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Investigation of Methanogenic Community Structures in Rural Biogas Digesters from Different Climatic Regions in Yunnan, Southwest China

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Abstract Understanding of the microbial community structures of the biogas digesters in different climatic regions can help improve the methane production in the fermentation process. The methanogenic archaeal diversity in four rural biogas digesters (BNA, JSA, LJA, and XGA) was investigated by a culture-independent rRNA approach in different climatic regions in Yunnan. Community structure composed of 711 clones in the all libraries. A total of 33 operational taxonomic units (OTUs) were detected, and major groups of methanogens were the orders *Methanosarcinales* and *Methanomicrobiales*. 63.2 % of all archaeal OTUs belong to the order *Methanosarcinales* which mostly contain acetotrophic methanogens. *Methanomicrobiales* (19.5 % in all OTUs) were detected in considerable number. Additionally, there were minor rates of uncultured archaea. The principal component analysis indicated that the genus *Methanosaeta* was mainly affected by the fermentation temperatures.

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Introduction

Biomass can biologically be converted to biogas using anaerobic digestion process and potentially reduce carbon dioxide emission [9]. Currently, it has become a global research hotspot that organic wastes are transformed to biogas, a kind of renewable energy [3, 32, 41]. Biogas was not only an important part of the development of renewable energy, but also an important aspect of sustainable development [14]. Simultaneously, the application of anaerobic digestion can treat various organic wastes in a rural area [29].

Moreover, due to the complex microbiological characteristics of the biogas fermentation process, various reactor designs, operating conditions, and fermentation substrates will result in changes within the microbial populations present in the system. Therefore, understanding the ecology and function of the microbial community in these processes was critical and can ultimately improve the conversion efficiency [26, 35]. So far, these characteristics of microbial community using culture-independent molecular techniques have been known in full-scale biogas digesters and plants [34, 39, 40]. To our knowledge, the community of methanogenic archaea has not yet been directly revealed in the household biogas digesters in different climatic regions in Yunnan.

Based on rRNA approach [2], an investigation of the diversity of methanogenic archaea of four rural biogas digesters was conducted in this study. To relate the community structures and process parameters such as total solids (TS), volatile solid (VS), total phosphorus (TP), chemical oxygen demand (COD), ammonia nitrogen (NH_3-N), and fermentation temperatures, principal component analysis (PCA) was used. These rural biogas digesters were usually fed with the local single or mixed substrates in the different climatic regions of Yunnan. The construction of

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16S ribosomal RNA gene clone libraries was carried out to determine the community structures of methanogenic archaea.

Materials and Methods

Rural Biogas Digesters and Samples

Total of 26 rural biogas digesters were sampled in different climate regions of Yunnan in July, 2012. For the molecular analysis of archaeal diversity, four of them (BNA, JSA, LJA, and XGA stand for the digesters in the tropical climate region, subtropical climate region, south temperate climate region, and north temperate climate region of Yunnan, respectively) were typically selected to research. These 6 m^3 digesters were operated at natural temperatures in wet fermentation conditions for several years. The activated sludge was collected in 50 ml sterile centrifuge tubes and preserved at −80 °C until DNA extraction in the laboratory. The fermentation temperatures of the digesters were directly measured. The process parameters have been obtained from these digesters in laboratory and given in Table 1.

DNA Extraction, PCR, and Library Construction

Total DNA was extracted from the samples using the PowerSoil DNA isolation kit (MO BIO-Laboratories, USA). PCR amplification of archaeal 16S rRNA genes was carried out using the primers ARC-8F/958R [12]. PCR reactions contained 50–100 ng DNA template, 1× GoTaq Green Master Mix (Promega), 1 mM $MgCl_2$, and 2 pmol of each primer. Amplification consisted of an initial 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension of 72 °C for 10 min. All PCR products were purified using the QIAquick spin kit (Qiagen). PCR products were visualized on agarose gels using standard electrophoresis procedures.

The purified DNA products were ligated into pMD19T vectors using a rapid ligation kit according to the instructions of the manufacturer (TaKaRa), and then transformed into competent *Escherichia coli* DH5α cells. For the gene clones, the vector-specific primers M13f/M13r were used to avoid co-amplification of *E. coli* host-cell DNA. Amplicons were sequenced with ABI 3730 (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China).

Phylogenetic and Statistical Analyses

Clone sequences were checked for chimeras using Chimera Check [23]. The sequences were compared pairwise using a Blast search and alignment with the sequences of related species retrieved from the GenBank using the multiple alignment program Clustal_X version 2.0 [1, 21]. Phylogenetic trees were constructed by the neighbor-joining method with the MEGA5 [28, 36]. Evolutionary distances were calculated according to the algorithm of the Kimura's two-parameter model [20]. Bootstrap analysis was used to evaluate the tree topology by means of 1,000 resamplings [16]. DOTUR was used to assign sequences to an OTU with a minimum sequence similarity of 97 % [30].

Analytic Rarefaction 1.3 was used for rarefaction analysis. Shannon diversity was calculated using the Bio-Dap software [31, 37]. A PCA of the data from the clone libraries was performed at the genus level using software Canoco 4.5 [6]. The 33 partial 16S rRNA gene sequences obtained in this study have been deposited in the GenBank under accession number KJ806520-KJ806552.

Results and Discussion

Species Richness and Diversity

711 clones retrieved from four digesters were retrieved, and 33 archaeal OTUs was obtained (Table 2). The rarefaction analyses showed that the single clone library size was sufficient (Fig. 1). Good's coverage ranged from 97 to 99 %, also suggesting that cloning captured the dominant genotypes and also supported the tendency of the asymptotes of rarefaction curves. Conspicuously, Shannon diversity of the

Table 1 Altitude and operating process parameters of the four rural biogas digesters

Digester	Substrate	Temperature (°C)	pH	TS (%)	VS (%)	TP (mg/L)	NH_3-N (mg/L)	COD (mg/L)	Altitude (m)
BNA[a]	Pig and chicken manure	28	7.4	11.2	75.1	190.2	231.9	349.5	838.9
JSA[a]	Pig manure	25	7.9	8.3	74.1	261.3	281.9	1183.0	1295.9
LJA[a]	Pig and horse manure	18	7.3	3.3	68.4	320.6	167.9	667.8	2450.6
XGA[a]	Cattle manure	10	7.6	10.5	53.9	433.3	249.9	1297.4	3219.5

[a] BNA (22°51′N, 101°6′E), JSA (23°70′N, 102°89′E), LJA (26°85′N, 100°14′E), and XGA (27°83′N, 99°73′E) stand for the digesters in the tropical climate region, subtropical climate region, south temperate climate region, and north temperate climate region of Yunnan, respectively

Table 2 Diversity indices of four 16S rRNA gene clone libraries of the rural biogas digesters in different climatic regions of Yunnan

Clone library	Number of clones	Number of OTUs	Shannon (H')	Evenness (E)	Chao I[a]	Coverage (%)
BNA	169	17	1.24	0.46	18 (17, 23)	97
JSA	162	18	1.63	0.56	22 (19, 44)	97
LJA	176	23	2.18	0.70	32 (22, 14)	97
XGA	204	12	1.76	0.72	13 (12, 19)	99

[a] Values in parentheses are given for 95 % confidence interval

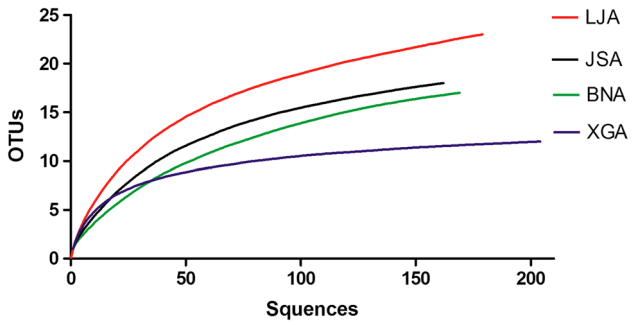

Fig. 1 Rarefaction analyses of four archaeal 16S rRNA gene clone libraries of the rural/household biogas digesters in different climate regions in Yunnan

clone libraries ranged from 1.2 to 2.2 (Table 2). The high Shannon index suggests that archaeal taxa in these habitats were significantly abundant. The results are similar to results of other researchers [4, 10].

Phylogenetic Analysis

BLAST search results showed that all the clones were assigned 33 OTUs to three phylogenetic groups: *Methanosarcinales*, *Methanomicrobiales*, and uncultured archaea, respectively (Table 3). Phylogenetic tree was constructed to determine the relationships of all OTUs (Fig. 2). About 63.2 % of the clones grouped into the order *Methanosarcinales* having 4 OTUs, which was represented by these genera *Methanosaeta*, *Methanosarcina*, and *Methanolobus*, and showed a 97–99 % similarity with them. 2 OTUs belonged to the genus *Methanosaeta* were the most frequently detected clones in all digesters with 48.0 %. The order *Methanomicrobiales* was represented by 4 families: 4 OTUs of *Methanomicrobiaceae* (9.4 %), 2 OTUs of *Methanocorpusculaceae* (5.3 %), 4 OTUs of *Methanospirillaceae* (4.0 %), and 1 OTU of *Methanoregula* (1.4 %). *Methanosaeta* was dominant group in three digesters except XGA. The major group was *Methanosarcina* in the XGA digester. It only contained an extremely small population of *Methanomicrobiales* (1.2 and 4.7 %) in the BNA and JSA digesters. It is obviously different consequences that the population of *Methanomicrobiales* was up to 39.8 and 29.4 % in LJA and XGA digesters. Here also contained a minor number of uncultured archaea (17.3 % in all clones).

The order *Methanosarcinales* was acetotrophic methanogens for majorly utilizing acetate as substrate for energy metabolism [5]. There is a general assumption that 70 % of methane was produced by acetotrophic methanogens [24].

Table 3 Results of four 16S rRNA gene clone libraries analyses of the rural biogas digesters in different climate regions of Yunnan

	BNA (%)	JSA (%)	LJA (%)	XGA (%)	Total (%)
Methanosarcinales	78.7	67.3	46.6	61.3	63.2
Methanosaeta	76.3	66.7	43.2	13.7	48.0
Methanolobus	1.2	0.6	0.6	ND	0.6
Methanosarcina	1.2	ND	2.8	47.5	14.6
Methanomicrobiales	1.2	4.3	39.8	29.4	19.5
Methanospirillum	1.2	4.3	7.4	ND	3.1
Methanogenium	ND	ND	11.4	6.4	4.6
Methanoplanus	ND	ND	8.5	6.4	3.9
Methanoregula	ND	ND	2.3	2.9	1.4
Methanocorpusculum	ND	ND	6.8	12.7	5.3
Methanoculleus	ND	ND	3.4	ND	0.8
Methanosphaerula	ND	ND	ND	1.0	0.3
Uncultured archaea	20.1	28.4	13.6	9.3	17.3

ND not detected

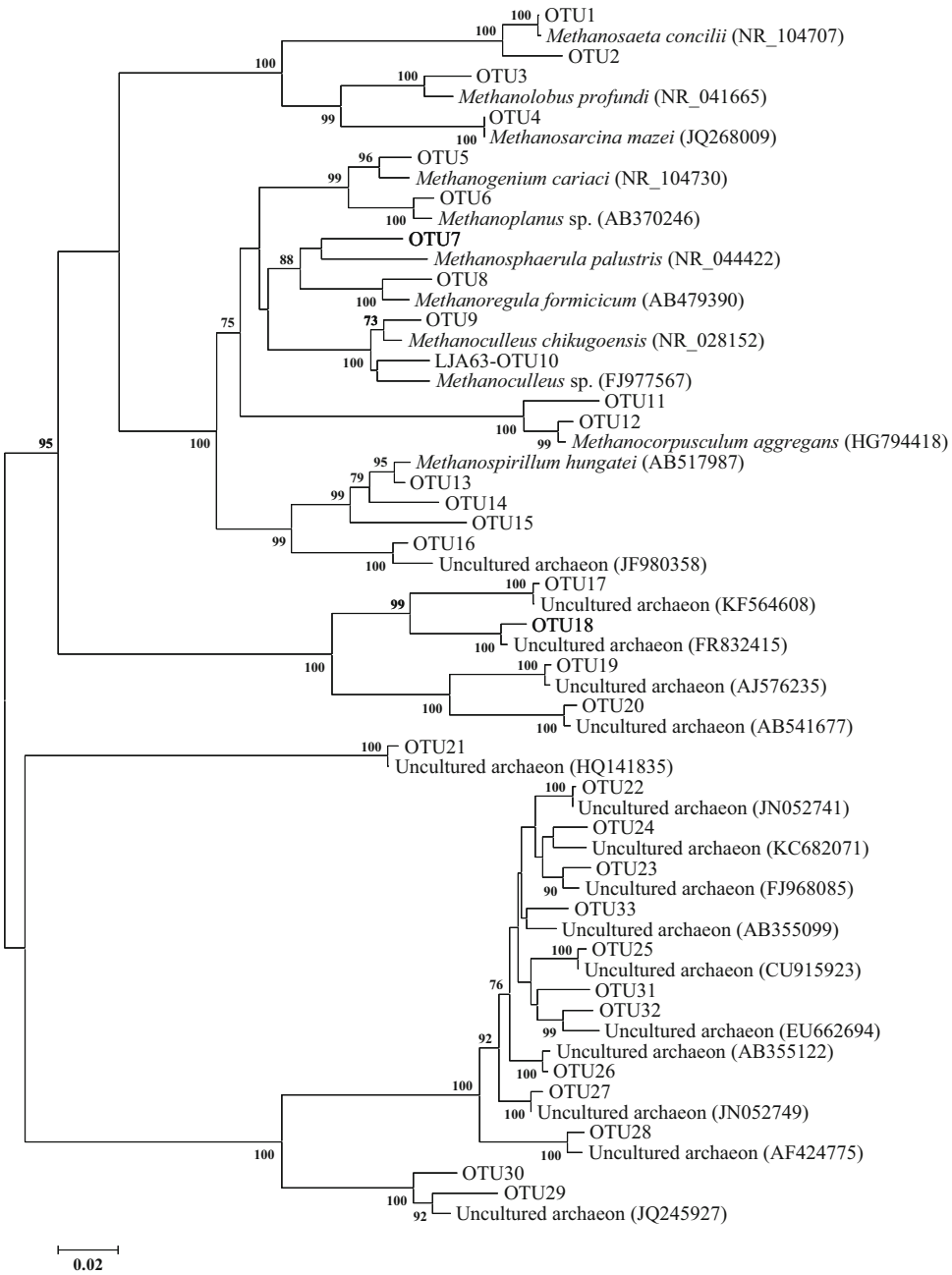

Fig. 2 Phylogenetic tree of 16S rRNA gene sequences constructed with evolutionary distances calculated based on the Kimura-2 model and the neighbor-joining method. The topology of the tree was estimated by bootstraps based on 1000 replication

The composition of the order *Methanosarcinales* ranged from 46.6 to 78.7 % among the digesters; therefore, the main methane metabolic pathway is acetate pathway in them. The genus *Methanosaeta* was found to be the dominant acetotrophic methanogens in a variety of anaerobic reactors at low acetate concentrations during quantification of *Methanosaeta* in anaerobic bioreactors [42]. The proportion of the genus *Methanosaeta* (13.7–76.3 %) in different digesters should be affected by comprehensive ecological environmental factors [19, 43]. In addition to hydrolysis of polymeric substances, the activity and performance of the methanogenic archaea was of paramount importance during methanogensis [13].

The presence of a considerable proportion of hydrogenotrophic methanogens in the LJA and XGA digesters suggested a high production of hydrogen by syntrophic bacteria during the organic matter degradation, because the acetate-oxidizing bacteria have a competitive relationship with the acetotrophic methanogens for converting acetate to $H_2 + CO_2$ [24]. At temperature as low as 15 °C, syntrophic acetate oxidation has been reported for natural anoxic environments in subtropical lake sediments [27]. Furthermore, the syntrophic relationship between hydrogenotrophic methanogens and acetate-oxidizing organisms could be the main route of acetate degradation in biogas fermentation [38].

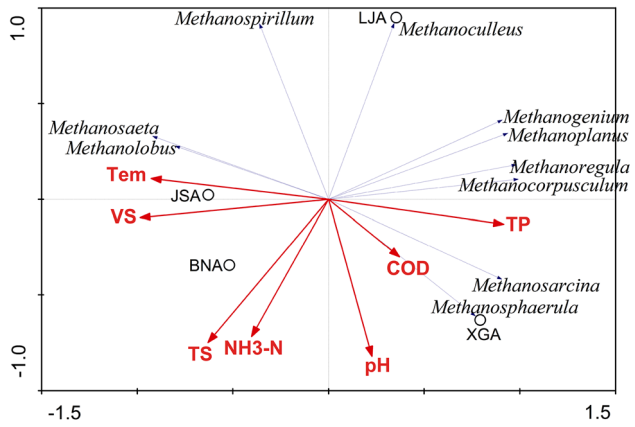

Fig. 3 Principal component analysis (PCA) of archaeal communities affected by environmental factors in the biogas digesters. *Tem* fermentation temperatures, *VS* volatile solid, *TS* total solids, *TP* total phosphorus, *COD* chemical oxygen demand, *NH_3-N* ammonia nitrogen

Effect of Environmental Factors on Community Structure

The PCA biplot gave three major clusters for archaeal community at the genus level (Fig. 3). The first cluster was dominated by sequences belonging to the acetotrophic methanogens *Methanosaeta* and *Methanolobus* closing with the major fermentation temperatures (Tem) and VS. The second and third clusters were *Methanosarcina* and the major hydrogenotrophic methanogens relevance with the TP, respectively.

The results implied that the fermentation temperatures and high VS were to determine the scale of *Methanosaeta* according to the PCA results. The proportion of *Methanosaeta* sharply declined with the fermentation temperatures of the digesters dropped from 28 to 10 °C. Some study also implied that *Methanosaeta* was observed to be the most abundant in mesophilic anaerobic digesters [17]. More importantly, temperature change will alter methanogenic community structure and diversity in the ecosystems, which make the succession of the dominant groups of methanogenic archaea [18]. We newly know that *Methanosaeta* is strongly influenced by fermentation temperatures in the digesters of this study, which explain a large proportion of the variance in methanogenic archaeal community at different climatic regions. Furthermore, *Methanosarcina* was found usually in digesters fed with cattle manure and often more tolerant than other methanogens against harsh conditions [7, 11, 15]. *Methanosarcina* consumed acetate at high acetate concentration, and usually indicated that the digester was unstable performance [25]. The archaeal community involved in the biogas fermentation process can be affected by change of environmental factors and made a reduction of the reactor efficiency and lead to process errors like acidification or decrease of gas production rate [8, 22]. The optimum habitat of *Methanospirillum* was the mesophilic condition [33]. That is the reason that *Methanospirillum* only absent in the XGA digester which of the fermentation temperatures was only 10 °C and even frozen in the winter.

Conclusion

In conclusion, the community structures of methanogenic archaea in the rural digesters study demonstrated a high level of archaeal diversity. The archaea were identified and included three phylogenetic groups: *Methanosarcinales* (63.2 %), *Methanomicrobiales* (19.5 %), and uncultured archaea (17.3 %). The PCA indicated that the dominant taxon *Methanosaeta* was mainly affected by the fermentation temperatures. The community structures of methanogenic archaea in this study will be helpful to recognize the effect of the environmental factors during the methane production in biogas digesters. Therefore, further investigation is required to define the interactions of methanogens and syntrophic bacteria during methanogensis and to improve the efficiency of biogas digesters in rural household digesters.

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