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Phylogenetic Analysis of Methanogens in the Pig Feces

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Abstract In order to assess methanogen diversity in feces of pigs, archaeal 16S rRNA gene clone libraries were constructed from feces of the pig. After the amplification by PCR using primers Met86F and Met1340R, equal quantities of PCR products from each of the five pigs were mixed together and used to construct the library. Sequence analysis showed that the 74 clones were divided into ten phylotypes as defined by RFLP analysis. Phylogenetic analysis showed that three phylotypes were most closely affiliated with the genus *Methanobrevibacte*r (46% of clones). The library comprised 55.4% unidentified euryarchaeal clones. Three phylotypes (LMG4, LMG6, LMG8) were not closely related to any known Euryarchaeota sequences. The phylogenetic analysis indicated that the archaea found in the libraries were all clustered into the Euryarchaeota. The data from the phylogenetic tree showed that those sequences belonged to three monophyletic groups. Phylotypes LGM2 and LGM7 grouped within the genus *Methanobrevibacter*. Phylotypes LGM4, LGM6, LGM8 and LGM9 grouped within the genus *Methanosphaera*. Other phylotypes grouped together, and formed a distantly related sister group to *Aciduliprofundum boonei* and species of the Thermoplasmatales including *Thermoplasma volcanium* and *Thermoplasm acidophilum*. Our results showed that methanogens belonging to the genus *Methanobrevibacter* were predominant in pig feces, and that many unique unknown archaea sequences were also found in the library. Nevertheless, whether these unique sequences represent new taxonomic groups and their role in the pig gut need further investigation.

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Introduction

Methanogens are members of the domain Archaea, and fall within the kingdom Euryarchaeota [7]. They are obligate anaerobes and can be unambiguously differentiated from other organisms since they all produce methane as a major catabolic product [1]. Interest in methanogens from pigs has resulted from the role of methane in global warming and from the fact that pigs were estimated to typically lose 1.2% of ingested energy as methane [4]. So far, methanogens have been isolated from various animals [2, 3]. However, only one report was on the isolation of methanogens from pig feces [3]. Based on cell wall composition, the strains isolated from swine feces appeared to belong to the genus *Methanobrevibacter* [3]. To the best of our knowledge, analysis of the 16S rRNA genes of the methanogens from swine feces has not been reported. In this study, we investigated the phylogenetic diversity of methanogens in pig feces by the analysis of 16S rRNA gene clone libraries.

Materials and Methods

Feces Sampling and Animal Diets

Approximately 10 g of feces was collected from each of five pigs(Duroc × Landrace × Yorkshire) before the first morning feed in QiangSheng farm in JiangSu Province in June 2010. The five samples were maintained on ice for approximately 60 min while being transported to the

laboratory for processing. The pig feces were aliquoted into two 1.5-ml Eppendorf tubes. The remaining fecal samples were fixed with an equal volume of 100% ethanol and stored. The animals were fed a total mixed diet consisting of 60% corn, 35% soybean meal, 2.4% limestone, 1.6% $CaHPO_4$, and 1% premix (vitamins/minerals).

DNA Extraction, PCR Amplification, and Clone Library Construction

Before DNA extraction, samples were thawed. A portion of 0.5 g of each sample was used to extract DNA based on the bead-beating method described by Zoetendal et al. [12]. The extracted DNA was purified and then checked on agarose gel with most of DNA fragments around 50 kb based on the DNA marker ladder run on the same gel. The DNA was used for PCR to amplify the 16S rRNA gene.

The PCR primers used to amplify 16S rRNA genes were Met86F (5′-GCT CAG TAA CAC GTG G-3′) and Met1340R (5′-CGG TGT GTG CAA GGA G-3′), which were reported as methanogen-specific primers [9, 10]. The amplification conditions were: initial denaturation at 94°C for 3 min, then 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 90 s, and last extension at 72°C for 10 min. The PCR mixture (total volume of 50 ml) contained 200 nM of both primers, approximately 0.35 mg of the purified DNA extract, 1× Taq reaction buffer, 200 mM of each dNTP, 2 mM of $MgCl_2$, and four units of Taq DNA polymerase. After amplification, equal quantities of PCR products from the five pigs were mixed for clone library construction. Amplified DNA for clone libraries was purified using a PCR Clean-Up system (Promega, Madison, WI, USA) and cloned into *Escherichia coli* strain TOP10 using the pGEM-T Easy vector (Promega). All the recombinant plasmids were reamplified by PCR using the primers and parameters described above. Different restriction endonucleases (Hae III, Alu I, Hpa II) were used to digest PCR production of the recombinant plasmids. Digested PCR products were immediately separated by electrophoresis on 4% agarose gels. Phylotypes were defined based on RFLP patterns, and that this was confirmed by sequence analysis (Invitrogen, Beijing, China), using 99% sequence identity as threshold for phylotype definition. The relative abundance of each phylotype in the library was calculated by dividing the number of clones belonging to this phylotype by the total number of clones in the library (i.e., 74).

Phylogenetic Analysis

Phylogenetic analysis of sequences retrieved in this study was performed with sequences of the best "known hits" from BLAST searches, 16S rRNA gene sequences of validly described methanogens and species belonging to the Euryarchaeota. Two crenarchaeal reference sequences (*Sulfolobus acidocaldarius* and *Thermoproteus tenax*) were used as outgroups. Sequences were aligned using Clustalx1.8, and a phylogenetic tree was constructed by MEGA4 (http://www.megasoftware.net/mega.html) software to illustrate the evolutionary relationships. Nucleotide sequences have been deposited in the GenBank database under accession numbers: HM210844, HM210845, HM210846, HM210847, HM210848, HM210850, HM210851, HM998286, HM998287, and HM998288.

Results

Single-clone colonies of ampicillin resistant transformants were picked up and transferred to LB broth medium and incubated at 37°C overnight. After colony PCR with pGEM-T-specific primers Sp6 and T7 (Promega) to check the size of insert by their plasmid DNA on agrose gel, a total of 74 clones with right insert (16S rRNA gene, approximately 1.2 kb in length) were obtained and then subjected to RFLP analysis to identify phylotypes. Sequence examination of these clones revealed 10 different phylotypes (Table 1). The majority of phylotypes (34 clones) had their sequences closely related to *Methanobrevibacter spp*. (*Methanobrevibacter gottschalkii*, *Methanobrevibacter ruminantium*) with similarity ranging from 93 to 98%. Three phylotypes (16 clones) were related to *Methanosphaera stadtmanae* with similarity ranging from 96 to 97%. This library also showed three phylotypes (21 clones) with similarity ranging from 77 to 80% to *Aciduliprofundum boonei* and one phylotypes (three clones) related to *Thermoplasma acidophilum* with similarity of 80%.

The phylogenetic analysis confirmed BLAST searches, as all sequences were clustered into the Euryarchaeota (Fig. 1). Tree topology indicated that all sequences belonged to three monophyletic groups. Phylotypes LGM2 and LGM7 grouped within the genus *Methanobrevibacter*. Phylotypes LGM4, LGM6, LGM8, and LGM9 grouped within the genus *Methanosphaera*. Other phylotypes grouped together, and were the sister group to *Aciduliprofundum boonei* and species of the Thermoplasmatales including *Thermoplasma volcanium* and *Thermoplasm acidophilum*.

Discussion

Investigating the diversity and structure of methanogenic communities has been a long-standing challenge in animal gut ecology. In this study, PCR-retrieved methanogenic

Table 1 16S rRNA gene clones of the pig feces methanogen library

Phylotypes	Accession no.	Size of clone (bp)	No. of clones	Nearest valid taxon	
				Name	Percentage sequence similarity
LGM1	HM210844	1256	16	*Aciduliprofundum boonei*	80
LGM2	HM210845	1262	15	*Methanobrevibacter gottschalkii*	97
LGM3	HM210846	1256	4	*Aciduliprofundum boonei*	80
LGM4	HM210847	1260	10	*Methanosphaera stadtmanae*	96
LGM5	HM210848	1257	3	*Thermoplasma acidophilum*	80
LGM6	HM210850	1260	5	*Methanosphaera stadtmanae*	96
LGM7	HM210851	1261	17	*Methanobrevibacter gottschalkii*	98
LGM8	HM998286	1260	2	*Methanobrevibacter ruminantium*	93
LGM9	HM998287	1260	1	*Methanosphaera stadtmanae*	97
LGM10	HM998288	1218	1	*Aciduliprofundum boonei*	77

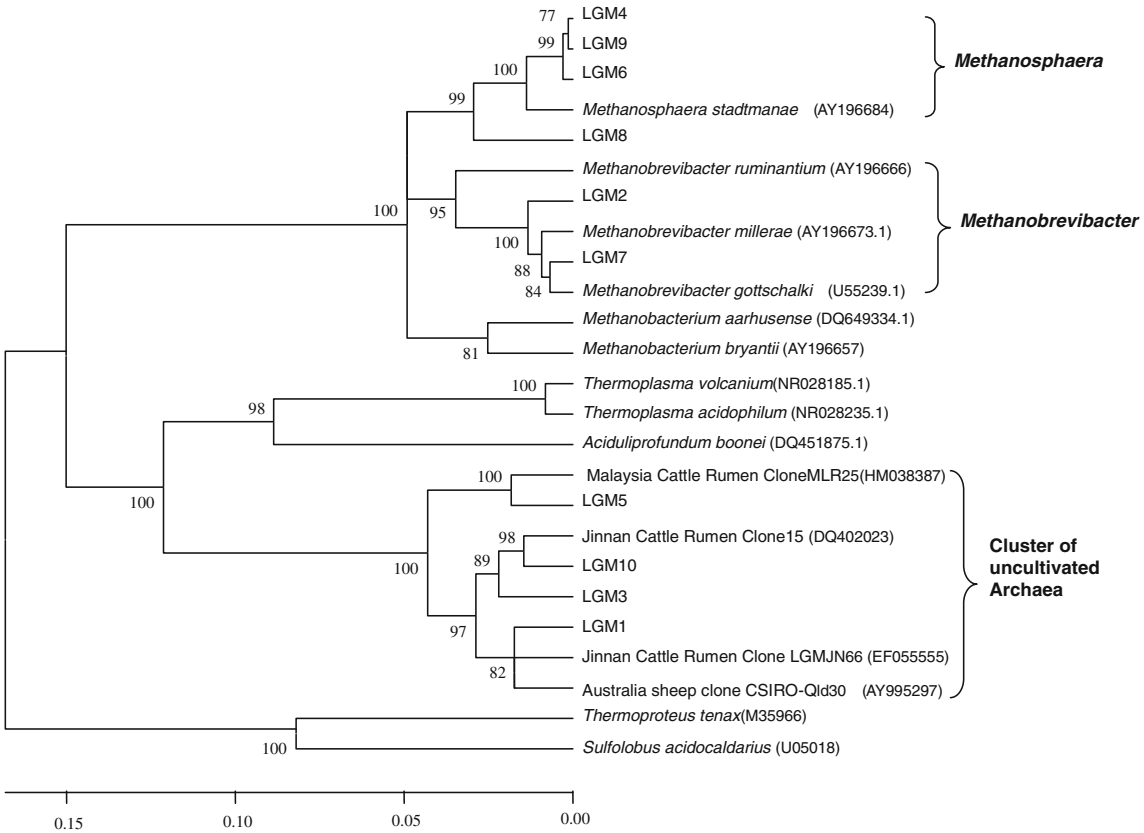

Fig. 1 Phylogenetic relationships of archaeal clones derived from 16S rRNA gene evolutionary distances produced by the Bootstrap model and constructed using the Unweighted Pair Group Method with Arithmetic mean method. The tree was bootstrap-resampled 1000 times

16S rRNA gene libraries were established from the pig feces. The results showed that the most predominant species of methanogens were related to the genus *Methanobrevibacter*. This is in agreement with previous studies indicating species belonging to the genus *Methanobrevibacter* as the major methanogens in the animal feces [3]. The predominance of this genus has also been suggested before based on cultivation studies [3].

In this study, our libraries uncovered some unidentified euryarchaeotic sequences. Three phylotypes (LGM4, LGM6, and LGM 8) were 93–96% identical to known methanogens, thus they are likely to be unknown methanogenic strains or species. Three phylotypes (LGM1, LGM3, and LGM10) were 77–80% similar and most closely related to *Aciduliprofundum boonei*, an euryarchaeotic species found in deep sea. All of them were clustered

within Euryarchaeota, in which three classes can be distinguished (*Methanobacteria*, *Methanosphaera*, a novel group of uncultivated archaea). All classes except for the methanogenic ones are hyperthermophilic or halophilic. In this study, four unique phylotypes (twenty four clones) clustered within a strongly documented phylogenetic group along with other clone sequences from uncultured methanogens. Interestingly, the sister group to this distinct branch is a branch containing two Thermoplasmatales species (*Thermoplasma volcanium* and *Thermoplasma acidophilum*) and a deep sea euryarchaeotic species (*Aciduliprofundum boonei*). The sister group of Thermoplasmatales species was also found in the rumen [5, 6, 8, 10]. Although it is unlikely that the uncultured and unidentified methanogens ultimately will be classified as *Aciduliprofundum boonei*, regarding their relatively low similarity, the consistency in our results in this study and the ones in our previous report on rumen by Pei et al. [5] is striking. This might indicate that the novel group represents a stable archaea group not only in the rumen, but also in the gut of monogastric animals.

From our survey literature, the primers (Met 86f/Met 1340r) used in this study have been reported as specific for methanogenic archaea and widely used to study the diversity of the methanogenic archaea in the rumen of dairy, sheep, or buffalo [5, 6, 8, 10, 11]. However, a probe match search at RDP website showed that both primers have matches not only with the methanogenic species within the Euryarchaeota, but also with a large number of sequences in the Crenarchaeota. Given the low similarity of sequences retrieved from the pig feces to these sequences, one cannot exclude that the organisms belong to the novel group detected in this study are derived from methanogens, but we do not know that until this activity has been experimentally shown. Although Wright et al. [10] indicated that research was underway in several laboratories attempting to cultivate species of this newly discovered novel group, to date no formal reports about the success of the isolation of these archaea have been reported. Therefore, further investigations on the isolation and its activity in the pig of the species belonging to the novel group are still needed.

Conclusions

This study has revealed the phylogenetic diversity of methanogens found in the pig feces. One of the methanogen groups is highly similar to the type strain of *Methanobrevibacter gottschalkii*, and other groups represent methanogens not previously recovered from the rumen or monogastric animal gut or not yet represented in culture collections. Many unique unknown euryarchaeotic sequences were also found. Nevertheless, whether these unique sequences represent new taxonomic groups and their role in the rumen or monogastric animal gut need further investigation.

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