Study of Methane Producing Bacteria from Rice Paddy Field Soil and Rice Roots by Fluorescent In Situ Hybridization Technique

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ABSTRACT

One of the sources of gas methane in the atmosphere causing the world warming comes from paddy fields which are the habitats of methane producing bacteria. Root zone soil and rice roots cv. Suphanburi 90 and Homsuphanburi provided by Rice Research Center Pathum Thani were studied for characterization of methane producing bacteria. The samples were inoculated in basal broth medium in serum vials under anaerobic condition and incubated at 37°C for 40 days. Methane in the vials was determined by gas chromatography. The results showed the methane producing bacteria from the soil of the paddy field of the rice cv. Suphanburi 90 and Homsuphanburi could produce methane at maximum rate of 0.7 and 0.6 ml/g/day at 17 days of incubation, respectively. The microorganisms from the rice root cv. Suphanburi 90 and Homsuphanburi could produce methane at maximum rate of 0.6 and 0.5 ml/g/day at 22 days of incubation, respectively. On determination the type of methane producing bacteria by FISH technique, it was found that the microbacteria from rice root was characterized to be *Methanosarcina* sp. while the microbacteria from the soil was characterized to be *Methanosatae* sp. The number of the methanogenic bacteria from the soil and rice root of the rice cv. Suphanburi 90 determined by MPN method was found to be 3.49×10^7 and 2.01×10^3 cell/g, respectively, while the number of the methanogenic bacteria from the soil and rice root of the rice cv. Homsuphanburi was 3.75×10^5 and 5.22×10^3 cell/g, respectively.

Key words: methane, methanogen, rice, root

INTRODUCTION

Global warming induced by increasing greenhouse gases concentrations in the atmosphere is a matter of great environmental concern. Methane is the greenhouse gases, which has strong infrared absorption bands and traps a part of the thermal radiation from the earth's surface. Atmospheric concentrations of methane increased from 1.50 to 1.72 ppm in the last decade. The paddy field environment is considered to be a major source of atmospheric methane, and it is estimated that approximately 5-30% of methane released into the atmosphere originated from paddy fields (Rasmussen and Khalil, 1986; Battle *et al*., 1996).

Methane is produced in the terminal step of several anaerobic degradation chains. Thebiochemical pathways leading to the production of methane include fermentation of methylated compounds (e.g. acetate, methanol, trimethylamine, and dimethylsulfide), CO_2

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reduction with molecular hydrogen, and, with minor significance than the other pathways, the reduction of CO and formic acid. Accordingly, methanogenic bacteria can be subdivided into methylotrophic, obligate chemolithotrophic, and "quasi"-chemolithotrophic microorganisms.

Methane is produced by the activity of methanogens. Although the mechanism of methanogenesis in paddy soils has been studied (Banik *et al*.,1985, Conrad *et al*., 1989), there was no report on the predominant methanogen type in rice cultivar in paddy field or analysis of methanogenic flora in root zone soil and rice cultivar. The relative contribution of different methanogenic processes to the total methane production in rice paddies is unknown. The aim of this study was to enumerate and characterise the population of active methanogen in the root zone soil and rice root of rice cv. Suphanburi 90 and Homsuphanburi, by using most probable number (MPN) technique and Fluorescent In Situ Hybridization(FISH). Methane production by root zone soil and rice root enrichment culture were also determined.

MATERIALS AND METHODS

Soil and rice root

The soils used for methanogen analysis were collected from the paddy fields of rice cv. Suphanburi 90 and Homsuphanburi at Rice Research Center, Prathumthani province. The rice plants were grown in the same ecosystem in the field.

Soil samples were taken from the anoxic layer (30-50 cm deep). Soil from paddy field before rice growing was water saturated. Root zone soil (rhizosphere: area contact between soil and root) was collected from a flooded rice paddy field.

The rice plants were grown for approximately 70 d. The plants were carefully removed from the soil together with the roots. The soil was loosely washed of the roots using

tap water. Then, all remaining soil particles were thouroughly removed from the roots by several washing steps using demineralized and autoclaved water. The roots were cut into 1-2 cm in length with a razor blade, washed twice again, and about 1-g fresh weight sub- samples were mixed to make homogenous samples for the determination of methanogenic activity.

Dry weights of the soil and rice root were obtained by weighing before and after drying at 105°C for 24 h.

Media for isolation and culture of methanogens

Methanogens were grown in defined basal medium containing 0.4 g/l KH_2PO_4, 0.4 g/l K_2HPO_4, 1.0 g/l NH_4Cl, 0.21 g/l $MgCl_2.6H_2O$, 10 ml Resazurine, 10 ml vitamin solution, 4.0 g/l $NaHCO_3$, 0.5 g/l cysteine $HCl.H_2O$, 0.25 g/l $Na_2S.9H_2O$, 0.002 g/l mneral solution, as described by Li and Noike (1992). The pH of the medium was adjusted to 7.3 with 2.0 N HCl. 0.1% Acetate was added to the medium as a carbon source and also as growth-stimulating reagent.

Methane analysis

One gram of sample with 7.5 ml of basal medium in serum vial with minimal headspace 1.5 cm^3 was used for the anoxic incubation of soil and rice root samples as described by Zhang and Noike(1991). The vials were sealed with rubber cap and the air inside was drawn out to make it anaerobic and incubated at 37°C. Each sample was prepared in triplicate. Methane concentration was measured every two days. The headspace content of the vials were sampled with 0.02 ml gas-tight syringes (Hamilton, USA). Methane production was calculated from the linear increase in methane volume. Methane was determined by gas chromatography (Shimadzu Model GC 9A) Parapak-N column and Thermal Conductivity Detector (TCD). The temperature of the column, injector and TCD was set at 70°C, 120°C and120 °C, respectively, with current Bridge of 100 mA.

Helium was used as carrier gas at the flow rate of 50 ml/min.

Counts of methanogens

The population of the methanogens were estimated by the most probable number (MPN) method (3 tubes per dilution). Successive 10-fold serial soil suspension dilutions were inoculated in the media described above. Counts were duplicated by using two composite soil samples for each soil type. Methanogen growth was assayed by measuring CH_4 produced after 60 days of incubation at 37°C. Inoculated vials containing medium supplemented with 1 g/l of yeast extract and 1 g/l of bio-trypticase, where no substrate added, were control. A serum vial was considered positive when CH_4 produced was as least 5% higher than the control. Populations of methanogens were expressed as MPN per gram dry soil or rice root.

All determinations were replicated three times to estimate mean values and standard deviations.

Morphology and classification of methanogen by Fluorescent In Situ Hybridization technique (FISH)

The culture media from the inoculated serum vials were fixed with 3% paraformaldehyde for 2 h and resuspended in the mixture of Tris buffer and ethanol (1:1) as described by Raskin *et al.* (1995). The cell suspensions were stored at -20°C. Hybridization was performed on poly-L-lysine-coated slides as described by Amann *et al.* (1996). Probe concentration was 50 ng in 10 µl of hybridization solution. The following oligonucleotide probes complementary to specific regions of 16S rRNA were used (i) ARC915, specific for the domain *Archaea* (Stahl *et al.*, 1988) and (ii) MSMX860, specific for *Methanosaeta* and *Methanosarcina* spp. (Raskin *et al.*, 1994). Oligonucleotide probes were synthesized and 5′ labeled with CY3 dye by Thermo Hybrid

(Germany). The slides were viewed on an Olympus Microscope BX60 with appropriate filters. Images were captured with an Olympus DP50 digital camera system and final images were prepared with Adobe PhotoShop 7.0 software (Adobe, Mountain View, CA, USA).

RESULTS

Determination of methane production

Methane production from root zone soil and rice root of cv. Homsuphanburi are shown in Figure 1. It was found that the methanogen from soil before rice growth (Soil) and rice root (Root) produced maximum rate of methane on days 24 and 28 of incubation at 0.42 and 0.45 ml/g/day, respectively, after that the production rate of gas decreasesd to 0.25 and 0.36 ml/g/day on 40 days of incubation, respectively. The methanogen from root zone soil produced methane more than from the soil before rice growth and from rice root. The maximum rate of methane produced on 20 days of incubation at 0.48 ml/g/day till on 32 days, the methane production rate decreased to 0.36 ml/g/day on 40 days of incubation.

Methane production from root zone soil and rice root cv. Suphanburi 90 are shown in Figure 2. It was found that the methanogen from soil before rice growth (Soil) produced maximum rate of methane on 34 days of incubation at 0.5 ml/g/day after that the rate of methane production decreasesd to 0.3 ml/g/day on 40 days of incubation. The methanogen from rice root (Root) produced the maximum rate of methane on 26 - 30 days of incubation at 0.52 ml/g/day and then the rate of methane production decreased to 0.42 ml/g/day on 40 days of incubation. The methanogen from root zone soil produced methane more than soil before rice growth and rice root. The maximum rate of methane production was 0.55 ml/g/day on 20 days of incubation, and then the methane production rate decreased to 0.3 ml/g/day on 40 days of incubation.
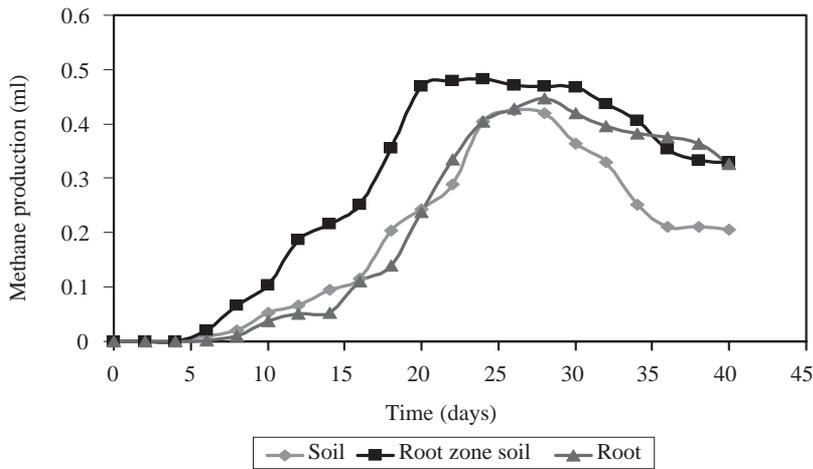
Figure 1 Accumulation of methane production by enriched culture of soil before growing rice (Soil), root zone soil and rice root of rice cv. Homsuphanburi.
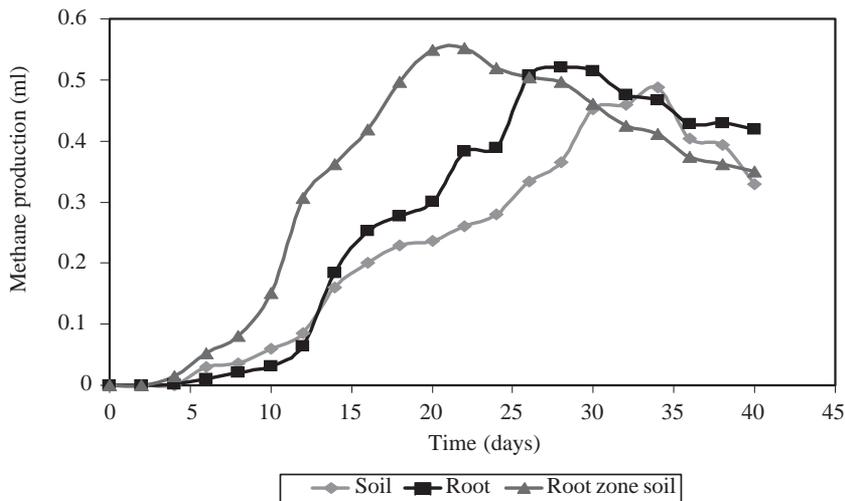

Figure 2 Accumulation of methane production by enriched culture of soil before growing rice (Soil), root zone soil and rice root of rice cv. Suphanburi 90.

Fluorescent In Situ Hybridization technique (FISH)

As shown in Figure 3 the morphology of microorganisms enriched by 0.1% acetic acid specific for probe ARC 915 were different.

After incubation in the culture medium under anaerobic condition at 37°C, the culture was microscopically observed (Figure 3). Figure 3 shows specific detections of two methanogens by

FISH in the culture of 30 day of incubation. The culture from soils before rice growing of rice cv Homsuphanburi, and Suphanburi 90, contained predominantly fluorescent bacilli resemble to *Methanosaeta* spp. The culture from root zone soils of rice cv Homsuphanburi, and Suphanburi 90 also contained fluorescent bacilli resemble to *Methanosaeta* spp. and fluorescent cocci similar to *Methanosarcina* spp. However, both of the
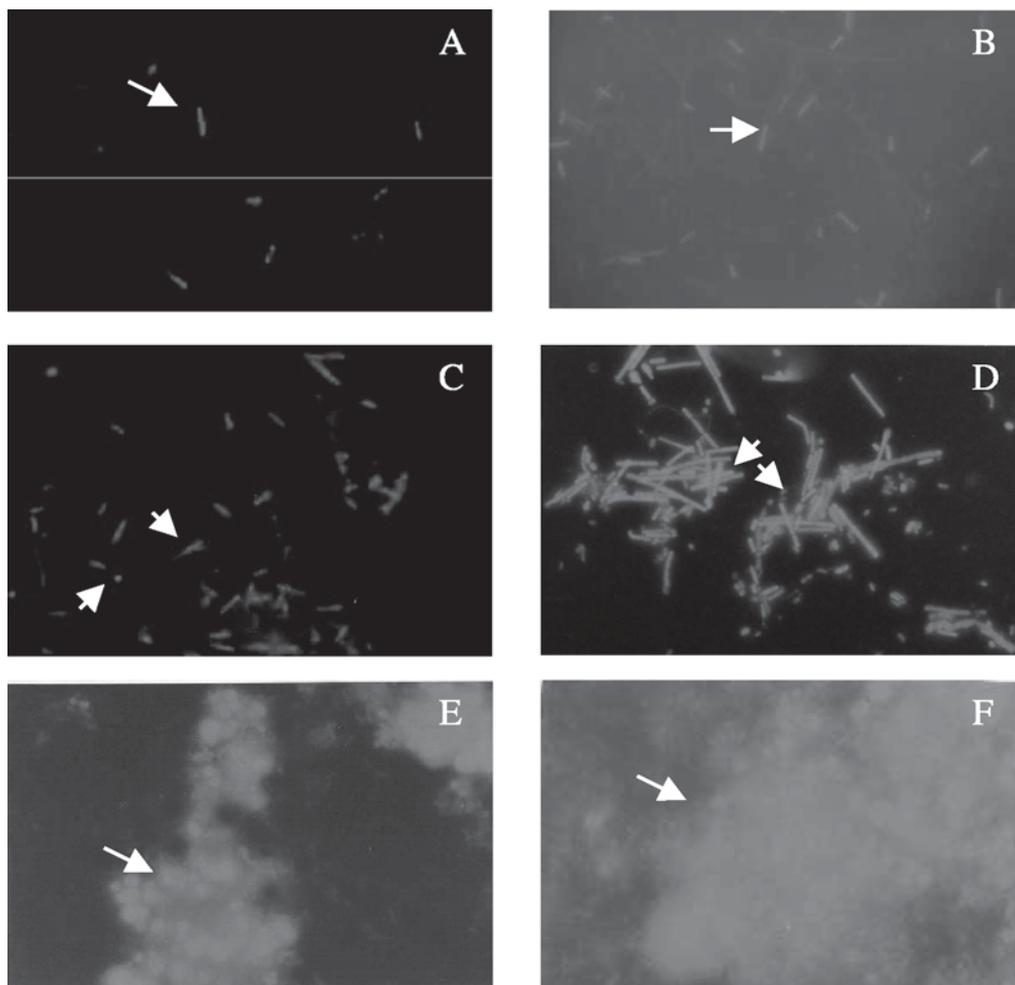
Figure 3 FISH micrographs of methanogen with CY3 red-labeled probe ARC915 and MSMX860 (specific for *Methanosaeta* and *Methanosarcina* spp.) from soil before rice growing of rice cv. Homsuphanburi (A) and Suphanburi 90 (B), methanogen from root zone soil of rice cv. Homsuphanburi (C) and Suphanburi 90 (D), methanogen from rice root cv. Homsuphanburi (E) and Suphanburi 90 (F).

culture from root of rice cv Homsuphanburi and Suphanburi 90 contained predominantly fluorescent cocci that similar to *Methanosarcina* spp.

From Figure 3, it was found that acetoclastic methanogens from soils before rice growing of rice cv Homsuphanburi and Suphanburi 90 were bacilli that hybridised with probe specific for methanogen so, the cells were *Methanosaeta* spp. The cells hybridised with probe specific for methanogen from soils during rice growing from

both rice cultivars were divided into 2 groups, bacilli and cocci, which were represented for *Methanosaeta* spp. and *Methanosarcina* spp., respectively. Methanogens from rice root of both rice cultivars hybridised with the specific probe in cluster of cocci, so the cells were *Methanosarcina* spp.

Enumeration of methanogen

The numbers of methanogen from soil before rice growing, root zone soil and rice root

were determined by MPN technique as shown in Table 1.

DISCUSSION

In this study, the methane production, cell numbers and inferred the groups of methanogenic populations in paddy field soils and rice root of two rice cultivars in the resemble location and ecosystem were estimated by culture method and the method of FISH.

The rate of methane production correlated to the number of methanogen. As shown in Figure1 and Table 1, the more number of methanogen, the more methane production. Soil and rice root might contain other microorganisms that degraded the large macromolecule to be acetic acid which was necessary to the growth of methanogen. So the methane production might depend on these microorganisms. In addition, the rate of methane production also involves with the type of soil and the organic contents (Lloyd *et al.*, 1998).

Since acetate was demonstrated to be a major methanogenic substrates in wetland rice fields, enumerations were performed on selective media containing acetate as energy sources (Schutz *et al.*, 1989, Conrad *et al.*, 1989). In this study, MPN counts of methanogens on acetate ranged from 10^3 to 10^7 cell per gram dry weight. Populations were in a range similar to those reported in Senegalese rice fields by Garcia *et al.* (1974) (10^2-10^7 cell per gram dry weight). In an Italian rice field, Schutz *et al.* (1989) and Mayer and Conrad (1990) counted 10^4-10^5 acetotrophs per gram dry weight. In most soils, acetotrophs were mostly sarcinae which are known to develop

as dense aggregates, difficult to separate into individual cells, thus their populations are underestimated by MPN counts (Fetzer *et al.*, 1993).

On the basis of phenotypic studies, all the isolated bacilli were affiliated to the genus *Methanosaeta*. The isolated sarcinae were related to the species *Methanosarcina barkeri* and *Methanosarcina mazei*, which were isolated from a broad range of environments, including sediments, digesters and rice field (Asakawa *et al.*, 1995).

These results indicated that, in rice fields, *Methanosaeta* and *Methanosarcina* spp. are mostly responsible for CH_4 production from acetate. Although results from isolation of methanogens from rice fields suggested the ubiquity and dominance of *Methanosarcina* spp. among culturable organisms, Kudo *et al.* (1997) reported *Methanobacterium* in rice field soils, where it was not dominant but reported the presence of members of the genera *Methanosarcina*- including *Msr. mazei* and *Msr. barkeri*- and *Methanogenium* or *Methanoculleus* and concluded that *Methanosarcina*, *Methanogenium* and *Methanosaeta* were dominant species.

Methanogens from soil and rice root of rice cv. Homsuphanburi and Suphanburi 90 enriched in acetate culture specific for probe ARC 915 were *Methanosarcina* and *Methanosaeta* spp. corresponsed to the studies of Chin *et al.* (1998) and Grobkopf *et al.* (1998).

From this study, it was concluded that the methanogen from soil before growing rice cv. Suphanburi 90 was *Methanosaeta* spp. The methanogen from root zone soil found to be

Table 1 The numbers of methanogen by MPN.

Sample	Rice cv. Homsuphanburi (cell/g)	Rice cv. Suphanburi 90 (cell/g)
Soil before rice growing	2.14×10^3	1.02×10^4
Root zone soil	3.75×10^5	3.49×10^7
Rice root 5.22×10^3	2.01×10^3	

Methanosarcina and *Methanosaeta* spp. while the rice root contained only *Methanosarcina* spp. The methanogen from soil before growing rice cv. Homsuphanburi was found to be *Methanosaeta* spp. but root zone soil found to be *Methanosarcina* and *Methanosaeta* spp. having the more ratio of *Methanosaeta* spp. For the rice root, the methanogen was only *Methanosarcina* spp. It was found that the methane produced more from the soil than from the root and the number of the methanogen from the soil was more the from the rice root.

From the results, methane production depended on the number of methanogen, so the decreased number of methanogen in the field might decrease the methane emission from the paddy field.

CONCLUSION

From this experiment, two isolates of methane-producing bacteria were found from paddy soil, root zone soil and rice root and partially characterized them. Based on the specific probe of FISH, morphological and nutritional characteristics the isolates were tentatively identified as *Methanosaeta* spp. (of the bacilli-shaped isolates) and *Methanosarcina* spp. (of the sarcina-shaped isolates) both which are acetate-using methanogens. Populations of methanogens were enumerated by most probable numbers (MPN) on the media in sample of soils and rice roots. The counts of methanogen from root zone soil were higher than counts of methanogen from the others. Methane production increased with the incubation time and organic matter. From this study, the methane production depended on the population of the methanogens. Naturally, methane generation, having high-temperature forcing potential (approximately $26 \times$ that of CO_2) responsible for global thermal warming, should be reduced by decreasing the amount of methanogen and appropriate drainage of the paddy

soil may reduce methane production.

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