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ORIGINAL ARTICLE

The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis

F.H. Liu[1,2], S.B. Wang[1], J.S. Zhang[1], J. Zhang[1,2], X. Yan[1], H.K. Zhou[1], G.P. Zhao[1] and Z.H. Zhou[1]

1 Key Laboratory of Synthetic Biology, Institute of Plant Physiology & Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
2 Graduate School of the Chinese Academy of Sciences, Beijing, China

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Correspondence
Zhihua Zhou, Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai, 200032, P.R. China.
E-mail: zhouzhihua@sippe.ac.cn

F.H. Liu and S.B. Wang have contributed equally to this study.

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Abstract

Aims: To identify the bacterial and archaeal composition in a mesophilic biogas digester treating pig manure and to compare the consistency of two 16S rDNA-based methods to investigate the microbial structure.
Methods and results: Sixty-nine bacterial operational taxonomic units (OTU) and 25 archaeal OTU were identified by sequencing two 16S rDNA clone libraries. Most bacterial OTU were identified as phyla of *Firmicutes* (47·2% of total clones), *Bacteroides* (35·4%) and *Spirochaetes* (13·2%). *Methanoculleus bourgensis* (29·0%), *Methanosarcina barkeri* (27·4%) and *Methanospirillum hungatei* (10·8%) were the dominant methanogens. Only 9% of bacterial and 20% of archaeal OTU matched cultured isolates at a similarity index of ≥97%. About 78% of the dominant bacterial (with abundance >3%) and 83% of archaeal OTU were recovered from the denaturing gradient gel electrophoresis (DGGE) bands of V3 regions in 16S rDNAs.
Conclusions: In the digester, most bacterial and archaeal species were uncultured; bacteria belonging to *Firmicutes*, *Bacteroides* and *Spirochaetes* seem to take charge of cellulolysis, proteolysis, acidogenesis, sulfur-reducing and homoacetogenesis; the most methanogens were typical hydrogenotrophic or hydrogenotrophic/aceticlastic; DGGE profiles reflected the dominant microbiota.
Significance and Impact of the Study: This study gave a first insight of the overall microbial structure in a rural biogas digester and also indicated DGGE was useful in displaying its dominant microbiota.

Introduction

As the biggest developing country in the world, China has a population of 1·3 billion. Among these people, 72·3% of them are living in the rural areas. Because of the rapid growth of industry and energy consumption in the city, energy shortage becomes a big problem [The Priority Programme for China's Agenda 21; see the World Wide Web (WWW) site: http://www.acca21.org.cn/indexe8.html]. Furthermore, the demands of energy in the rural areas also increase daily. On the other hand, there is a great deal of waste biomass produced in the countryside, including a large amount of lignocellulose byproducts in agriculture and excrement of poultry and cattle, which creates problems for the rural environment. To improve the environmental and living conditions in the countryside and to create a sustainable development in rural economy (Gupta 2003), it is necessary to resolve the energy and sanitary problems in the rural areas.

Anaerobic fermentation of waste biomass not only generates biogas fuel for cooking, lighting and heating, but also reduces waste biomass, potentially providing a mutually beneficial situation for the environmental, social and cycling-economic development in the rural areas. The technology for constructing biogas digesters at different scales and for different applications for treating rural wastes is well established (Chynoweth *et al.* 1999), while the efficiency of biogas production needs to be improved

urgently. The composition of the microbial community in a biogas digester directly determines its efficiency and biogas yield. The process of anaerobic conversion of different biomasses to methane usually includes four steps: hydrolysis, acidogensis, acetogenesis and methanogenesis, in which hydrolytic, fermentative bacteria, acetogens and methanogens play distinct roles, respectively (Pretty *et al.* 2002; Angenent *et al.* 2004).

Hydrolytic and acidogenic bacterial strains identified as *Clostridium thermocellum*, *Clostridium leptum*, *Clostridium botulinum*, *Bacteroides termitidis*, *Desulfovibrio desulfuricans*, *Treponema palladium* and *Pirochaeta aurantia,* acetogens such as *Syntrophobacter wolinii* and *Syntrophomonas wolfei* and archaeal methanogens belonging to *Methanosaeta* sp., *Methanocorpusculum* sp., *Methanoculeus sp.* and *Methanobrevibacter* sp. *Methanobacterium* sp., *Methanosarcina* sp. and *Methanobrevibacter* sp. were isolated from biogas digesters and anaerobic storages of animal manure in several studies (Boone and Bryant 1980; Zhao *et al.* 1986; Ney *et al.* 1990; Meher and Ranade 1993; Ohkuma and Kudo 1996; Whitehead and Cotta 1999; Cotta *et al.* 2003; Snell-Castro *et al.* 2005; Drake *et al.* 2006). A number of studies have indicated that only a small portion of the microorganisms (0·1–25%) were cultured (Cotta *et al.* 2003).

Culture-independent approaches, mainly 16S rDNA-based methods, e.g. cloning and sequencing, D/TGGE (denaturing/temperature gradient gel electrophoresis) fingerprinting, FISH (fluorescence *in situ* hybridization), RFLP [restriction fragment length polymorphism, or ARDRA (amplified rDNA restriction analysis)] and T-RFLP (terminal-RFLP), are applied to elucidate the diversity and composition of microbial communities in anaerobic methanogenic digesters widely used for the treatment of municipal and industrial wastewater (Karakashev *et al.* 2005; Connaughton *et al.* 2006; Mladenovska *et al.* 2006; Cirne *et al.* 2007; Klocke *et al.* 2007; Sousa *et al.* 2007a; Ye *et al.* 2007). Connaughton *et al.* (2006) and Sousa *et al.* (2007a) reported changes over time of the bacterial and archeal population in an anaerobic digester, the former compared the biomass composition with the activity of the digester and the latter compared the composition of microbiota in the presence and absence of long chain fatty acids. Ye *et al.* (2007) reported changes in digesters operated at several pH. Several studies involved investigation of microbial compositions in anaerobic methanogenic digesters treating rural waste (Mladenovska *et al.* 2006; Cirne *et al.* 2007; Klocke *et al.* 2007). By TGGE and cloning library and sequencing, Mladenovska *et al.* (2003) found that the most dominant methanogens in lab-scale anaerobic digesters with cattle manure or a mixture of cattle manure with glycerol trioleate were phylogenetically related to *Methanosarcina siciliae*. The bacterial and archaeal composition identified by T-RFLP analysis of 16S rRNA genes were found to be identical in two thermophilic continuously stirred tank reactors (CSTR), treating nontreated manure and pretreated manure for 40 min at 140°C, respectively (Mladenovska *et al.* 2006). However, to our knowledge, no previous studies characterized the overall microbial communities in a biogas digester treating rural waste.

Compared with the sequencing analysis of 16S rDNA genes in clone libraries, DGGE profiles of the V3 region was simpler and less costly for analysing the structural variation between different microbial systems or the spatio-temporal dynamics of the same system (Muyzer *et al.* 1993). It could also be useful in tracing the variation of dominant microbial organism in a biogas digester. However, previous studies have indicated that DGGE profiles were not consistent with the results from the sequencing of clone libraries in different microbial systems (Krave *et al.* 2002; Freeman *et al.* 2008; Wakase *et al.* 2008). It is important to ensure whether the results from DGGE profiles are consistent with clone library analyses before they are applied in biogas digesters.

For the purpose of both basic research and biogas biotechnology, there is considerable interest in elucidating the microbial composition and metabolic diversity involved in biogas production, as well as setting up an applied and less costly method to trace the variation of microbial structure in anaerobic bio-digesters. In this study, the overall microbial communities in a mesophilic anaerobic biogas digester were investigated by analysing the diversity of 16S rDNA using DGGE and sequence analysis. Such an investigation for the composition of the microbial community in the biogas digesters would be the first step to elucidate the relationship between the efficiency of biogas production in the digesters and the structure and variation of the microbiota. The purpose of this study was twofold: (i) to characterize the microbial diversity of an anaerobic biogas digester using culture-independent methods and (ii) to compare the discriminatory power of DGGE *vs* 16S library screening.

Materials and methods

Sample collection

A biogas slurry sample (3 l) was collected in June 2005 from a biogas digester in Qianwei Village, Chongming County, Shanghai, China, which was built in the early of 1980s and kept running for about 25 years. The digester has a volume of 600 m^3 for anaerobic fermentation and produces 150 m^3 biogas per day. The main fermentation substrate is pig manure from a nearby piggery breeding 1500 pigs. The digester can treat 1800 tonnes of pig manure from the piggery every year (Hao and Liu 2006).

DNA extraction

To remove extracellular DNA and soluble organic contaminants, the fresh slurry samples (50 ml) were washed thrice in five volumes of TENP buffer [50 mmol l^{-1} Tris-HCl, 20 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 0·01 g ml^{-1} polyvinylpyrrolidone (PVP), pH 10], twice in five volumes of sterile phosphate-buffered saline (PBS buffer, 137 mmol l^{-1} NaCl, 2·7 mmol l^{-1} KCl, 1·5 mmol l^{-1} KH_2PO_4, 8·1 mmol l^{-1} Na_2HPO_4 in distilled water, pH 7·4) and vortexed for 5 min and centrifuged for 10 min at 10 000*g*. The washed cell pellets were resuspended in a suitable volume of sterile PBS buffer containing glycerol at a final concentration of 20%, divided into 4-ml aliquots, and stored at −70°C until nucleic acid extraction.

After thawing and centrifugation of subsamples (1 ml), the supernatants were removed. The genomic DNA was then extracted using QIAamp DNA stool mini kit (Qiagen, Heidelberg, Germany) according to the manufacturer's instruction with minor modification. The treated sample was carefully disrupted by Cell Disruptor Genie (Scientific Industries Inc., New York) in the 2·0-ml microcentrifuge tube after adding the extraction buffer. The purified DNA was quantified with a Biophotometer (Eppendorf), and stored at −20°C until use.

Establishment of bacterial and archaeal 16S rDNA libraries

Bacterial and archaeal clone libraries were generated from polymerase chain reaction (PCR)-amplified 16S rDNA using bacterial primers 27f (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Bianciotto *et al.* 1996) and the archaeal primers 1Af (5′-TCY GKT TGA TCC YGS CRG AG-3′) and 1100Ar (5′-TGG GTC TCG CTC GTT G-3′) (Embley *et al.* 1992). Reaction mixtures (25 μl) contained 2·5 μl of 10 × PCR buffer (TaKaRa Inc., Dalian, China), 0·2 mmol l^{-1} each of deoxyribonucleotide triphosphates (dNTP), 0·5 μmol l^{-1} of each primer, 1 U of Ex *Taq* DNA polymerase (TaKaRa Inc.) and 5 ng of template DNA. PCR reactions were performed on a Flexigene thermal cycler (Techne Flexigene, Cambridge, UK). The PCR reaction for archaeal 16S rDNA was performed using the following programme: initial denaturation for 3 min at 94°C; 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (2 min at 72°C) with a final extension of 72°C for 10 min. The optimized PCR amplification conditions for bacterial 16S rDNA were as follows: initial denaturation at 95°C for 1·5 min; 5 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1·5 min; 5 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1·5 min; 15 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1·5 min and a final extension of 72°C for 10 min.

To minimize PCR artefacts, 'reconditioning PCR' was performed as described by Thompson *et al.* (2002) after the initial amplification of the bacterial and archaeal 16S rDNA as described before. The initial PCR-amplified reaction was diluted 10-fold in a fresh reaction mixture of the same composition and cycled thrice using this programme. ssDNA and heteroduplex DNA could be minimized by adding excess primer during the 'reconditioning PCR' (Zhang *et al.* 2005).

Cloning and sequencing

Exactly 100 μl of bacterial and archaeal 16S rDNA reconditioning PCR products were electrophoresed on 1·0% agarose and the bands of the correct size (*c.* >1·5 kb for bacteria, and 1·1 kb for archaea) were purified using 3S PCR Product Purification Kit V2·0 (Shenergy Biocolor Biological Science & Technology Co., China), respectively. Finally, the purified product was cloned into the pMD18-T plasmid vector (TaKaRa Inc.) following the manufacturer's instructions. The ligated products were transformed into *Escherichia coli* TOP10 competent cell (Invitrogen) with ampicillin and blue/white screening, and positive clones were arrayed in 96-well plates and stored at −80°C for long-term storage. Plasmid inserts were checked by PCR amplification using the M13 PCR set. Exactly 310 bacterial and 192 archaeal positive insert-containing clones were randomly selected for gene sequencing. The template DNA was prepared from overnight cultures of selected clones using an alkaline miniprep kit (Qiagen), sequencing were performed on an ABI 3730 DNA sequencer with Big Dye terminator chemistry as specified by the manufacturer (Applied Biosystems).

16S rDNA V3 region amplification

The V3 regions of bacterial and archaeal 16S rDNA from the biogas slurry DNA extract were amplified by PCR and the amplified products were used for analysis by DGGE. The bacterial primer sets EubacVf (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and Vr (5′-ATT ACC GCG GCT GCT GG-3′), archaeal primer sets PARCH340f (5′-CCC TAC GGG GYG CAS CAG-3′) and PARCH519r (5′-TTA CCG CGG CKG CTG-3′), GC clamp and PCR amplification used were the same as described by Muyzer *et al.* (1993) and Øvreås *et al.* (1997). Reconditioning PCR products (200 μl) were concentrated with two volumes of ethanol and finally dissolved in 20 μl of water for further DGGE analysis. PCR product was observed on agarose gel (2·0%) with 1 × TAE buffer (40 mmol l^{-1} Tris-HCl,

40 mmol l^{-1} acetate, 1·0 mmol l^{-1} EDTA) and ethidium bromide (0·5 μg ml^{-1}) under ultraviolet (UV) light.

Denaturing gradient gel electrophoresis

DGGE of the PCR products was performed by the method described by Muyzer *et al.* (1993) with the DCode Universal Mutation Detection system (Bio-Rad Laboratories, Hercules, CA < USA). Denaturing gradient gel [1 mm thickness × 160 × 160 mm; 1 × TAE (40 mmol l^{-1} Tris base with 1·0 mmol l^{-1} EDTA and 20 mmol l^{-1} sodium acetate at pH 7·4), 8% acrylamide-bisacrylamide (37·5 : 1), and 25–60% (35–70% for archaea, based on our unpublished results of perpendicular DGGE) denaturant (100% = 7 mol l^{-1} urea with 40% formamide)] was poured with a gradient delivery system (model 475; Bio-Rad Laboratories). Electrophoresis was performed at a constant temperature of 60°C, first for 10 min at 25 V and then for 5 h at 200 V in 1 × TAE buffer. After the electrophoresis, the gels were stained with $AgNO_3$ as described by the manufacturer.

Clone library construction of single DGGE bands

Each gel slice that contained an obvious DNA band was excised with a clean razor blade and placed in an 1·5-ml Eppendorf tube. The gel slice was crushed and incubated with 50 μl of TE buffer at 4°C overnight. The 3-μl supernatant was subjected to a second PCR under the same conditions as described before. The re-amplified PCR products were examined by DGGE to confirm that single bands were present at the same positions. The PCR products were then purified with Mini-DNA Rapid Purification Kit (BioDev, Beijing, China) and cloned into the pMD18-T Vector (TaKaRa Inc.) to construct the clone libraries. Five clones were picked from each library and sent to Invitrogen (Shanghai, China) for sequencing.

Sequence analysis

Sequences were edited manually to remove vector and ambiguous sequences at the ends by scanning of the individual chromatograms using Chromas software ver.2.23 (Technelysium, Shanghai, China). Chimeras were checked by the CHIMERA_CHECK programme (Cole *et al.* 2003) in Ribosomal Database Project (RDP) at first, and then were further firmed by Bellerophon programme on the Greengenes website (DeSantis *et al.* 2006). All reference sequences were obtained from the GenBank and RDP. Then all the sequences and their closest relatives were fitted into an alignment using the automated tools of the ARB software package (Ludwig *et al.* 2004). Aligned sequences were added to the ARB neighbour-joining tree (based on pairwise distances with Olsen correction) with the parsimony insertion tool as described by Ley *et al.* (2005). Sequences with internal regions of poor quality leading to alignment problems were excluded from further analysis. Dotur (Schloss and Handelsman 2005) was used to cluster sequences into operational taxonomic units (out) by % pairwise identity (%ID, using a furthest-neighbour algorithm and a precision of 0·03). The stability of tree branches was assessed by the bootstrap method using 1000 replicates.

Nucleotide sequence accession numbers

Bacterial nucleotide sequences obtained in this study are available in the GenBank database under accession numbers: EU358617–EU358650 and EU358676–EU358744. Archaeal nucleotide sequences obtained in this study are available in the GenBank database under accession numbers: EU358606–EU358616 and EU358651–EU358675.

Results

Microbial community analysed by clone library-bacterial community

The 16S rDNA genes were amplified from the total DNA extracted from the biogas slurry sample with a bacterial primer set 27f/1495r and amplicons were ligated to pMD-18 T vector to construct a library. In total, 310 clones were randomly selected and sequenced. After eliminating low-quality (68 clones) sequences and chimeric sequences (53 clones), 189 sequences were used for the following analyses. The coverage of the library was 81·0%, indicating that the library was large enough for further analyses. The 189 sequences of 16S rDNA genes were classified into 69 OTU (Table 1). The abundances of all OTU in the library were less than 7%, where only nine OTU were more than 3%. The accession number, sources and described functions of their phylogenetically closest matched organisms are also listed in Table 1.

Only 14 of the 69 OTU in the bacterial library were matched to the closest related known sequences deposited in NCBI and RDP at a similarity index of more than 97%, which was regarded as an experiential index for differentiating species (Table 1). Twenty-nine of the 69 OTU were matched to the closest related known sequences deposited in the databases at a similarity index of between 90% and 96%, while 26 of the 69 OTU were matched to the closest related known sequences at a similarity index of between 80% and 90%. Nearly 80% of the bacteria in this digester may be new, previously undescribed species. Even among the 14 OTU most closely related to known sequences, only 6 OTU matched with the cultured

Table 1 Taxonomic relationship of bacterial 16S rDNA sequences from Chongming biogas digester compared (BLAST) with public databases (RDP, Greengenes and NCBI)

OTU (%)	% of total	Accession number	Phylogenetically most closely related organism (accession no.)	Sm (%)	Phylum	Functional group	Source
BS01	6·9	EU358676	Uncultured anaerobic bacterium (AY953213)	87	*Firmicutes*	–	USA: swine lagoon
BS02	6·3	EU358677	*Alkaliflexus imshenetskii* (AJ784993)	90	*Bacteroidetes*	Acidogenic	Russia: soda lake
BS03	6·3	EU358678	*Petrimonas sulfuriphila* (AY570690)	90	*Bacteroidetes*	Acidogenic	Canada: biodegraded oil
BS04	5·3	EU358679	*Proteiniphilum acetatigenes* (AY742226)	94	*Bacteroidetes*	Proteolytic	China: sludge of UASB reactor
BS05	4·8	EU358680	*Spirochaeta* sp. SPN1 (AJ698092)	88	*Spirochaetes*	–	Germany: hindgut of the termite
BS06	4·2	EU358681	Uncultured Clostridiaceae (DQ069192)	94	*Firmicutes*	–	USA: SA Au mine
BS07	3·7	EU358682	*Clostridium quinii* (X76745)	99	*Firmicutes*	Acidogenic	UK: type strain DSM6736
BS08	3·2	EU358683	Uncultured bacterium (EF559197)	99	*Bacteroidetes*	–	France: mesophilic digester
BS09	3·2	EU358684	*Ruminofilibacter xylanolyticum* (DQ141183)	91	*Bacteroidetes*	–	China: rumen
BS10	2·6	EU358685	*Clostridium thermocellum* (L09173)	86	*Firmicutes*	Cellulolytic	DSM 1237
BS11	2·6	EU358686	Uncultured *Clostridium* sp. (DQ309375)	93	*Firmicutes*	–	India: effluent treatment
BS12	2·1	EU358687	*Treponema brennaborense* (Y16568)	91	*Spirochaetes*	Acidogenic	FRG: dairy cow
BS13	2·1	EU358688	*Treponema brennaborense* (Y16568)	91	*Spirochaetes*	Acidogenic	FRG: dairy cow
BS14	2·1	EU358689	uncultured Fibrobacteres (EF454806)	90	*Fibrobacteres*	–	USA: termite hindgut
BS15	2·1	EU358690	*Clostridium thermocellum* (L09173)	88	*Firmicutes*	Cellulolytic	DSM 1237
BS16	2·1	EU358691	*Paludibacter propionicigenes* (AB078842)	88	*Bacteroidetes*	Acidogenic	Japan: rice straw in paddy soil
BS17	2·1	EU358692	Uncultured Bacteroidetes(EF111167)	89	*Bacteroidetes*	–	Colombia: bogota river
BS18	1·6	EU358693	Uncultured spirochete (EF562545)	94	*Spirochaetes*	–	Canada: biodegraded oil
BS19	1·6	EU358694	*Clostridium orbiscindens* (Y18187)	89	*Firmicutes*	Acidogenic	DSM 6740
BS20	1·6	EU358695	*Clostridium bartlettii* (AY438672)	92	*Firmicutes*	Acidogenic	USA: human feces
BS21	1·6	EU358696	*Anaerovorax odorimutans* (AJ251215)	92	*Firmicutes*	Acidogenic	Germany: strain NorPut
BS22	1·6	EU358697	*Paludibacter propionicigenes*(AB078842)	88	*Bacteroidetes*	Acidogenic	Japan: rice straw in paddy soil
BS23	1·6	EU358698	uncultured Cytophaga sp. (EF562564)	94	*Bacteroidetes*	–	USA: paper pulp column
BS24	1·1	EU358699	uncultured Verrucomicrobia (AM040118)	86	*Verrucomicrobia*	–	Germany: sandy sediments
BS25	1·1	EU358700	*Tissierella praeacuta* (X77848)	96	*Firmicutes*	–	DSM 5675
BS26	1·1	EU358701	*Clostridium orbiscindens* (Y18187)	87	*Firmicutes*	Acidogenic	DSM 6740
BS27	1·1	EU358702	Uncultured Clostridiales (AB234509)	89	*Firmicutes*	–	Japan: gut of termites
BS28	1·1	EU358703	*Clostridium chartatabidum* (X71850)	99	*Firmicutes*	Cellulolytic	DSM 5482
BS29	1·1	EU358704	*Tissierella praeacuta* (X80833)	93	*Firmicutes*	–	UK: type strain ATCC 25539
BS30	1·1	EU358705	*Aminobacterium colombiense* (AF069287)	86	*Firmicutes*	–	Australia: anaerobic sludge
BS31	1·1	EU358706	Uncultured Leptospiraceae (EF454914)	88	*Spirochaetes*	–	USA: termite hindgut
BS32	0·5	EU358707	Uncultured spirochete (EF562545)	94	*Spirochaetes*	–	Canada: biodegraded oil
BS33	0·5	EU358708	*Sphaerochaeta* sp. RCcp2 (DQ833401)	89	*Spirochaetes*	–	USA:TCE-dechlorinating
BS34	0·5	EU358709	*Spirochaeta* sp. SPN1 (AJ698092)	88	*Spirochaetes*	–	Germany: hindgut of the termite
BS35	0·5	EU358710	*Xanthomonas vasicola* (Y10755)	95	*Proteobacteria*	–	FRG: Strain LMG 736 T
BS36	0·5	EU358711	Uncultured planctomycete (DQ206406)	98	*Planctomycetes*	–	USA: soda lake water
BS37	0·5	EU358712	*Anaerovorax odorimutans* (AJ251215)	92	*Firmicutes*	Acidogenic	Germany: strain NorPut
BS38	0·5	EU358713	*Clostridium straminisolvens* (AB125279)	88	*Firmicutes*	Cellulolytic	Japan:cellulose-degrading
BS39	0·5	EU358714	Clostridiaceae bacterium 80Wc (AB078860)	95	*Firmicutes*	–	Japan: rice straw in paddy soil
BS40	0·5	EU358715	Uncultured Clostridiales (AB234479)	95	*Firmicutes*	–	Japan: gut of termites
BS41	0·5	EU358716	*Sporobacter termitidis* (Z49863)	91	*Firmicutes*	Homoacetogenic	Australia: wood-feeding termite
BS42	0·5	EU358717	*Desulfotomaculum guttoideum* (Y11568)	93	*Firmicutes*	Acetogenic	DSM 4024
BS43	0·5	EU358718	*Garciella nitratireducens* (AY176772)	89	*Firmicutes*	Acidogenic	Mexico: oilfield separator
BS44	0·5	EU358719	*Streptococcus alactolyticus* (AF201899)	99	*Firmicutes*	Acidogenic	Denmark: ATCC 43077
BS45	0·5	EU358720	Uncultured bacterium (AY976000)	98	*Firmicutes*	–	USA: human colon mucosal
BS46	0·5	EU358721	*Clostridium nexile* (X73443)	93	*Firmicutes*	–	DSM 1787
BS47	0·5	EU358722	*Tissierella praeacuta* (X80833)	93	*Firmicutes*	–	UK: type strain ATCC 25539
BS48	0·5	EU358723	*Clostridium* sp. (X75909)	98	*Firmicutes*	–	UK: strain BN II
BS49	0·5	EU358724	*Sporobacter termitidis* (Z49863)	92	*Firmicutes*	Homoacetogenic	Australia: wood-feeding termite
BS50	0·5	EU358725	*Lactobacillus reuteri* F275(CP000705)	99	*Firmicutes*	Acidogenic	DSM 20016

Table 1 *(Continued)*

OTU (%)	% of total	Accession number	Phylogenetically most closely related organism (accession no.)	Sm (%)	Phylum	Functional group	Source
BS51	0·5	EU358726	*Leuconostoc citreum*(AF111949)	100	*Firmicutes*	Acidogenic	South Korea: fermented cabbage
BS52	0·5	EU358727	*Guggenheimella bovis* (AY272039)	89	*Firmicutes*	Proteolytic	USA: bovine dermatitis digitalis
BS53	0·5	EU358728	*Desulfotomaculum thermocisternum* (U33455)	83	*Firmicutes*	Acetogenic	Norway: hot North Sea oil
BS54	0·5	EU358729	Uncultured bacterium (EF559146)	97	*Firmicutes*	–	France: mesophilic digester
BS55	0·5	EU358730	*Gracilibacter thermotolerans* (DQ117465)	88	*Firmicutes*	Acidogenic	USA: acid sulfate wetland
BS56	0·5	EU358731	*Moorella glycerini* (U82327)	81	*Firmicutes*	Homoacetogenic	USA: strain YS6
BS57	0·5	EU358732	Uncultured Thermoanaerobacteriales (AY684076)	92	*Firmicutes*	–	Germany: methanogenic enrichment
BS58	0·5	EU358733	Uncultured bacterium (EF559145)	97	*Firmicutes*	–	France: mesophilic digester
BS59	0·5	EU358734	*Dethiobacter alkaliphilus* (EF422412)	94	*Firmicutes*	Sulfur-reducing	Russia: soda lakes
BS60	0·5	EU358735	*Clostridium* sp. (AB186360)	88	*Firmicutes*	–	Japan: methanogenic bioreactor
BS61	0·5	EU358736	Uncultured bacterium (EF559145)	99	*Firmicutes*	–	France: mesophilic digester
BS62	0·5	EU358737	Uncultured bacterium (CR933151)	99	*Firmicutes*	–	France: naerobic sludge digester
BS63	0·5	EU358738	*Alkaliflexus imshenetskii* (AJ784993)	90	*Bacteroidetes*	Acidogenic	Russia: soda lake
BS64	0·5	EU358739	*Proteiniphilum acetatigenes* (AY742226)	89	*Bacteroidetes*	Proteolytic	China: sludge of UASB reactor
BS65	0·5	EU358740	Uncultured Bacteroidetes (AB234401)	90	*Bacteroidetes*	–	Japan: gut of termites
BS66	0·5	EU358741	*Owenweeksia hongkongensis* (AB125062)	85	*Bacteroidetes*	–	China: strain UST20020801
BS67	0·5	EU358742	Uncultured Bacteroidetes (AF529128)	89	*Bacteroidetes*	–	USA: trichloroethene-contaminated
BS68	0·5	EU358743	Bacteroidetes bacterium (AY548787)	98	*Bacteroidetes*	–	Finland: SRB reactor
BS69	0·5	EU358744	*Alkaliflexus imshenetskii* (AJ784993)	89	*Bacteroidetes*	Acidogenic	Russia: soda lake

OTU, operational taxonomic unit.

bacterial strains, which were *Leuconostoc citreum*, *Clostridium quinii*, *Clostridium chartatabidum*, *Streptococcus alactolyticus*, *Lactobacillus reuteri* and *Clostridium* sp. The remaining eight OTU were from uncultured bacteria. These results indicated that more than 91% bacteria in this digester were uncultured.

Within the 69 OTU, 41 were classified as *Firmicutes*, 16 as *Bacteroides* and 8 as *Spirochaetes*. In a phylogenetic tree (Fig. 1), 24 OTU of *Firmicutes* were clustered with the pure cultures belonging to *Clostridia*, the most of which belonged to the family *Clostridiaceae*. Three *Firmicutes* OTU were assigned to different families of *Bacilli*. All of the 16 *Bacteroides* OTU clustered together and divided into four subgroups, belonging to family *Porphyromonadaceae*, *Rikenellaceae* and two uncultured *Bacteroidetes*. In the eight *Spirochaetes* OTU, two clustered together with genus *Treponema*, five with genus *Spirochaeta* and the remaining one with uncultured *Leptospiracheae*. The remaining four sequences were grouped together with uncultured *Fibrobacteres*, *Xanthomonas vasicola*, uncultured *planctomycete* and uncultured *Verrucomicrobia*, respectively.

The phylogenetically most closely matched bacteria were mostly detected from the intestine of pig or other animals, waste-water treatment plant (sludge or biofilm), anaerobic reactor or digester or landfill leachate and compost, which were all related to anaerobic fermentation (Table 1). The matching micro-organisms of most OTU belonged to phylum *Firmicutes*, *Bacteroides* and *Spirochaetes*, which were fermentative acidogens. Several OTU from *Firmicutes* were cellulolytic and those mainly from *Bacteroides* were proteolytic. The most closely matched micro-organisms of three OTU from *Firmicutes* were homoacetogens and that of another one was the sulfur-reducing bacteria (Table 1).

Microbial community analysed by clone library-archaeal community

The 16S rDNA genes amplified from the total DNA extracted from biogas slurry sample with a specific archaeal primer set of 1Af/1100Ar were ligated into the pMD-18 T vector to construct a library. Exactly 186 clones were used for the following analyses and the length of the amplified genes was approximately 1·1 kbp. All of the sequences were classified into 25 OTU (Table 2). The coverage of the library was 94·1%. OTU AS01, AS02 and AS04 accounted for 27·4%, 29·0% and 10·8% of the sequenced clones in the archaeal library, respectively.

Only 6 of the 25 OTU had matches to most closely related sequences in the databases at a similarity more

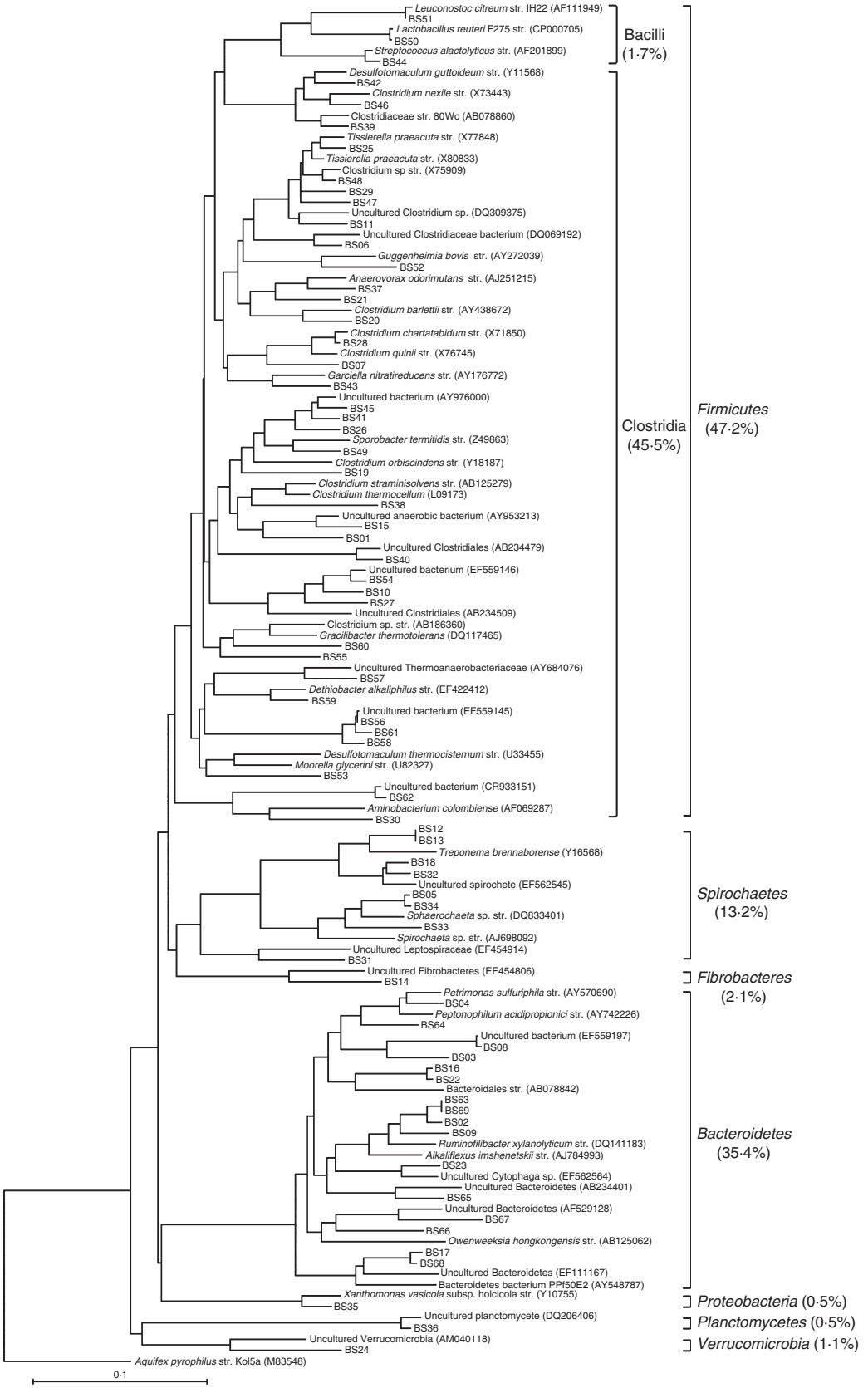

Table 2 Taxonomic relationship of archaeal 16S rDNA sequences from Chongming biogas digester compared (BLAST) with public databases (RDP, Greengenes and NCBI)

OTU (%)	% of total	Accession number	Phylogenetically most closely related organism (accession no.)	Sm (%)	Genus	Functional group	Source
AS01	27·4	EU358651	*Methanosarcina barkeri* (AJ002476)	97	*Methanosarcina*	Hydrogenotrophic/ aceticlastic	New Zealand: cow
AS02	29·0	EU358652	*Methanoculleus bourgensis* (AB065298)	97	*Methanoculleus*	Hydrogenotrophic	DSM 6216
AS03	0·5	EU358653	*Methanospirillum hungatei* (M60880)	93	*Methanospirillum*	Hydrogenotrophic	–
AS04	10·8	EU358654	*Methanospirillum hungatei* (M60880)	97	*Methanospirillum*	Hydrogenotrophic	–
AS05	5·4	EU358655	Methanomicrobiales archaeon (DQ280483)	97	*Methanogenium*	-	USA: Skan bay
AS06	3·9	EU358656	*Methanospirillum hungatei* (M60880)	92	*Methanospirillum*	Hydrogenotrophic	–
AS07	1·1	EU358657	*Methanogenium marinum* (DQ177345)	95	*Methanogenium*	Hydrogenotrophic	USA: Skan bay
AS08	2·2	EU358658	*Methanogenium marinum* (DQ177344)	89	*Methanogenium*	Hydrogenotrophic	USA: Skan bay
AS09	0·5	EU358659	*Methanothrix soehngenii* (X51423)	99	*Methanosaeta*	Aceticlastic	Netherlands: Opfikon
AS10	0·5	EU358660	Uncultured archaeon (AY835414)	92	–	–	USA: sediment
AS11	0·5	EU358661	*Methanogenium cariaci* (M59130)	94	*Methanogenium*	Hydrogenotrophic	Library: DSM 1497
AS12	2·2	EU358662	Methanoculleus sp. (AJ550158)	94	–	Hydrogenotrophic	Germany: dm2
AS13	0·5	EU358663	*Methanoculleus bourgensis* (AB065298)	90	–	Hydrogenotrophic	DSM 6216
AS14	0·5	EU358664	Methanosarcina sp. HB-1 (AB288262)	91	–	Hydrogenotrophic/ aceticlastic	Japan: sedimentary rock
AS15	1·6	EU358665	*Methanosarcina mazei* (NC_003901)	90	–	Hydrogenotrophic/ aceticlastic	USA: strain Go1
AS16	0·5	EU358666	*Methanosarcina barkeri* (AF028692)	91	–	Hydrogenotrophic/ aceticlastic	France: Sar
AS17	0·5	EU358667	*Methnosarcina siciliae* (U89773)	96	*Methanosarcina*	Hydrogenotrophic/ aceticlastic	USA: C2J
AS18	1·1	EU358668	*Methanoculleus bourgensis* (AY196674)	93	–	Hydrogenotrophic	Australia: MS2
AS19	4·9	EU358669	Uncultured euryarchaeote (EF552190)	98	*Methanosarcina*	–	France: digester
AS20	0·5	EU358670	*Methanospirillum hungatei* (CP000254)	92	*Methanospirillum*	Hydrogenotrophic	USA: JF-1
AS21	1·6	EU358671	Methanospirillum sp. (AJ133792)	92	*Methanospirillum*	Hydrogenotrophic	Germany: Bremen
AS22	0·5	EU358672	Uncultured Methanomicrobiales (AB353214)	95	–	–	Japan: mesophilic sludge
AS23	1·1	EU358673	*Methanoculleus bourgensis* (AB065298)	94	–	Hydrogenotrophic	DSM 6216
AS24	0·5	EU358674	*Methanoculleus bourgensis* (AB065298)	95	–	Hydrogenotrophic	DSM 6216
AS25	2·2	EU358675	*Methanoculleus bourgensis* (AY196674)	94	–	Hydrogenotrophic	Australia: MS2

than 97% (Table 2), among which 5 OTU were matched to cultured isolates. Eighteen of the twenty-five OTU were matched to the closest related known sequences at a similarity index of between 90% and 96%; the remainders were matched to the closest related known sequences at a similarity index of 89%. More than 80·0% of the archaeal OTU in this digester may be uncultured.

All of the archaeal OTU were classified into *Methanomicrobia* of phylum *Euryarchaeota* and assigned to two branches: *Methanomicrobiales* and *Methanosarcinales* (Fig. 2). In branch *Methanomicrobiales*, four OTU were clustered with two isolates from genus of *Methanogenium*, one OTU was grouped to genus *Methanoculleus*, five to genus *Methanospirillum* and the remaining seven were divided into four different sub-branches, which might represent new genus or new families. Within the *Methanosarcinales* branch, three OTU clustered with genus *Methanosarcina*, one OTU belonged to genus *Methanosaeta* and the remaining four (AS10, AS14, AS15 and AS16) were clustered together but separated from genus *Methanosarcina*. It suggested that OTU AS10, AS14, AS15 and AS16 might represent a new genus of *Methanosarcinaceae*.

Figure 1 Phylogenetic tree of bacteria. The tree was constructed with the neighbour-joining method of the ARB programme package using nearly complete sequences of the 16S rRNA gene. Scale bar is 10% of the estimated difference in nucleotide sequence position. *Aquifex pyrophilus* was used as the outgroup.

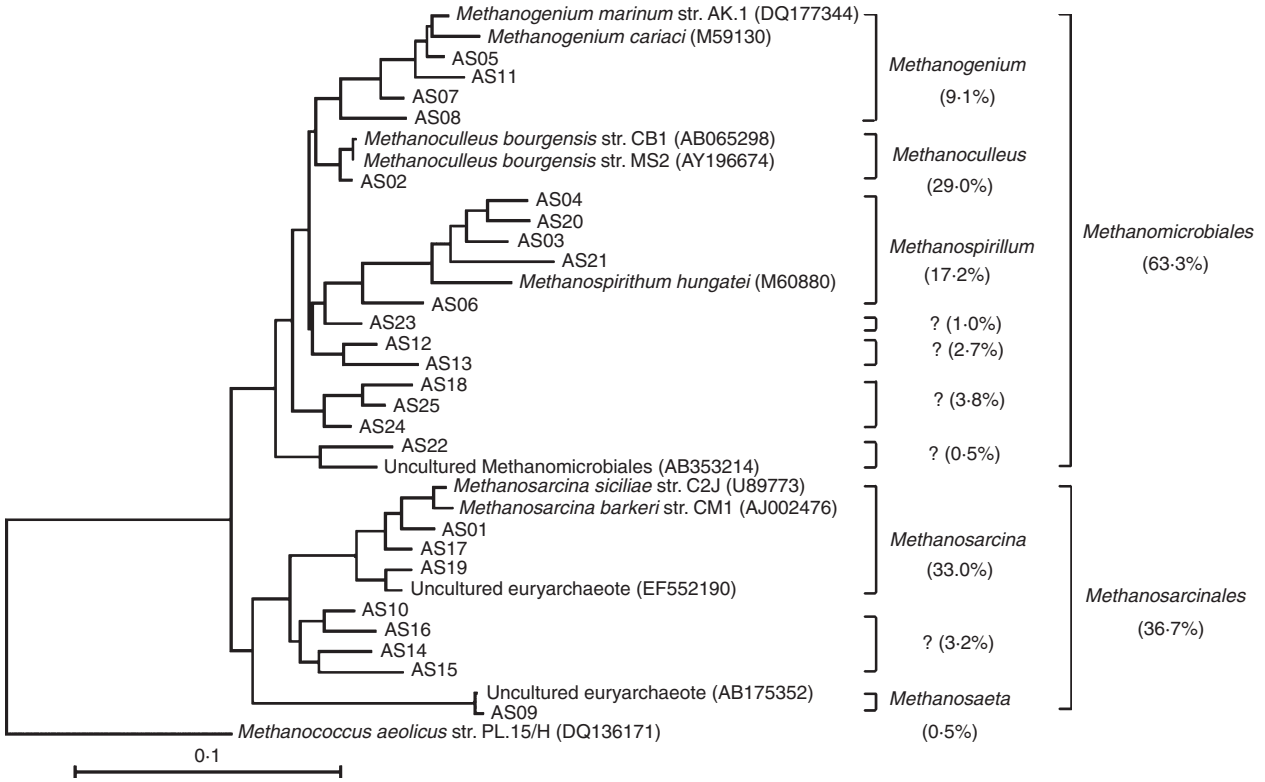

Figure 2 Phylogenetic tree of archaea. The tree was constructed using the neighbour-joining method of the ARB programme package using nearly complete sequences of 16S rRNA gene. Scale bar indicates 10% estimated difference in nucleotide sequence position. *Methanococcus aeolicus* was used as the outgroup.

However, the three most abundant OTU, AS02 (29·0%), AS01 (27·4%) and AS04 (10·8%), comprising more than 67% clones in the library, matched pure cultures of *Methanoculleus bourgensis, Methanosarcina barkeri* and *Methanospirillum hungatei* at a similarity index of 97%, respectively. This indicates that the four methanogens were the dominant archaeal species in this biogas digester.

The phylogenetically assigned archaea OTU were divided into three functional groups. Most OTU, including the most and the third most abundant OTU, were hydrogenotrophic methanogens (Table 2). The most closely matched archaea to five OTU, including the second most abundant OTU, were hydrogenotrophic/aceticlastic methanogens, and one OTU was an aceticlastic methanogen.

Structure of the dominant microbiota in the digester analysed by DGGE fingerprinting and sequencing the DGGE bands

The structures of the dominant bacteria and archaea in the slurry sample were analysed by DGGE fingerprinting (Fig. 3). Twenty-four detected bands were found in the bacterial DGGE profile (CB1–CB24) and nine bands (CA1–CA9) in the archaeal profile (Fig. 3). These results demonstrated a higher diversity of bacteria than archaea in the digester.

All of the detected bands from bacterial and archaeal DGGE fingerprinting were excised, amplified and cloned. The mobility of the inserted fragments of three clones randomly selected from each clone library (33 libraries in total) were checked via DGGE and compared with the original DGGE pattern. Clones of the inserts that migrated to the same locations as the original bands in the DGGE profile were sequenced. In total, 34 different sequences from the bacterial libraries and 11 sequences from archaeal libraries were obtained (Tables 3 and 4). Multi-fragments were found in a single band in at least 12 of the 33 recovered DGGE bands.

Nineteen of the bacterial sequences were assigned to *Firmicutes*, ten of the sequences were clustered to *Bacteroidetes*, three were grouped to *Spirochaetes* and one to *Proteobacteria* and *Verrucomicrobiales*, respectively (Table 3). Seven of the 34 bacterial sequences matched one isolate at a similarity index of ≥97%, the remainder

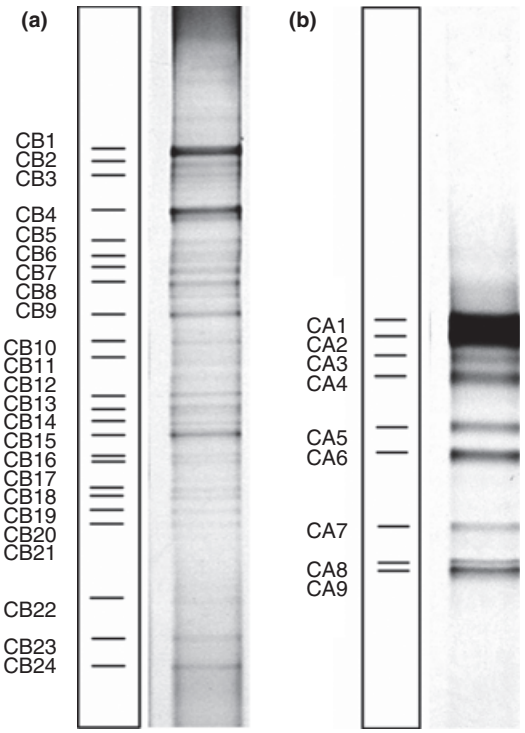

Figure 3 Denaturing gradient gel electrophoresis (DGGE) fingerprints of the bacterial (a) and archaeal (b) communities of biogas slurries obtained at Chongming in June 2005. UPGMA tree representing the genetic similarity of the microbial community profiles was obtained by polymerase chain reaction-DGGE.

matched isolates at a similarity index of <97% (Table 3), indicating that nearly 80% of the dominant bacteria in the biogas digester were unclassified.

In the DGGE profile, most of the fragments representing V3 regions of phylum *Firmicutes* were found to melt at high denaturant concentration areas, and that of phylum *Bacteroidetes* tended to melt at low denaturant concentration areas (Table 3 and Fig. 3).

Three archaeal sequences were assigned to genera *Methanoculleus* and *Methanosarcina*, respectively; one belonged to genus *Methanospirillum*, three grouped to order *Methanomicrobiales* and the remaining was grouped to order *Methanosarcinales* (Table 4). About half of the sequences were matched to uncultured taxa.

The consistency of dominant bacterial and archaeal composition revealed by clone library and DGGE profile analysis

Eighteen bacterial sequences representing 16 of the 24 recovered DGGE bands were also present in the clone library. On the other hand, seven of the nine OTU with abundance >3% were matched with the V3 sequences recovered from the DGGE bands (Table 3). This means that 78% dominant OTU (with abundance >3%) in the bacterial clone library could be also detected by DGGE profile analysis.

Ten archaeal sequences representing all of the nine recovered DGGE bands were in the clone library. Five of the dominant six archaeal OTU with abundance more than 3% were found to match with the V3 sequences recovered from DGGE bands. Exactly 83% of the dominant OTU (with abundance >3%) in the archaeal library were observed in the DGGE profile.

Discussion

Cloning and sequencing the full length of 16S rDNA has been frequently applied to elucidate the exact composition of a microbial community. However, DGGE profiles of the V3 regions can be employed to reflect the dominant community structure and to analyse the structural variation between different systems or the dynamics of the same system (Muyzer *et al.* 1993). In this study, sequencing of V3 regions from recovered DGGE bands and 16S rDNA from cloning libraries were both used to analyse the microbial community in an anaerobic digester. Both analyses indicated that the community was mainly composed of phyla *Firmicutes*, *Bacteroides* and *Spirochaetes* for bacteria, and orders *Methanomicrobiales* and *Methanosarcinales* for archaea. The analyses also indicated that the diversity of bacteria was higher than that of achaera. Importantly, 78% of dominant OTU (with abundance >3%) in the bacterial library and 83% of dominant OTU in the archaeal library could be detected in the DGGE profile. These results indicate that the DGGE profile in this study clearly reflected the dominant composition of the microbial community in the Qianwei biogas digester. Additionally, the major components of this bacterial community could be separately located on the different areas in the DGGE profile, e.g. *Firmicutes* tended to appear at the high denaturant concentration area and *Bacteroidetes* at low denaturant concentration areas in the DGGE profile. The analysis of DGGE profiles may be useful in displaying the dominant microbial composition in biogas digesters in our further studies. However, more OTU were identified by sequencing 16S rDNA libraries, indicating that DGGE may underperform in elucidating the diversity or the exact composition of a complex microbial community.

Anaerobic digesters are widely used to treat different wastes, e.g. brewery and pulp industry wastewater containing different carbohydrates, long-chain fatty acids, volatile fatty acids, methanethiol, terephthalate (Mata-Alvarez *et al.* 2000; Yadvika *et al.* 2004). Many studies focus on analyses of microbial community in such anaerobic digesters by using different approaches (Cotta *et al.* 2003; Snell-Castro *et al.* 2005; Peu *et al.* 2006). In these studies, the

Table 3 Bacterial 16S rDNA sequence similarities of excised bands that appear in Fig. 3

Band ID	Accession number	Phylogenetically most closely related organism (accession no.)	Lg (bp)	Sm1 (%)	Corresponding OTU no.	Sm2 (%)	Functional group
CB1	EU358617	*Proteiniphilum acetatigenes* (AY742226)	189	95	BS04, BS64	100	Proteolytic
CB2	EU358618	*Eubacterium tortuosum* (L34683)	195	83	–	–	–
CB3	EU358619	*Petrimonas sulfuriphila* (AY570690)	189	89	BS03	99	Acidogenic
CB4-1	EU358620	Uncultured bacterium (EF559197)	189	100	BS08	100	–
CB4-2	EU358621	*Alkaliflexus imshenetskii* (AJ784993)	160	89	BS02, 63, 69	100	Acidogenic
CB5-1	EU358622	Uncultured bacterium (EF686929)	189	97	BS08	98	–
CB5-2	EU358623	Uncultured bacterium (AY816908)	190	95	–	–	–
CB6-1	EU358624	*Spirochaeta* sp. *grapes* (AF357917)	194	87	–	–	–
CB6-2	EU358625	Uncultured bacterium (AJ937700)	189	95	–	–	–
CB7	EU358626	Uncultured bacterium (AB290394)	189	99	–	–	–
CB8	EU358627	Uncultured bacterium (AJ628010)	189	94	BS23	100	–
CB9-1	EU358628	Uncultured spirochete (EF562545)	194	96	BS32	99	–
CB9-2	EU358629	*Clostridium orbiscindens*(Y18187)	174	95	–	–	Acidogenic
CB10-1	EU358630	*Syntrophomonas zehnderi* (DQ898277)	194	93	–	–	Acetogenic
CB10-2	EU358631	*Oscillospira guilliermondii* (AB040499)	171	100	–	–	–
CB11-1	EU358632	*Clostridium intestinale* (X76740)	168	96	–	–	–
CB11-2	EU358633	Uncultured bacterium (AY816908)	190	97	BS63, 69	98	–
CB12	EU358634	*Acetivibrio cellulolyticus* (L35516)	169	99	–	–	Cellulolytic
CB13-1	EU358635	Uncultured bacterium (EF559146)	171	98	BS54	100	–
CB13-2	EU358636	*Clostridium thermocellum* (L09173)	169	92	–	–	Cellulolytic
CB14	EU358637	*Succinivibrio dextrinosolvens* (Y17600)	171	96	–	–	Acidogenic
CB15	EU358638	Uncultured Clostridiaceae (DQ069192)	169	100	BS06	100	–
CB16-1	EU358639	*Clostridium hydroxybenzoicum* (L11305)	169	94	–	–	–
CB16-2	EU358640	*Tissierella praeacuta* (X80833)	169	96	BS47	99	–
CB17	EU358641	*Clostridium chartatabidum* (X71850)	169	98	BS28	98	Cellulolytic
CB18	EU358642	Uncultured Verrucomicrobiales (AJ853598)	194	96			–
CB19-1	EU358643	*Anaerovorax odorimutans* (AJ251215)	172	98	BS21	99	Acidogenic
CB19-2	EU358644	Uncultured bacterium (AY980698)	172	96	–	–	–
CB20	EU358645	*Clostridium quinii* (X76745)	169	100	BS07	100	Acidogenic
CB21-1	EU358646	*Clostridium quinii* (X76745)	169	98	BS07	98	Acidogenic
CB21-2	EU358647	*Tissierella praeacuta* (X80833)	169	95	BS29	98	–
CB22	EU358648	Uncultured bacterium (EF559145)	195	95	BS58	99	–
CB23	EU358649	Spirochaeta sp. SPN1 (AJ698092)	194	95	BS05	99	–
CB24	EU358650	*Dethiosulfovibrio acidaminovorans* (AY005466)	172	97	–	–	Sulfur-reducing

Sm1, similarity between the sequences of denaturing gradient gel electrophoresis (DGGE) band and its phylogenetically closely related organism.
Sm2, similarity between the sequences of DGGE band and its corresponding operational taxonomic unit (OTU).

microbial structures in these systems varied greatly owing to the difference of the substrates used. *Firmicutes*, *Nitrospira* and *Deferribacteres* were found to be the predominant bacteria and *Methanosaeta concilii* was the dominant methanogenic archaea in an anaerobic digester treating wastewater from a beer brewery (Diaz *et al.* 2006). In an anaerobic digester treating long-chain fatty acids, species of *Syntrophomonadaceae* and *Syntrophobacteraceae* families, which oxidize fatty acids, were the predominant bacteria (Sousa *et al.* 2007b). When degrading methanethiol from paper mill wastewater, methylotrophic methanogens *Methanomethylovorans hollandica* were enriched (de Bok *et al.* 2006). In a laboratory methanogenic digester amended with glucose, *Spirochaetes*-, eubacterium- and propionibacterium-like bacteria were found to be dominant (Fernandez *et al.* 1999, 2000). Pig manure, which was mainly comprised of undigested biomass and some fatty acids, such as acetic acid and propionic acid, was the sole substrate in Qianwei biogas digester analysed in this study. *Firmicutes* (47·2%), *Bacteroides* (35·4%) and *Spirochaetes* (13·2%) were found to be the three most abundant bacterial phyla in this study. Within phylum *Firmicutes,* class *Clostridia* was the most dominant of the bacterial community (45·5% of the clones).

Several other studies have shown that within the bacterial and archaeal community of a pig manure slurry and a manure storage pit, *Eubacterium*, *Clostidium*, *Bacillus–Lactobacillus–Streptococcus* subdivision, *Mycoplasma* and

Table 4 Archaeal 16S rDNA sequence similarities of excised bands that appear in Fig. 3

Band ID	Accession number	Phylogenetically most closely related organism (accession no.)	Lg (bp)	Sm1 (%)	Corresponding OTU no.	Sm2 (%)	Functional group
CA1-1	EU358606	*Methnosarcina siciliae* (U89773)	152	98	AS17	100	Hydrogenotrophic/ aceticlastic
CA1-2	EU358607	*Methanosarcina barkeri* (AJ002476)	152	97	AS01	98	Hydrogenotrophic/ aceticlastic
CA2-1	EU358608	*Methanoculleus bourgensis* (AB065298)	148	97	AS02, 18, 24, 25	99	Hydrogenotrophic
CA2-2	EU358609	Uncultured archaeon (AM712547)	132	98	–	–	–
CA3	EU358610	Uncultured Methanomicrobiales (AB353214)	148	96	AS22	99	–
CA4	EU358611	Uncultured Methanomicrobiales (AB353214)	148	97	AS22	99	–
CA5	EU358612	*Methanoculleus bourgensis* (AB065298)	148	98	AS13	99	Hydrogenotrophic
CA6	EU358613	*Methanoculleus bourgensis* (AB065298)	148	98	AS13	99	Hydrogenotrophic
CA7	EU358614	Methanospirillum sp. (AJ133792)	148	95	AS3, 4, 6, 15, 20, 21	99	Hydrogenotrophic
CA8	EU358615	Uncultured Methanosarcinales (AB353215)	152	99	AS17	99	Hydrogenotrophic/ aceticlastic
CA9	EU358616	Uncultured euryarchaeote (EF552190)	152	99	AS19	99	–

Sm1, similarity between the sequences of denaturing gradient gel electrophoresis (DGGE) band and its phylogenetically closely related organism. Sm2, similarity between the sequences of DGGE band and its corresponding operational taxonomic unit (OTU).

the *Flexibacter–Cytophaga–Bacteroides* were the main components of the bacterial communities (Snell-Castro *et al.* 2005; Peu *et al.* 2006). Hydrogenotrophic methanogens, such as *Methanoculleus*, *Methanogenium* and *Methanobrevibacter*, dominated the archaeal communities (Whitehead and Cotta 1999; Tang *et al.* 2004; Hori *et al.* 2006). This agrees with the results from our digester, suggesting most archaea in our digester might originate from pig manure.

Most phylogenetically closely matched bacteria to the OTU identified in the biogas digester were found in anaerobic environments, such as the guts of animal or insects, sediments, anaerobic digesters and faeces (Table 1). Many phylogenetically closest matched taxa were uncultured organisms or function-unidentified organisms. The metabolic functions of their related OTU in the biogas digester were unknown. Among the function-identified bacteria, most were acidogenic, producing H_2, CO_2, formate, acetate and other fatty acids as well as a small amount of ethanol from cellobiose, D-fructose, *N*-acetylglucosamine, D-glucose, maltose, mannose and saccharose (Vandamme *et al.* 1999); several OTU related to the *Clostridium* sp. might be cellulolytic and the other three ones were related to proteolytic, both of which might take charge of decomposing polymers in the pig manure to monomers; Only three OTU might be homoacetogenic. This seems to be consistent with the fact that most archaeal OTU or the most abundant OTU were hydrogenotrophic methanogens, and only one OTU at low frequency was identified as the aceticlastic methanogen (Table 2).

The phylum *Euryarchaeota* was the major methanogenic archaeal group in anaerobic fermentation environments. *Methanoculleus bourgensis*, *Methanosarcina barkeri*, *Methanospirillum hungatei* and *Methanomicrobiales archaeon* were the most abundant methanogenic species in our digester (Table 2). Each of them showed some specific characteristics in methanogenic metabolism. *Methanoculleus bourgensis* was reported to use H_2–CO_2 or formate as a substrate for growth and methanogenesis, and is a hydrogenotrophic methanogen (Blotevogel *et al.* 1992). *Methanospirillum hungatei* produces methane only from H_2–CO_2 or formate, but not from acetate or ethanol and methanol, being a strictly hydrogenotrophic methanogen (Ferry *et al.* 1974). *Methanosarcina barkeri* could be used in different substrates to produce methane, including H_2–CO_2, methanol, mono-, di- and trimethylamines, acetate and CO (Bryant and Boone 1987), and is a hydrogenotrophic or aceticlastic methanogen.

Chimeric sequences of the full length of 16S rDNA were usually found while analysing the compositions of complex microbial communities (Ashelford *et al.* 2006). Therefore, several programmes were designed to identify chimeric sequences (Cole *et al.* 2003; DeSantis *et al.* 2006). Ashelford *et al.* (2006) reported that the average chance to falsely identify a sequence as chimeric by the Bellerophon programme was 7·2%. Fifty-three 16S rDNA sequences from bacteria were identified to be putatively chimeric. To ensure most of them to be assigned rightly, the putative chimeric sequences were confirmed by two different programmes, and some of them were further confirmed by PCR amplification. However, it was still

possible that some of the 53 sequences be assigned as chimeric by wrong, and thus might underestimate the bacterial diversity in the biogas digester.

High diversities of microbial composition and metabolism were found in this microbiota in this study. It provides a pool of functional micro-organisms involved in biomass transformation. However, most micro-organisms, more than 91% bacteria and 80% of archaea, in this digester were uncultured. Presently it is impossible to elucidate the total metabolic process in the biogas digester using only the analysis of microbial composition. The recent application of metagenomic techniques suggest they may provide a new approach to obtain function genes related to biomass transformation within the assemblage. A lab anaerobic fermentation system and its control system are necessary in elucidating the relationship between the biogas-producing efficiency and microbiota composition under variable substrate conditions, with the addition of an inhibitor or accelerant, and/or adding specific micro-organisms for bio-augmentation.

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