solution²⁵ (9 ml), vitamin mixture solution⁶ (10 ml), 2 g l⁻¹ resazurin (0.5 ml), yeast extract (0.5 g, Difco), NaHCO_3 (4.8 g), L-cysteine hydrochloride (0.5 g) and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.5 g) in distilled water (1,000 ml). The pH was adjusted to 7.0. The LPBM medium (5 ml) was dispensed into 34-ml test tubes under $\text{N}_2\text{-CO}_2$ (4:1) gas-flushing, and the test tubes were sealed with W-shaped butyl rubber stoppers. After autoclaving, the top gas phase was exchanged

with an $\text{H}_2\text{-CO}_2$ (4:1) atmosphere as growth substrate. After inoculation, the tubes were incubated at 30°C under static conditions. Cell growth (colony formation in the bottom of the liquid culture) was observed and CH_4 production in the tube headspace was monitored by gas chromatography on day 0, 15, 23, 30 and 38 of incubation.

Co-culture of a methanogen and methanotroph on sterilized soil in 110-ml tubes

The characteristics of the soil (paddy field soil) used in this experiment have been described^{1,28}. The 110-ml tubes with 30 g of 1mm-sieved soil were sterilized twice at 121°C for 1 h with a 24 h interval to eliminate the indigenous microflora. LPBM medium (40 ml) supplemented with 2 g l⁻¹ of KNO_3 was then added under a $\text{N}_2\text{-CO}_2$ (4:1) gas atmosphere. The test tubes were sealed with rubber stoppers, and sterilized for 20 min at 121°C. The gas phase was then exchanged and the tubes were filled with $\text{H}_2\text{-CO}_2$ (4:1) gas aseptically using a needle and a 0.2- μm -pore filter. After the mixing of the methanogen liquid culture (15-day-old culture) with a vortex mixer, aliquots (4 ml) of the methanogenic strain SA culture were inoculated into the tubes and subsequently mixed with the soil solution using a vortex mixer, after which the tubes were incubated for 10 days at 30°C. On the 10th day of incubation, aliquots (4 ml) of the methanotrophic strain R16 culture (7-day-old culture) were inoculated into the tubes, and serial amounts of pure O_2 gas were injected, to give 0, 2, 5, 10 or 16% O_2 concentrations in the tube headspace. There were four replications. At approximately 10-day-intervals, headspace gas samples were taken and analyzed with the gas chromatograph described above.

Results and Discussion

For the preparation of inoculants of methanogen and methanotroph, methanotrophic strain R16 was grown in shaking cultures, and methanogenic strain SA in static cultures at 30°C. Growth (OD 620 nm) and CH_4 oxidation (percentage of oxidized CH_4) of methanotrophic strain R16 in about 18% CH_4 and about 16% O_2 is shown in Fig. 1. After 8 days incubation, optical density showed a maximum value and 98% of CH_4 was already oxidized. Hence the 7-day-old cultures were used as methanotroph inoculant in the co-culture experiment. Fig. 2 shows CH_4 production of methanogenic strain SA in pure culture under static conditions. After 15 days incubation, cell aggregates of the strain in the bottom of the liquid culture were formed, and CH_4 -producing activity was high in the pure culture. The 15-day-

Fig. 1. Growth (OD 620 nm) and CH₄ oxidation (percentage of oxidized CH₄) of a methanotrophic strain (*Methylosinus* sp. strain R16) in a shaking culture in the presence of 18% CH₄ and 16% O₂ at 30°C. Vertical bars show the standard deviations of three replications.

old cultures were used as methanogen inoculant.

In natural ecosystems such as waterlogged paddy soil microhabitats, where the partial pressure of O₂ is very low even though the redox potential is high, anaerobic bacteria may be maintained^{14,33}. In our design, the use of sterilized soil was aimed to limit O₂ toxicity for the methanogen in the co-culture as in a natural ecosystem.

Fig. 3 shows CH₄ emission, namely the balance of [(CH₄ production) – (CH₄ oxidation) – (CH₄ dissolved in the soil phase)], into the top gas phase in the system for soil-mediated co-culture. Thirty-six days after methanogen inoculation (26 days after methanotroph inoculation and O₂ addition), a gradual CH₄ emission was observed. The delay in emission may indicate that initial conditions were not suitable for methanogenic activity, even though the co-culture was set up with N₂-CO₂ (4:1) gas-flushing and the addition of reducing agents (L-cysteine hydrochloride 0.5 g l⁻¹ and Na₂S·9H₂O 0.5 g l⁻¹). Among the 5 treatments with both methanogen and methanotroph, the highest CH₄ emission was observed without O₂, while the lowest emission was found at 16% O₂. The production of CH₄ by the methanogen after methanotrophic inoculation was confirmed, and subsequent CH₄ oxidation by the methanotroph along with increasing O₂ concentrations was suggested, although the emission itself was indirect proof of CH₄-oxidizing activity. On the other hand, in the control, i.e. methanogen inoculation without methanotroph inoculation at different levels of

Fig. 2. CH₄ production of a methanogenic strain (*Methanobrevibacter arboriphilicus* strain SA) in a static liquid culture at 30°C. Vertical bars indicate the standard deviations of two replications.

O₂, no emission of CH₄ was observed (there was no activity to produce CH₄).

These results suggest a symbiotic relation in this co-culture system, because the methanogen provided CH₄ to the methanotroph, while the inoculation of methanotroph was necessary for subsequent production of CH₄ by the methanogen. The activity of the methanotroph probably created anaerobic conditions in the soil suitable for methanogenic activity, protecting the methanogen from O₂-toxicity. In natural environments, other aerobic bacteria or facultative anaerobic bacteria may play this kind of role (protection from O₂-toxicity) for methanogens.

As Espiritu *et al.*¹³ mentioned, the ecology (population fluctuations) of methanotrophs in paddy soil and rice rhizosphere is likely to be closely affected by the supply of O₂, namely reduced or oxidative conditions, at each stage of rice cultivation. The studies of Amaral and Knowles², and Amaral *et al.*³ provide the foundations for the establishment of a promising new model ecosystem based on the two-membered co-culture of a methanogen and methanotroph for the study of the production, oxidation and emission of CH₄ in paddy soil.

The authors also examined the effect of O₂ concentrations on CH₄ oxidation in the same system (mono-culture of the methanotroph), but relatively large standard deviations were obtained due to low replication numbers (n=2 or 3) (data not shown). Hence in future, elucidation of the effect of O₂ concentrations on CH₄ oxidation in mono-cultures of methan-

Fig. 3. Effect of methanotroph inoculation at different concentrations of O₂ in the presence of methanogen on CH₄ emission into headspaces in soil-mediated co-cultures. Vertical bars show the standard deviations of four replications. MGA, methanogenic archaeon, *Methanobrevibacter arboriphilicus* strain SA; MTB, methanotrophic bacterium, *Methylosinus* sp. strain R16. In the 5 treatments with MGA and without MTB at different concentrations of O₂, CH₄ concentrations in the headspaces were very low (within the range of 0.03 to 0.17 μmol ml⁻¹ culture solution ± 0.02) throughout the incubation period.

otrophs and enumeration of the methanogen and methanotroph in co-cultures from the onset to the end of the incubation period is necessary to confirm the methanotrophic capacity and the establishment of both microbes. This short report shows that we are still in the preliminary stages of developing a co-culture-system for methanogens and methanotrophs with which to estimate the activity of CH₄ production and oxidation, because the soil-mediated design was too simple. Further experiments are required for the establishment of an improved model ecosystem based on the co-culture of a methanogen and methanotroph with or without soil-mediation.

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