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Evaluation of Methanogenic Strains and Their Ability to Endure Aeration and Water Stress

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Abstract During periods of drainage, both water stress and oxygen can cause damage to indigenous methanogens. In the present study, we evaluated the tolerance of seven methanogenic strains (*Methanobrevibacter arboriphilicus*, *Methanobacterium formicicum*, *Methanococcus vannielii*, *Methanospirillum hungatei*, *Methanoculleus olentangyi*, *Methanoplanus limicola*, and *Methanosarcina mazei*) to long-term exposure to air/nitrogen and drying. We found that these methanogenic strains except for *M. limicola* and *M. olentangyi* in pre-dried cells offered more tenacious resistance to desiccation and oxygen exposure than those in enriched liquid cultures. In the case of *M. formicicum*, the liquid culture of this strain could remain viable when mixed well with fresh or sterile soil, but not when cultured without soil, or with agar slurry. These results suggest that indigenous methanogens localize within soil compartments to protect themselves from the damage caused by gradual drying under an oxic atmosphere.

Introduction

Methane is exclusively produced by methanogens that can metabolize only in the strict absence of free oxygen [8]. In wetland rice-based cropping systems, soils are cultivated by distinct cycles of flooding and drainage. Submerging a rice paddy field cuts off the oxygen supply from the atmosphere to the soil, and facultative and anaerobic microorganisms sequentially reduce soil substrates [15]. At the end of the growing season, fields are harvested, drained, and ploughed, remaining dry and oxic until the next cultivation period [13]. In addition to water stress, the presence of oxygen also causes damage to indigenous methanogens. Although methanogens are strict anaerobes and do not form spores, several experiments have indicated that oxygen does not kill methanogens instantaneously [5, 6, 10, 16] and they are able to survive the dry-oxic periods [5]. Oxygen-protecting enzymes such as superoxide dismutase and catalase have been found in some methanogenic strains and were proposed to defend such methanogens against the lethal effect of oxygen [3, 4, 11, 17, 18].

The effect of the combination of desiccation and exposure to oxygen or nitrogen on methanogenic strains has not been studied in detail. Consequently, we investigated the tolerance of diverse methanogenic strains to long-term exposure to air/ nitrogen and drying in this study. In addition, we evaluated the role of paddy soil in preserving indigenous methanogens from damage caused by oxic-desiccation.

Materials and Methods

Soil Samples

The soil samples used in this study were collected from paddy fields at Saitama Agricultural and Forest Research Center, Saitama prefecture, Japan. All visible roots were removed prior to further processing. The properties of the soil have been reported previously [12].

Methanogenic Strains

Methanosarcina mazei TMA strain (DSM 9195) and *Methanobrevibacter arboriphilicus* SA strain (DSM 7056)

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were isolated from paddy soils at Kyushu National Agricultural Experiment Station, Fukuoka pref., Japan [1, 2]. The following strains: *Methanobacterium formicicum* (DSM 1535T), *Methanococcus vannielii* (DSM1224T), *Methanospirillum hungatei* (DSM 864T), *Methanoculleus olentangyi* (DSM 2772T), *Methanoplanus limicola* (DSM 2279) were provided by Deutsche Sammlumg von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Media and Cultural Conditions

M. mazei strain TMA was cultivated at 30°C in DSM 120 medium [1]. *M. arboriphilicus* SA, *M. formicicum*, *M. vannielii*, *M. hungatei*, and *M. limicola* strains were cultivated in a modified NaCl-free DSM 141 medium, [12] at 37°C, except *M. limicola* strain, which was cultivated at 35°C. *Methanoculleus olentangyi* strain was cultivated in a modified DSM 141 medium containing 0.6 % NaCl. Each methanogenic strain was cultivated in a 70-ml vial with a butyl rubber stopper containing 20 ml of anoxic liquid medium (pH 7). All methanogenic strains were cultivated under a gas phase of H_2/CO_2 (80:20) with shaking, except for the *M. mazei* strain TMA, which was incubated statically under N_2.

Desiccation of Pre-Dried Methanogenic Pellets Under Oxic and Anoxic Atmospheres

In the oxic experiments, 1-ml aliquots of the cultured enrichment of methanogenic strains were harvested by centrifugation at 12,000 *g* for 3 min. The cell pellets were left in microtubes (1.5 ml) and desiccated quickly by a centrifugal evaporator (Sarvant, USA) for 30 min. The tubes were sealed with a paper filter as shown in Fig. 1a. The microtubes containing pre-dried cell pellets were placed in a desiccator at room temperature. The humidity was kept below 10% during the experimental period. In the anoxic experiments, 1-ml aliquots of the cultured enrichment of methanogenic strains were harvested by centrifugation, which was performed in an anaerobic glove box filled with N_2 gas. Then, the cell pellets were desiccated anaerobically for 30 min by a centrifugal evaporator. The microtubes with pre-dried cell pellets were then put into a glass tube with a butyl rubber stopper and the inside atmosphere was exchanged with N_2 gas using a modified Hungate technique [14]. All desiccating experiments above were conducted for 3, 14, and 30 days. Initiation of methane production was performed as described in the Media and Cultural Conditions section.

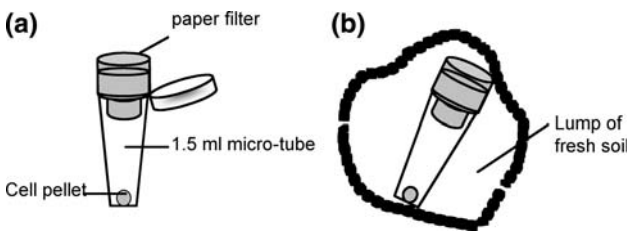

Fig. 1 **a** Pre-dried methanogenic pellet was placed in a microtube, which was then sealed with a paper filter. **b** The microtube containing the wet cell pellet was enclosed within fresh paddy soil

Oxygen Sensitivity Test for Enriched Cultures

One-milliliter aliquots of the cultured enrichments of *M. formicicum* strain were put into 1.5-ml microtubes. Each tube was sealed with a tiny filter as described above and left in an oxic atmosphere at room temperature. To test the time required for initiation of methane production, 10-ml aliquots of medium were added to glass test tubes with butyl rubber stoppers under the anoxic atmosphere specific to each methanogenic strain and incubated. Initiation experiments were conducted at least in triplicate.

Tolerance to Oxic-Desiccation of Methanogens Surrounded by Paddy Soil

Ten grams of fresh or sterilized paddy soil sampled at Saitama was submerged with 25 ml of liquid medium in a 120-ml vial. Alternatively, the paddy soil was replaced by agar powder (~3 g) as a comparison trial. The *M. formicicum* strain was then inoculated to the above suspension and cultivated at 37°C with shaking under a gas phase of H_2/CO_2 (80:20). While methane production was detected by gas analysis, the soil or agar slurry was centrifuged at 1000 *g* for 10 min, and the pellet was dried in a desiccator for 14 days at room temperature. The oxic-desiccated soil/agar pellet was then placed into a vial (120 ml) and submerged with 25 ml of anoxic liquid medium and incubated under conditions previously described. Production of methane was determined periodically for 2 months. As a control experiment, cultured enrichment of *M. formicicum* (25 ml) was harvested by centrifugation at 3000 *g* for 10 min. The cell pellet was retained in the 1.5-ml microtube sealed with a tiny filter (Fig. 1a). The microtube was enclosed within bulk fresh paddy soil as shown in Fig. 1b (i.e., no direct contact between the methanogens and soil particles, and then dried in a desiccator at room temperature for 14 days). The microtube was then taken out by crushing the soil lump, and the cell pellet was placed in a vial (120 ml), submerged with 25 ml of proper liquid medium and incubated as described above. Production of methane was determined periodically for 2 months.

Measurement of Methane

The gas chromatographic measurements of methane indicated survival for varying time periods. Gas samples were taken with gas-tight syringes from the headspace and were analyzed for methane in a gas chromatograph with flame ionization detector (GC-14, Shimadzu, Japan).

Results and Discussion

Desiccation of Methanogenic Strains Under Oxic/Anoxic Atmospheres

When the growing season of paddy is over, indigenous methanogens have to endure water stress and oxygen toxicity for extended periods of time [13]. In this study, we conducted experiments to evaluate the effect of the combination of desiccation and exposure to oxygen or nitrogen on some methanogenic strains.

In a preliminary experiment, we found that almost all of the pre-dried methanogenic strains underwent rapid desiccation (the pre-dry process, by centrifugal evaporator for 30 min as described in Materials and Methods) and survived longer than those grown in liquid culture (Table 1). For example, the pre-dried cell pellet of *M. formicicum* strain showed viability for at least 1 month of desiccation under oxic conditions. However, liquid culture of *M. formicicum*, which was desiccated aerobically for 14 days, had already lost its viability. The pre-dried pellet contained less medium than the liquid culture and, consequently, there was less damage to the methanogenic strains. Alternatively, the rapid desiccating process may have killed the outer layers of pre-dried methanogen cells, resulting in the formation of a protective layer from the outer conditions. In the present study, seven methanogenic strains from diverse orders of methanogens were used as the experimental materials. *Methanobacterium formicicum* and *Methanobrevibacter arboriphilicus* belong to *Methanobacteriales*; *Methanospirillum hungatei*, *Methanoculleus olentangyi*, and *Methanoplanus limicola* are included in *Methanomicrobiales*; *Methanosarcina mazei* is a member of *Methanosarcinales*, and *Methanococcus vannielii* is associated with *Methanococcales*. We evaluated the potential tolerance of each methanogenic strain to drying and oxygen stress for longer periods, and conducted the durability test in pre-dried conditions.

The periods of desiccation were 3, 14, and 30 days, and the dried cell pellets of each methanogenic strain were then suspended in suitable media and incubated at the appropriate temperature. The viability of the methanogens was

Table 1 Viability of each methanogenic strain after desiccating under oxic/anoxic atmosphere

Strains	Desiccating duration (days)	Methanogenesis[a]		
		In liquid culture	In pre-dried condition	
		Oxic-desiccation	Oxic-desiccation	Anoxic-desiccation
Methanobacterium formicicum	3	+ (3–5)[a]	+ (3–5)	+ (3–5)
	14	—[b]	+ (3–5)	+ (7–8)
	30	—	+ (5–7)	—
Methanobrevibacter arboriphilicus SA	3	+ (1–3)	+ (1–3)	+ (1–3)
	14	—	+ (1–3)	+ (3–5)
	30	—	+ (3–5)	+ (3–5)
Methanococcus vannielii	3	—	—	+ (3–5)
	14	—	—	+ (3–5)
	30	—	—	+ (3–5)
Methanospirillum hungatei	3	—	+ (8–10)	+ (8–10)
	14	—	—	—
	30	—	—	—
Methanoplanus limicola	3	—	—	—
	14	—	—	—
	30	—	—	—
Methanoculleus olentangyi	3	—	——	
	14	—	—	—
	30	—	—	—
Methanosarcina mazei TMA	3	+ (3–5)	+ (10–12)	+ (5–7)
	14	+ (13–15)	+ (23–25)	+ (14–17)
	30	—	+ (11–13)	+ (7–9)

[a] +, methanogenesis. The numbers in parentheses indicate the recovery period (days) of methane production. —, no methanogenesis. The experiments were conducted at least in triplicate.

determined by methane production. As shown in Table 1, the *M. arboriphilicus* SA and the *M. mazei* TMA strains remained viable for 1 month of desiccation under either oxic or anoxic atmospheres. The *M. formicicum* strain remained viable for 1 month of desiccation only in the oxic conditions. The *M. vannielii* strain could not endure desiccation under an oxic atmosphere, but could survive for 1 month under anoxic-desiccation. The *M. hungatei* strain showed viability for 3 days of desiccation under both oxic and anoxic atmospheres. We noticed that the *M. mazei* TMA strain recovered more quickly from anoxic-desiccation than from oxic-desiccation. On the other hand, the recovery period of the *M. formicicum, M. arboriphilicus* SA, and *M. hungatei* strains showed little difference between oxic and anoxic desiccation.

Although methanogens are well known as the strictest anaerobes, specific methanogenic strains such as *Methanobacterium bryantii* [11], *Methanobacterium thermoautotrophicum* [18], *Methanobrevibacter arboriphilicus* [4], *Methanosarcina barkeri* [3], etc. equipped the detoxification enzymes (superoxide dismutase, catalase) can survive in the presence of oxygen. The methanogenic strains used in this study (*M. formicicum*, *M. arboriphilicus* SA strain, and *M. mazei* TMA strain), all of which showed strong resistance to oxic-desiccation (Table 1), all belong to the following three genera: *Methanobacterium*, *Methanosarcina*, and *Methanobrevibacter*. It seems reasonable to suppose that the ability to survive oxic-desiccation for a long period of time is partially associated with the activity of detoxification enzymes, although the methanogenic species used in this study were not all consistent with those reported in published works.

Fetzer et al. indicated that oxic drying had a stronger effect than anoxic drying, i.e., drying and oxygen toxicity have a cumulative detrimental effect on the viability of methanogenic strains [5]. This is true for most of the methanogenic strains used in this study. However, we found that pre-dried *M. formicicum* strain showed stronger survival capacity in oxic-desiccation (30 days) than in anoxic-desiccation (14 days) (Table 1). Kendrick et al. have indicated that *M. formicicum* can survive anoxic-desiccation for at least 25 days [9]. It is possible that *M. formicicum* could not sustain anoxic-desiccation for more than 30 days under conditions used in this study. Otherwise, the initiation time for producing methane may be beyond our measurement time period (2 months).

Protection of Methanogens Against Oxic-Desiccation by the Presence of Soil Particles

In this study, we employed a set of experiments to assess the role of paddy soil in tolerance to oxic-desiccation (14 days).

Table 2 Tolerance of the *Methanobacterium formicicum* strain under oxic atmosphere for 14 days

	Whether other soil-born reducing microbes existed	Viability of *M. formicicum*[a]
Cultured enrichment	No	No
Mix with fresh soil	Yes	Yes (3–5)
Mix with sterile soil	No	Yes (4–5)
Mix with agar	No	No
Encapsulated in tubes, wrapped with fresh soil	Yes	No

[a] The numbers in parentheses indicate the recovery period (days) of methane production. The experiments were conducted at least in triplicate.

As shown in Table 2, the cultured enrichment of *M. formicicum* strain could not endure 14 days of oxic-desiccation. On the other hand, the liquid culture of *M. formicicum* strain could remain viable when mixed well with fresh or sterile soil. In natural habitats, indigenous methanogens generally rely on other fermentative bacteria to produce the strong reducing conditions necessary for growth and methane production [7]. However, in the case of the sterile soil experiment, the effect of other fermentative bacteria was ruled out. In contrast, when *M. formicicum* strain was mixed with agar slurry before conducting the same test, the methanogen could not survive (Table 2). Consequently, we assume that paddy soil may serve as an oxygen scavenger as a result of the presence of reduced minerals. Furthermore, argilliferous soil (Saitama paddy soil) may also retain water to some degree during drying.

If paddy soils truly play roles as oxygen scavengers and moisturizers, we presumed that they should also shield the indigenous methanogens from oxic-desiccation even without direct contact. We designed an experiment to verify this assumption. We encapsulated the pellet of *M. formicicum* strain in a microtube and wrapped the tube with bulk fresh soil as shown in Figure 1b. We then desiccated it aerobically for 14 days. However, there was no methane production detected during the period of observation (2 months) (Table 2). Taken together, we suggest that in addition to its reducing capacity and moisture-retaining ability, paddy soil also provides numerous compartments that act as shelters for indigenous methanogens during the long-term drainage period.

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