Short Communication

Methanogenesis from Dried and Heat Treated Paddy Soil

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Methane is one of the greenhouse gases and is generated by methanogen in flooded paddy fields. Since paddy fields are under oxic and dry conditions during off-crop and winter cropping season, methanogens in the paddy soil are exposed to oxygen and become dry. The following spring, methanogenesis is observed again after the flooding of the paddy fields. However, methanogens are strictly anaerobic. Some experiments have showed that only a small concentration of oxygen inhibited methanogenesis in culture medium. The growth of methanogens was delayed by 0.004% (v/v) of oxygen in the head space of the culture medium¹⁷⁾ and that methane production was inhibited by 0.03–0.1% (v/v) of oxygen^{14,15,17)}. Therefore, that methanogens, that are strictly anaerobic bacteria, survive in paddy soil during the oxic and drying period is contradictory to its basic characteristics. By contrast, several reserches indicate that oxygen dose not kill methanogen instantaneously^{8,9,12,15,19)}. When a methanogenic population was exposed to oxygen for 4 h, about 40% of the initial population survived¹⁹⁾. Incubation of methanogen with air for 200 min did not decrease methanogen viability⁸⁾. A 30 h exposure to air did not have any effect upon the number of colony forming units of methanogen¹²⁾. In spite of these results, more than 30 h exposure to oxygen decreased the viability of methanogen¹²⁾. It appears that the viability of methanogen in paddy soil is not influenced by air-drying treatment unlike

methanogen in a cultural medium. The methanogenic population did not change before or after the initiation of methane production¹³⁾ and was almost constant irrespective of flooded conditions (wet and anaerobic) or wheat cropping conditions (dry and aerobic)²⁾.

Spore-formation has not been reported for any methanogens. That many anaerobic protozoa keep methanogenic symbionts has recently been reported^{3–7,10)}. The protozoa that live in landfill spend much of the time encysted under a dry condition and autofluorescence of methanogens is observed in the squashed cysts⁶⁾. It is highly probable that methanogens in air-dried paddy soil keep their viability within the protozoal cysts. The protozoal cysts are generally separated from vegetative cells by heat treatment as they are considered to be heat tolerant¹⁾. Therefore, heat tolerance of methanogens is presumed to be related to the heat tolerance of protozoal cysts.

This study attempts to understand the mechanisms of the tolerance of methanogens in air-dried paddy fields. With respect to air-dry tolerance, two kinds of experiments were carried out. Experiment I was performed to reveal the effects of short period drying on methanogenesis. In experiment II, methanogenesis from soils which have been dry for very long period, 20 to 30 years, were observed. Two kinds of experiments were also carried out on heat tolerance. Experiment III concerns the effect

of repeated heating at 80°C under a flooded condition and experiment IV concerns the effect of heat at 80°C on dry soil.

Experiment I was performed to reproduce the air-dry tolerance of methanogen in a paddy field *in vitro*. For this study, the soil was collected from a paddy field of Saitama Agricultural Experiment Station, Kumagaya, Saitama at wheat cropping season. This soil was Gray lowland soil. Soil samples were dried at room temperature for 14 days and passed through a 2-mm-mesh sieve. Total carbon and nitrogen contents of the soil were 1.49% and 0.15%, respectively. Soil moisture was 3.63% and pH(H₂O) was 4.9. Ten grams of the soil was submerged with 25ml of 20mM sodium acetate solution in a 120ml serum bottle. The submerged soil was incubated at 30°C under anaerobic condition (O₂-free N₂ atmosphere) with constant shaking for 20 days. After incubation, the soil was precipitated by centrifugation at 3000 × *g* for 10 min and was dried for 20 days in a desiccator. In order to dry the soil completely, the soil had been well broken up. Since organic matter was consumed by microorganisms during incubation, nutrients were supplied by adding the following soil extract. Eighty grams of the same soil was extracted with 200 ml of 20 mM sodium acetate solution by boiling for 15 min. The extract was then filtrated with filter paper. The resulting solution was sterilized by autoclaving. The 20-days dried soil was submerged with 25ml (this volume corresponded with the extract from 10 g soil) of this solution, and was incubated under same conditions as described above for 40 days. The methane production from the soils was determined by gas chromatograph (model GC-8A; Shimadzu Co) with a thermal conductivity detector (TCD). As shown in Fig.1, the soil that was dried twice did not show any delay of methane production compared with the soil that was dried once. Methane production from soil seems not to be affected by short period (20 days) drying. It is also supposed that the methanogenic population in the soil did not decrease after drying. Asakawa and Hayano analyzed the methanogen population in natural paddy

Fig. 1. Total CH₄ production from air-dried soil after submersion.

○; once dried soil
●; twice dried soil

fields by the MPN counting method and reported that the population of methanogen in paddy soil was constant under flooded conditions and dry conditions²⁾. Our result corresponds with that report²⁾.

Experiment II was carried out by using the following 20-30 years long period air-dried paddy soils: Fukushima soil (Gley soils) collected from Fukushima Agricultural Experiment Station in Aizu region, Aizubange, Fukushima on 6 May 1976. Total C and N contents were 2.45% and 0.23%, respectively. Soil moisture was 2.73% and pH(H₂O) was 5.2; Kounosu soil (Gray lowland soils) obtained from the Central Agricultural Experiment Station, Kounosu, Saitama on 19 April 1977. Total C and N contents were 1.52% and 0.13%, respectively. Soil moisture was 3.25% and pH(H₂O) was 6.8; Yamanashi soil (Gray lowland soils) collected from Yamanashi Agricultural Experiment Station, Koufu, Yamanashi on 6 December 1966. Total C and N contents were 1.00% and 0.10%, respectively. Soil moisture was 2.08% and pH(H₂O) was 6.3. All soils had been sealed in bottles for at least 10 years. Five grams of each soil and newly collected Saitama air-dry soil were submerged separately and incubated for 30 days under the same conditions as described above. To test the probability of contamination with methanogen from the environment, another experiment was performed as follows: glass

sand, 5 g, had been left outside the clean room with no cover for 4 days to become contaminated with environmental dust. The dusty glass sand was submerged and reduced with Titanium (III) citrate solution¹⁸⁾. This submerged glass sand was incubated under same conditions as other long period air-dried soils. If environmental dust included methanogens, methanogenesis should be detected in the contamination test. However, no methanogenesis was detected during the incubation period. Fig.2 shows methane production from long period dried soils after submersion and 30 days of incubation. The initial methane production rate of long period dried soils were as high as that of Saitama soil. The final amount of methane produced from long period dried soils and Saitama soil were approximately the same (18 mmol/100 g of dry soil) except for Fukushima soil. Fukushima soil produced a larger amount of methane (24 mmol/100 g of dry soil) as compared with Saitama soil. Methane production from long period dried soils showed a little longer lag period than Saitama soil. These results show that methanogens retain their viability for 20–30 years under oxic and dry conditions.

In experiment III, to estimate the heat tolerance of methanogens in paddy soil, newly collected Saitama soil was submitted to various treatments. Three grams of the soil was submerged with 8 ml of 20 mM

Fig. 2. Total CH_4 production from 20–30 years long period air-dried soils after submersion.

- ; Fukushima soil
- △; Kounosu soil
- ; Yamanashi soil
- ; Saitama soil sampled as the control

sodium acetate solution in an 18×180 mm test tube under anaerobic conditions. The submerged soil was heated at 80°C for 10 min in four different ways, as follows: i) heated once before incubation. ii) heated once before incubation and four times every two days during the initial 8 days of incubation. iii) heated once after methanogenesis was observed. iv) heated five times every two days during incubation after methanogenesis was observed. Submerged untreated soil was used as a control. Then, the submerged soils were incubated statically at 30°C for 30 days. The result is shown in Fig.3. The methane production from the control soil started after 4 days lag period. When the submerged soil was heated once before incubation (i), methanogenesis started after 8 days lag time (Fig.3A). When the soil was heated once after methane generation

Fig. 3. Total CH_4 production from submerged soils. The soils were heated at 80°C for 10 min under flooded condition before (A) and after (B) methanogenesis was observed.

- ; heated once before incubation
- ; heated once before and four times every two days during an incubation
- ▲; heated once after methanogenesis was observed
- △; heated five times every two days after methanogenesis was observed
- ; no heat (control).

had started (iii), the methanogenesis stopped and restarted after 8 days of the heat treatment (Fig.3B). Methane was not generated from soils that were heated five times during the initial 10 days (ii) and after methanogenesis was observed (iv) (Fig.3A,B).

Experiment IV was carried out to estimate the effects of heat treatment on dry soil. Three grams of the soil was sealed in an 18×180 mm test tube with a butyl rubber stopper. One was sealed with O₂-free N₂ and another was sealed with air. The soils were heated at 80°C for 24 h. After heat treatment, the soils were submerged and incubated under anaerobic conditions as described above. As shown in Fig.4, methane production from soils that were heated for 24 h in N₂ or air started after 8 days of lag period. The amounts of methane (8 mmol/100g of dry soil) produced from these two soils were larger than that in the control soil. The amounts were also larger than that produced from soils which were submerged and heated for 10 min. That dried and heat-treated soil generated more methane than control soil was explained by the air-dry effect¹¹⁾ and heating effect¹⁶⁾.

Experimental results indicate that methanogen in

paddy soil was tolerant to heat and behaved like spore-forming bacteria. Heat treatment at 80°C for 10 min and heat treatment at 80°C for 24 h delayed the initiation of methanogenesis. However, repeated heat treatment at 80°C for 10 min inhibited methanogenesis completely. This effect of repeated heat treatment is well explained by the tyndallization theory. The theory of tyndallization is that vegetative cells and some spores are killed at the first heating and more heat resistant spores germinate and are killed at later heating.

From the experiments described above, we have shown that methanogens in paddy fields are not particularly affected by long periods of oxic and dry conditions. It is suggested that methanogens in paddy fields keep their viability against heat in some special microhabitat such as heat resistant protozoal cysts.

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Fig. 4. Total CH₄ production from soils after submer-sion. These were heated once at 80°C.

- △; heated for 24 h in N₂ under dry condition
 ▲; heated for 24 h in air under dry condition
 ●; heated for 10 min under flooded condition
 □; no heat (control)

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