Methylo-trophic methanogenesis governs the biogenic coal bed methane formation in Eastern Ordos Basin, China

Hongguang Guo · Zhisheng Yu · Ruyin Liu ·
Hongxun Zhang · Qiding Zhong · Zhenghe Xiong

Received: 23 October 2011 / Revised: 29 December 2011 / Accepted: 2 January 2012 / Published online: 28 January 2012
© Springer-Verlag 2012

Abstract To identify the methanogenic pathways present in a deep coal bed methane (CBM) reservoir associated with Eastern Ordos Basin in China, a series of geochemical and microbiological studies was performed using gas and water samples produced from the Liulin CBM reservoir. The composition and stable isotopic ratios of CBM implied a mixed biogenic and thermogenic origin of the methane. Archaeal 16S rRNA gene analysis revealed the dominance of the methylo-trophic methanogen *Methanobus* in the water produced. The high potential of methane production by methylo-trophic methanogens was found in the enrichments using the water samples amended with methanol and incubated at 25 and 35 °C. Methylo-trophic methanogens were the dominant archaea in both enrichments as shown by polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE). Bacterial 16S rRNA gene analysis revealed that fermentative, sulfate-reducing, and nitrate-reducing bacteria inhabiting the water produced were a factor in coal biodegradation to fuel methanogens. These results suggested that past and ongoing

biodegradation of coal by methylo-trophic methanogens and syntrophic bacteria, as well as thermogenic CBM production, contributed to the Liulin CBM reserves associated with the Eastern Ordos Basin.

Keywords Coal bed methane · Methanogenesis · Deep subsurface environment · Ordos Basin

Introduction

Coal bed methane (CBM) is a major clean energy resource worldwide and which is being intensively developed in many countries. CBM is generally understood to be formed by microbial degradation of coal (biogenic origin) or thermal decomposition of coal (thermogenic origin), which is typically analyzed by such standard geochemical technologies as plotting hydrocarbon gas composition $C_1/(C_2+C_3)$ (C_1 – C_3 , methane, ethane, propane) versus stable carbon isotope ratios of methane $\delta^{13}C$ – CH_4 (Bernard et al. 1978). Biogenic CBM has been reported to be present in various reservoirs with mixed biogenic and thermogenic CBM or predominantly biogenic CBM, e.g., in the San Juan Basin (Scott et al. 1994), in the Illinois Basin (Strapoć et al. 2007), and in the Powder River Basin (Flores et al. 2008). It is generally accepted that biogenic CBM is the end product of coal biodegradation by methanogenic archaea and syntrophic bacteria inhabiting coal beds. These microorganisms are essential for artificially stimulating the regeneration of biogenic CBM (Green et al. 2008; Harris et al. 2008; Pfeiffer et al. 2010). Thus, it is essential to explore the existence of biogenic CBM to more fully understand the microorganisms involved in the formation of biogenic CBM.

Some systemic studies have evaluated the microbial communities inhabiting the CBM reservoirs containing biogenic

H. Guo · Z. Yu (✉) · R. Liu · H. Zhang
College of Resources and Environment,
Graduate University of Chinese Academy of Sciences,
19 A Yuquan Road,
Beijing 100049, People's Republic of China
e-mail: yuzs@gucas.ac.cn

H. Zhang
Research Center for Eco-Environmental Sciences,
Chinese Academy of Sciences,
18 Shuangqing Road,
Beijing 100085, People's Republic of China

Q. Zhong · Z. Xiong
Food Analysis using Isotope Technology Lab, China National
Research Institute of Food and Fermentation Industries,
32 Xiaoyun Road,
Beijing 100027, People's Republic of China

CBM. Sampling gas and water produced in the Illinois Basin, Strapoc et al. (2007, 2008) demonstrated the predominance of biogenic CBM formed via hydrogentrophic methanogenesis by a population of *Methanocorpusculum* using geochemical and microbiological methods. It was found that methanogens were also present in water produced from other reservoirs, e.g., the Powder River Basin (Green et al. 2008), the Gippsland Basin (Midgley et al. 2010), and the Alberta Basin (Penner et al. 2010). Furthermore, enrichment studies demonstrated that native methanogens indicated the production of methane by supplementing water samples with nutrients and substrates for methanogens (Green et al. 2008; Midgley et al. 2010). It has been reported that acetotrophic and hydrogenotrophic methanogenesis were the dominant pathways for the production of methane in CBM reservoirs (Strapoc et al. 2011). However, as one of the pathways for producing methane by methanogens, methylotrophic methanogenesis was rarely reported in CBM formation (Shimizu et al. 2007; Strapoc et al. 2010).

The Ordos Basin, situated in central China within the Yellow River drainage basin (Fig. 1), is one of the largest CBM reservoirs and a principal target area for CBM exploration and development in China. However, there is limited understanding on the origin of CBM and of the microbial communities related to CBM in the Ordos Basin. In this study, we sought preliminary identification of the origin of CBM using the chemical and stable isotopic signatures of CBM. Culture-independent and culture-dependent methods were also used to explore the pathway of biogenic CBM formation in a deep CBM reservoir associated with the Ordos Basin.

Materials and methods

Study site and sample collection

Water and gas samples were collected from deep wells in the Liulin District which is located in the middle part of the

Fig. 1 Location of the Liulin District in China. The map is modified from Ding et al. (2011)

Eastern Ordos Basin (Fig. 1). It is the first experimental base of CBM exploration and development in China (Su et al. 2003). The wells were drilled down to about 700 m below the ground surface and constructed with tight steel pipes. Water samples were collected from five CBM-producing wells (SXLL-2, SXLL-3, SXLL-4, SXLL-6, and SXLL-9). To prevent contamination by air and steel pipes, the water produced was continuously pumped for more than 5 days before sampling. Water samples were collected into autoclaved glass bottles, carefully maintaining air in the headspaces clean. Bottles were then tightly sealed and immediately transported on ice to the laboratory. The gas samples from four CBM-producing wells (SXLL-2, SXLL-3, SXLL-6, and SXLL-9) were collected at the well sites through water displacement into inverted bottles. The bottles were tightly sealed with butyl rubber stoppers underwater to prevent air contamination.

Physical and chemical analysis

Cations were determined by utilizing an inductively coupled plasma optical emission spectrometer (Spectro Analytical Instruments, Kleve, Germany). Anions were determined by ion chromatography (Metrohm Ltd, Herisau, Switzerland), except that NH_4^+ was analyzed by utilizing a colorimetric method. Concentrations of H_2 , O_2 , CO_2 , N_2 , CH_4 , C_2H_6 , and C_3H_8 in the gas samples were determined using the Agilent 3000A Micro gas chromatograph (GC) (Agilent Technologies).

The stable isotopic ratios of the ^{13}C ($\delta^{13}\text{C}$) of the CH_4 and CO_2 in the gas samples were measured by an online CH_4 extraction system, Trace GC UltraTM (Thermo Electron Corporation, Austin, TX, USA), a combustion furnace (ceramic tube packed with CuO , NiO , and Pt wires), and ThermoQuest Delta^{plus}XL isotope ratio mass spectrometer (Thermo Finnigan). The $\delta^{13}\text{C}$ derived was determined with reference to Vienna Pee Dee Belemnite samples. The precision of the determination was $\pm 0.1\%$.

Archaeal and bacterial 16S rRNA gene analysis

Aliquots of water sample (1 L) were aseptically filtered with a 0.22- μm membrane filter (Whatman, Japan). The filters were maintained at -70°C until extraction. Bulk DNA was extracted using a FastDNA SPIN kit for soil (Bio101 Systems, Carlsbad, CA, USA) according to the manufacturer's instructions. Archaeal 16S rRNA gene fragments were amplified by PCR using the archaea-specific primers AR-8F and AR-958R (DeLong 1992), and bacterial 16S rRNA gene fragments were amplified by PCR using the bacteria-specific primers BAC-27F and BAC-1492R (Lane 1991). These reactions were performed according to the following protocol: 94°C for 4 min, 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and finally 72°C for 10 min.

The PCR products of the archaeal and bacterial 16S rRNA genes were cloned and transformed into competent *Escherichia coli* DH5 α cells using pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA). Recombinant pGEM-T Easy vectors were checked for 16S rRNA amplicon inserts by PCR using M13 primers. For the archaeal library, the right-inserted recombinant clones were sequenced. For the bacterial library, the correct clones were analyzed by amplified ribosomal DNA restriction analysis (ARDRA) using restriction enzyme Msp I (NEB) as described by Steven et al. (2007). For each restriction fragment length polymorphism (RFLP) type, one representative clone was chosen at random and sequenced.

Sequencing and phylogenetic analyses

The sequences of inserted PCR products selected from recombinant colonies were determined on an Applied Biosystems 3730xl (Applied Biosystems, Darmstadt, Germany) using M13 primers. The operational taxonomic units (OTUs) for the archaeal clone library were determined using Mothur software (Schloss et al. 2009). A 3% distance level between sequences was used as the cutoff to define distinct OTUs. Sequences for each archaeal OTU and bacterial RFLP type were then compared with NCBI nucleotide sequence database using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences were aligned by Clustal X (Thompson et al. 1997). A phylogenetic tree was constructed in the MEGA 4 software using the neighbor-joining method and bootstrap resampling analysis for 1,000 repeats (Tamura et al. 2007).

Real-time PCR analysis

The 16S rRNA genes from *Methanobrevibacter* species and *Clostridium* species were respectively amplified using primer sets AR-8F and AR-958R (DeLong 1992) and BAC-27F and BAC-1492R (Lane 1991) to construct the plasmids to be used for a standard curve in real-time PCR. The PCR products were cloned and transformed according to the protocol described above. The plasmids were extracted using a Tianprep Mini Plasmid Kit (Tiangen Biotech, China) and quantified based on absorbance at 260 nm then sequenced as described above to confirm the correction of the insertion. The plasmids were then tenfold serially diluted to 10^2 – 10^9 copies per microliter for both archaeal and bacterial 16S rRNA genes. Copies per microliter were calculated according to the formula mentioned by Smith et al. (2006).

SYBR Green real-time PCR was performed in Mx3000P Real-time QPCR System (Stratagene) using MaximaTM SYBR Green qPCR Master Mix (Fermentas, Hanover, MD, USA) following the modified protocols as described by the manufacturer. Archaea-specific primers AR-344F

and AR-519R (Lane 1991; Raskin et al. 1994) and bacteria-specific primers BAC-338F and BAC-518R (Lane 1991; Muyzer et al. 1993) were respectively used to determine the abundance of archaea and bacteria in the samples. Amplification was carried out in a total volume of 25 μ L containing 12.5 μ L of 2 $\times$ ROX, 1 μ L of each primer (10 μ M), and 1 μ L of sample DNA. The thermocycling program was as follows: initial denaturation for 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. Each template DNA was analyzed in triplicate.

Measurements of potential microbial methane

To determine the potential of methane production by methanogenic archaea, enrichments were performed using water samples amended with methanogenic substrates and an anaerobic medium. The anaerobic medium was modified according to Strapoć et al. (2008). The equivalent of five samples was mixed and aliquots of water samples (20 ml) were anaerobically injected into autoclaved 100-ml serum bottles with an anaerobic medium, supplemented with acetate (20 mM), methanol (20 mM), formate (20 mM), $H_2 + CO_2$ (80/20, V/V ; 10^5 Pa), or bromoethanesulfonate (BES) (20 mM), an inhibitor of methanogens (Gunsalus et al. 1978). Except for $H_2 + CO_2$ -supplemented bottles, the headspaces of the serum bottles were filled with pure N_2 gas at 10^5 Pa. The enrichments were anaerobically incubated without shaking at 15, 25, and 35 °C, respectively. The concentration of CH_4 in the headspace was measured using GC-7890A equipped with a flame ionization detector (Agilent Technologies). All enrichments were carried out in triplicate.

PCR–DGGE analysis of enrichments

The enrichments in which methane production was observed were centrifuged. Bulk DNA extraction and PCR amplification were carried out according to the method

described above using primers AR-344F with a GC clamp at the 5' end and AR-519R (Lane 1991; Raskin et al. 1994) for archaeal 16S rRNA gene PCR–DGGE analysis. PCR–DGGE was performed using DCode™ system (Bio-Rad Laboratories). The optimized denaturing gradient extended from 40% to 60% in 10% acrylamide gels. Electrophoresis was performed at 60 V and 60 °C for 18 h. After electrophoresis, DNA was stained with EB and visualized under UV light. Individual DGGE bands were excised and re-amplified with the same primer sets without the GC clamp. The PCR products were purified using Gel Extraction Pure Kit (Fermentas) according to the manufacturer's instructions then sequenced and compared to the 16S rRNA gene sequences in NCBI as described above.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in GenBank under accession No. JN223421 to JN223444.

Results

Properties of water produced

The temperature of the water samples ranged from 15 to 19 °C; pH values were from 7.5 to 7.8. The concentration of Cl^- and Na^+ (Table 1) in the water samples was the same as reported by Shimizu et al. (2007). The K^+ , Ca^{2+} , Mg^{2+} , NO_3^- , and NH_4^+ were similar to those of groundwater from the coal bed in the Powder River Basin (Bates et al. 2011).

Composition and stable isotopic ratios of CBM

Methane was the predominant component of CBM (>95%). Other main components were N_2 and CO_2 at 0.04–3.15%

Table 1 Physicochemical parameters of water samples

	SXLL-2	SXLL-3	SXLL-4	SXLL-6	SXLL-9
Temperature (°C)	15	19	17	17	18
pH ^a	7.62	7.54	7.49	7.55	7.78
SO_4^{2-} (mmol/L)	NA ^b	1.75	1.48	1.49	1.56
Cl^- (mmol/L)	51.66	55.91	52.10	54.02	55.58
NO_3^- (mmol/L)	0.011	0.016	0.022	0.015	0.019
NO_2^- (10^{-3} mmol/L)	0.14	0.07	<0.05	<0.05	<0.05
NH_4^+ (mmol/L)	0.13	0.20	0.11	0.13	0.14
Na^+ (mmol/L)	91.74	86.52	86.09	84.78	80.43
K^+ (mmol/L)	0.21	0.22	0.21	0.22	0.21
Ca^{2+} (mmol/L)	0.15	0.35	0.29	0.30	0.18
Mg^{2+} (mmol/L)	0.84	1.01	1.23	1.24	1.30

^apH units

^bNot analyzed

and 0.55–1.77%, respectively. H_2 , O_2 , and C_3H_8 in the CBM were below the detection limits. The $C_1/(C_2+C_3)$ ratios of the CBM were all $>10^3$. Stable carbon isotope ratios of methane ($\delta^{13}C-CH_4$) and carbon dioxide ($\delta^{13}C-CO_2$) ranged from -42.28‰ to -39.78‰ and from -6.88‰ to 19.74‰ , respectively (Table 2). The difference between the stable carbon isotope ratios of carbon dioxide ($\delta^{13}C-CO_2$) might be because wells SXLL-2 and SXLL-3 aimed at the no. 5 coal seam while wells SXLL-6 and SXLL-9 aimed at the no. 8 coal seam. The no. 5 coal seam is located in the Shanxi Formation of the Lower Permian while the no. 8 coal seam is located in the Taiyuan Formation of the upper Carboniferous (Su et al. 2003).

Phylogenetic analysis of archaeal and bacterial 16S rRNA genes

A total of 48 clones in the archaeal 16S rRNA gene clone library were sequenced and divided into three OTUs (SXLL-A01 to SXLL-A03) (Fig. 2). Phylogenetic analysis showed that all of the clones in the library belonged to the family *Methanosarcinaceae*, one of the most common methanogenic lineages found in coal beds (Strapoć et al. 2011). Additionally, 70.83% of the clones were affiliated to *Methanobolus* sp. Specifically, both SXLL-A01 and SXLL-A02 were closely related to the 16S rRNA gene from the cultured organisms *Methanobolus chelonpuianus* (98% identity), *Methanobolus psychrophilus* (96% identity), and *Methanobolus profundus* (95% identity), which are all methylophilic methanogens (Mochimaru et al. 2009; Wu and Lai 2011; Zhang et al. 2008). SXLL-A03 showed the highest identity to the 16S rRNA gene from the cultured organism *Methanosarcina mazei* (98% identity), which metabolizes a wide variety of substrates (Simankova et al. 2003).

A total of 159 clones in the bacterial 16S rRNA gene clone library were examined and 21 unique RFLP types were detected (Fig. 3). The majority of clones belonged to *Epsilonproteobacteria* and *Betaproteobacteria*. The clones of *Epsilonproteobacteria* accounted for 43.4% of the clones in the library and all of the phylotypes were closely

Fig. 2 Diagram of gas composition $C_1/(C_2+C_3)$ [methane/(ethane + propane)] versus methane carbon isotope of the CBM from Ordos Basin. The categorization of methane origin is according to Bernard et al. (1978)

affiliated ($>96\%$ similarity). They showed utmost identity to the 16S rRNA gene from the cultured organism *Arco-bacter defluvii* ($>97\%$ 16S rRNA sequence identity), which is able to anaerobically reduce nitrate (Collado et al. 2011). The *Betaproteobacteria* (39% of the clones) consisted of three phylotypes. SXLL-B06 was closely related to the 16S rRNA gene from the cultured organism *Azonexus hydrophilus* (99% identity), known as a nitrate-reducing bacterium (Chou et al. 2008). Both SXLL-B11 and SXLL-B13 showed the closest match to the 16S rRNA gene from the cultured organism *Thauera* sp. (98% identity), which is a denitrifying bacterium and able to degrade aromatic compounds (Mechichi et al. 2002; Shinoda et al. 2004). Sequences related to the *Firmicutes* (10.7% of the clones) were clustered into eight phylotypes (Fig. 3), which were all closely related to the 16S rRNA gene from the cultured sulfate-reducing or fermentative bacteria. Both SXLL-B17 and SXLL-B20 were closely related to the 16S rRNA gene from the cultured sulfate-reducing bacterium *Desulfosporosinus lacus* (100% identity) (Ramamoorthy et al. 2006) and also related to *Desulfosporosinus* sp. (100% identity) which is able to metabolize such aromatic compounds as syringic acid, ferulic acid, phenol, benzoate, and toluene (Liu et al. 2004). Both SXLL-B16 and SXLL-B21 showed the closest match to the 16S rRNA gene from the cultured organism *Desulfotomaculum* sp. (90% identity) which is a sulfate reducer and able to degrade *o*-xylene, *m*-xylene, and toluene (Morasch et al. 2004) and from *Pelotomaculum terephthalicum* (90% identity) which was isolated from methanogenic sludges as syntrophic phthalate isomer-degrading microbes (Qiu et al. 2004). Both SXLL-B02 and SXLL-B18 were closely related to the 16S rRNA gene from the cultured organism *Saccharofermentans acetigenes* (91%

Table 2 Chemical compositions and stable isotope analysis of gas samples obtained from the CBM-producing wells

No.	Composition (%)			$\delta^{13}C$ (‰ PDB)		$C_1/(C_2+C_3)^a$
	N_2	CO_2	CH_4	CH_4	CO_2	
SXLL-2	1.25	0.74	97.94	-40.78	-1.90	1,399.14
SXLL-3	3.15	1.28	95.5	-41.72	-6.88	1,364.29
SXLL-6	0.62	0.55	98.74	-39.78	17.41	1,097.11
SXLL-9	0.04	1.77	98.11	-42.28	19.74	1,226.38

^a Methane/(ethane + propane)

Fig. 3 Phylogenetic tree of archaeal 16S rRNA gene sequences from the water samples together with related sequences of environmental clones and cultured archaea. Bootstrap support values (1,000

replicates) above 50% are shown at nodes. The scale bar represents 1% estimated sequence divergence

identity), which was isolated from an upflow anaerobic sludge blanket reactor and could ferment polysaccharides (Chen et al. 2010). They were also related to the anaerobic cellulolytic bacterium *Clostridium straminisolvens* (90% identity) (Kato et al. 2004). SXML-B05 was closely related to the 16S rRNA gene from several cultured organisms of genus *Acetobacterium* which are acetogens and also reported to utilize H_2 , e.g., *Acetobacterium carbinolicum* (99% identity) and *Acetobacterium psammolithicum* (98% identity) (Krumholz et al. 1999; Paarup et al. 2006). SXML-B15 showed the closest match to the cultured fermentative bacteria *Alkalibacter saccharofermentans* (98% identity), known as saccharolytic bacteria in the cellulolytic process (Garnova et al. 2004). Only two phylotypes, i.e., SXML-B09 and SXML-B19, belonged to *Deltaproteobacteria* (4.4% of the clones). SXML-B09 showed the highest identity to the 16S rRNA gene from the cultured sulfate-reducing bacteria *Desulfofustis glycolicus* (96% identity) growing with glycolate (Friedrich et al. 1996) and SXML-B19 showed the highest identity to the 16S rRNA gene from several strains of the genus *Desulfomicrobium* (99% identity) which are all sulfate reducers (Hippe et al. 2003). The residual phylotypes SXML-B04 and SXML-B07 were defined as unclassified bacteria, with <85% identity to the cultured organism.

Real-time PCR assay

Quantification of the total archaeal and bacterial community in the water produced was carried out. All of the samples were quantified in triplicate, and the average value was converted to

16S rRNA gene copies per milliliter of water. The copy number of archaeal 16S rRNA genes in the water sample was 7.2×10^4 , while that of bacterial 16S rRNA genes was $3.43 \times 10^7/\text{ml}$, which was two orders of magnitude higher than the CBM reservoir in Hokkaido (Shimizu et al. 2007).

Potential for microbial methane production

Methane production was observed only in the enrichments supplemented with methanol, and high rates of methane production were found in the enrichments incubated at 25 and 35 °C (Fig. 4). The maximum methane production rate for 25 °C was 83.2 $\mu\text{mol}/\text{day}$ (between 20 and 24 days) compared to 203.7 $\mu\text{mol}/\text{day}$ (between 6 and 8 days) at 35 °C, as increasing temperature leads to increasing methane production rates.

The archaeal communities in the enrichments were analyzed by PCR–DGGE. Five to seven DGGE bands were detected in each enrichment (Fig. 5) and a total of nine distinct archaeal bands were excised and sequenced (Table 3). The DGGE profiles were very similar between the enrichments, especially the enrichments incubated at 25 and 35 °C (Fig. 5). A few bands present in enrichments were not detected in the water sample, which might be due to the strong selective pressure caused by the amended anaerobic medium and the high concentration of supplemented methanol. Five bands showed high similarity (>98%), i.e., SLG-A01, SLG-A02, SLG-A05, SLG-A07, and SLG-A08. They were all closely related to the 16S rRNA gene from the cultured mesophilic methanol and trimethylamine-using methanogen

Fig. 4 Phylogenetic tree of bacterial 16S rRNA gene sequences from the water samples together with related sequences of environmental clones and cultured bacteria. Bootstrap support values (1,000

replicates) above 50% are shown at nodes. The scale bar represents 5% estimated sequence divergence

M. chelungpuianus (>95% identity) (Wu and Lai 2011). The sequences of these bands were >98% identical to the SXLL-A01-derived sequence from the archaeal clone library from the water produced. SLG-A03 was closely related to the 16S rRNA gene from the cultured methanogen *Methanosarcina horonobensis* (94% identity), which grew on methanol, dimethylamine, trimethylamine, dimethylsulfide, and acetate (Shimizu et al. 2011). Both SLG-A04 and SLG-A06 showed the closest identity to the 16S rRNA gene from the cultured ammonia-oxidizing archaeon *Candidatus nitrosopumilus* (91% identity) (Matsutani et al. 2009). SLG-A09 showed the highest match to the 16S rRNA gene from the hydrogenotrophic methanogen *Methanobacterium congolense* (95% identity) (Cuzin et al. 2001).

Considering the enrichments at 25 and 35 °C, only two bands, SLG-A01 and SLG-A02, were different. However, both bands were closely related to the same cultured organism, suggesting that the same archaeal communities existed

in both enrichments. The same archaeal communities in the enrichments incubated at 25 and 35 °C and the dominance of methylotrophic methanogen were in accordance with the observation of methane production. Although the DGGE analysis showed that methanogens existed in the enrichment incubated at 15 °C (SLG-A02, A03, A08, and A09), significant methane production was not observed (Fig. 4). This might be because that the temperature was not optimal for the production of methane, given that the temperature range for growth was 20 to 42 °C for *M. horonobensis* (Shimizu et al. 2011), 25 to 50 °C for *M. congolense* (Cuzin et al. 2001), and 24 to 45 °C for *M. chelungpuianus* (Wu and Lai 2011).

Discussion

We determined the chemical composition and stable isotopic ratios of CBM derived from the CBM reservoir associated

Fig. 5 Methane production from the water samples supplemented with acetate (open square), formate (open circle), H₂ + CO₂ (open triangle), methanol (open inverted triangle), and BES (open diamond). The enrichments were incubated at 15 °C (a), 25 °C (b), and 35 °C (c). Values are the mean of three replicates indicating the amount of methane accumulated in the headspaces of the culture bottles. Errors bars (where visible) represent the standard deviation

Table 3 Archaeal 16S rRNA gene sequences derived from DGGE bands

Band	Phylogenetic group	Closest cultivated species (% identity)
SLG-A01	<i>Methanosarcinaceae</i>	<i>Methanolobus chelungpuianus</i> (99%)
SLG-A02	<i>Methanosarcinaceae</i>	<i>Methanolobus chelungpuianus</i> (97%)
SLG-A03	<i>Methanosarcinaceae</i>	<i>Methanosarcina horonobensis</i> (94%)
SLG-A04	<i>Nitrosopumilaceae</i>	<i>Candidatus Nitrosopumilus</i> (91%)
SLG-A05	<i>Methanosarcinaceae</i>	<i>Methanolobus chelungpuianus</i> (99%)
SLG-A06	<i>Nitrosopumilaceae</i>	<i>Candidatus Nitrosopumilus</i> (91%)
SLG-A07	<i>Methanosarcinaceae</i>	<i>Methanolobus chelungpuianus</i> (99%)
SLG-A08	<i>Methanosarcinaceae</i>	<i>Methanolobus chelungpuianus</i> (97%)
SLG-A09	<i>Methanobacteriaceae</i>	<i>Methanobacterium congolense</i> (95%)

with the Ordos Basin and the cross-plot of C₁/(C₂+C₃) over δ¹³C–CH₄ typifies methane of mixed biogenic and thermogenic origin (Bernard et al. 1978) (Fig. 6).

To identify methanogenic archaea in the water samples, we determined the archaeal community using 16S rRNA gene clone library. The phylogenetic analysis indicated that all archaea were related to methanogens belonging to the family *Methanosarcinaceae* (Fig. 2). The limited archaeal diversity was not surprising as coal beds are nutrient-limited habitats. Similarly, only one RFLP-type closely related to *Methanobacterium alcaliphilum* was detected in the water produced in the CBM reservoir in the Gippsland Basin (Midgley et al. 2010). The majority of the species were closely related to *Methanolobus*, an obligate methylotrophic methanogen (Mochimaru et al. 2009; Wu and Lai 2011). The remainder of the species were closely related to *Methanosarcina*, which is also known to utilize the methylotrophic pathway (Simankova et al. 2003). Furthermore, high rates of methane production were observed in the enrichments amended with methanol and incubated at 25 and 35 °C (Fig. 4). The primary methanogenic archaea propagated on these enrichments were closely related to the cultured methanogens *M. chelungpuianus* and *M. horonobensis* (Table 3; Fig. 5), which are both methylotrophic methanogens (Shimizu et al. 2011; Wu and Lai 2011). *M. chelungpuianus* was also the dominant archaea detected in the water samples (Fig. 2). Taken together, the current phylogenetic and physiological evidence strongly suggests that the biogenic CBM in the Liulin District associated with the Eastern Ordos Basin is produced by methylotrophic methanogenesis.

Fig. 6 DGGE profiles of archaeal 16S rRNA genes amplified from microbial community DNA obtained from water sample and enrichments: *a* water sample, *b* enrichment incubated at 15 °C, *c* enrichment incubated at 25 °C, *d* enrichment incubated at 35 °C. Marked bands were selected for sequencing

The question remains as to why methylotrophic methanogenesis predominates in that site as it was rarely reported in other CBM reservoirs (Shimizu et al. 2007; Strapoć et al. 2010). We examined the bacterial community in the water produced using a 16S rRNA gene clone library to better understand the coal biodegradation by syntrophic bacteria at that site.

The prevailing view is that the growth of methanogens associated with CBM reservoirs was dependent on coal biodegradation by syntrophic bacteria. Coal is extremely rich in complex organic matter often dominated by aromatic and largely lignin-derived macromolecules, which are attractive carbon sources for microbial biodegradation (Strapoć et al. 2008). Several intermediates are factors in the biodegradation of coal, e.g., aromatic and aliphatic monomers, propionate, butyrate, valerate, succinate, alcohols, and various other substrates, which are precursors for methanogenesis (Strapoć et al. 2010).

Several clones in the library were closely related to fermentative bacteria. The biodegradation of coal in this site could be potentially performed by the clones associated with *Firmicutes*. Various representative clones are found in our library, e.g., *Clostridium* sp. degrades cellulose, xylan, and

polysaccharides (Chen et al. 2010; Kato et al. 2004; Zhilina et al. 2005), *Desulfosporosinus* sp. utilizes aromatic compounds (Liu et al. 2004), and *Acetobacterium* sp., *Alkalibacter* sp., and *Desulfotomaculum* sp. use monosaccharide and monoaromatic hydrocarbons as substrates (Garnova et al. 2004; Morasch et al. 2004; Paarup et al. 2006). Methanol, acetate, CO₂, H₂, and other substrates for methanogens are thereby produced. Certain sulfate-reducing bacteria found in our library might also grow by fermentation of coal components at the site as *Desulfotomaculum* sp. is able to degrade *o*-xylene, *m*-xylene, and toluene (Morasch et al. 2004).

Various relatives of the fermentative bacteria detected in our library were related to acetogens or facultative acetogens using H₂ as an electron donor such as clones related to *Acetobacterium* (Krumholz et al. 1999; Paarup et al. 2006), *Alkalibaculum* (Allen et al. 2010), and *Desulfosporosinus* (Ramamoorthy et al. 2006). The presence of acetogens might explain the non-detection of hydrogenotrophic methanogens. However, the product acetate would simulate sulfate reduction at the site because sulfate-reducing bacteria were also found in our library, e.g., *Desulfotomaculum*, *Desulfofustis*, *Desulfomicrobium*, and *Desulfosporosinus*. Sulfate-reducing bacteria would outcompete methanogens for acetate, resulting in the non-detection of acetoclastic methanogens (Oremland and Polcin 1982). Sulfate-reducing bacteria would not compete with methanogens with methanol, allowing methanogenesis and sulfate reduction to operate simultaneously at the site (Oremland and Polcin 1982). The presence of both acetogens and sulfate-reducing bacteria led to the predominance of methylotrophic methanogens. Methanol might be produced in the process of biodegrading the lignin component of coal as it has been reported as a biodegradation product of the natural methoxylated aromatics which are the building blocks of lignin (Donnelly and Dagley 1980).

Corresponding to other CBM reservoirs (Penner et al. 2010), an abundance of clones affiliated with nitrate-reducing bacteria was also detected in the bacterial library (SXLL-B06 and all clones related to *Epsilonproteobacteria*), which are potentially involved in the cycling of inorganic nitrogen together with denitrifying bacteria (SXLL-B11 and SXLL-B13). ARDRA might underestimate the actual bacterial diversity and all of the metabolic considerations above were solely obtained from DNA sequences. A deeper evaluation of bacterial diversity using next-generation sequencing, e.g., 454 pyrosequencing, and further determination of the activities and functions of these bacteria using stable isotope probing and cultivation are necessary for further investigation.

In conclusion, the composition and stable isotopic ratios of the CBM implied a mixed biogenic and thermogenic origin for the Liulin CBM. The available microbiological data showed that fermentative, sulfate-reducing, and nitrate-reducing bacteria were a factor in coal biodegradation,

providing substrates for methylotrophic methanogenic archaea to produce methane. These results suggested that past and ongoing biodegradation of coal by methylotrophic methanogens and syntrophic bacteria, as well as thermogenic CBM production, contributed to the Liulin CBM reserves associated with Eastern Ordos Basin.

Acknowledgements We are grateful to China United Coalbed Methane Co., Ltd., for helping with sampling opportunities on the water produced. This work was supported by National Science and Technology Major Project, China (2011ZX05060-005 and 2009ZX05039-003).

References

- Allen TD, Caldwell ME, Lawson PA, Huhnke RL, Tanner RS (2010) *Alkalibaculum bacchi* gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil. Int J Syst Evol Microbiol 60:2483–2489
- Bates BL, McIntosh JC, Lohse KA, Brooks PD (2011) Influence of groundwater flowpaths, residence times and nutrients on the extent of microbial methanogenesis in coal beds: Powder River Basin, USA. Chem Geol 284:45–61
- Bernard BB, Brooks JM, Sackett WM (1978) Light hydrocarbons in recent Texas continental shelf and slope sediments. J Geophys Res 83:4053–4061
- Chen S, Niu L, Zhang Y (2010) *Saccharofermentans acetigenes* gen nov, sp nov, an anaerobic bacterium isolated from sludge treating brewery wastewater. Int J Syst Evol Microbiol 60:2735–2738
- Chou JH, Jiang SR, Cho JC, Song J, Lin MC, Chen WM (2008) *Azonexus hydrophilus* sp nov, a *nifH* gene-harboring bacterium isolated from freshwater. Int J Syst Evol Microbiol 58:946–951
- Collado L, Levican A, Perez J, Figueras MJ (2011) *Arcobacter defluvii* sp nov, isolated from sewage samples. Int J Syst Evol Microbiol 61:2155–2161
- Cuzin N, Ouattara AS, Labat M, Garcia JL (2001) *Methanobacterium congolense* sp nov, from a methanogenic fermentation of cassava peel. Int J Syst Evol Microbiol 51:489–493
- DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685–5689
- Ding S, Zhu J, Zhen B, Liu Z, Liu Q (2011) Characteristics of high rank coal bed methane reservoir from the Xiangning Mining Area, Eastern Ordos Basin, China. Energ Explor Exploit 29:33–48
- Donnelly M, Dagley S (1980) Production of methanol from aromatic acids by *Pseudomonas putida*. J Bacteriol 142:916–924
- Flores RM, Rice CA, Stricker GD, Warden A, Ellis MS (2008) Methanogenic pathways of coal-bed gas in the Powder River Basin, United States: the geologic factor. Int J Coal Geol 76:52–75
- Friedrich M, Springer N, Ludwig W, Schink B (1996) Phylogenetic positions of *Desulfotomaculum glycolicum* gen. nov., sp. nov. and *Syntrophotulus glycolicus* gen. nov., sp. nov., two new strict anaerobes growing with glycolic acid. Int J Syst Evol Microbiol 46:1065–1069
- Garnova ES, Zhilina TN, Tourova TP, Kostrikina NA, Zavarzin GA (2004) Anaerobic, alkaliphilic, saccharolytic bacterium *Alkalibacter saccharofermentans* gen. nov., sp. nov. from a soda lake in the Transbaikalian region of Russia. Extremophiles 8:309–316
- Green MS, Flanagan KC, Gilcrease PC (2008) Characterization of a methanogenic consortium enriched from a coal bed methane well in the Powder River Basin, USA. Int J Coal Geol 76:34–45
- Gunsalus RP, Romesser JA, Wolfe RS (1978) Preparation of coenzyme M analogs and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum*. Biochemistry 17:2374–2377
- Harris SH, Smith RL, Barker CE (2008) Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. Int J Coal Geol 76:46–51
- Hippe H, Vainshtein M, Gogotova G, Stackebrandt E (2003) Reclassification of *Desulfobacterium macestii* as *Desulfomicrobium macestii* comb. nov. Int J Syst Evol Microbiol 53:1127–1130
- Kato S, Haruta S, Cui ZJ, Ishii M, Yokota A, Igarashi Y (2004) *Clostridium straminisolvans* sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. Int J Syst Evol Microbiol 54:2043–2047
- Krumholz LR, Harris SH, Tay ST, Suflita JM (1999) Characterization of two subsurface H₂-utilizing bacteria, *Desulfomicrobium hypogaeum* sp nov and *Acetobacterium psammolithicum* sp nov., and their ecological roles. Appl Environ Microbiol 65:2300–2306
- Lane D (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–175
- Liu A, Garcia-Dominguez E, Rhine E, Young L (2004) A novel arsenate respiring isolate that can utilize aromatic substrates. FEMS Microbiol Ecol 48:323–332
- Matsutani N, Nakagawa T, Nakamura K, Takahashi R, Yoshihara K, Tokuyama T (2009) Enrichment of a novel marine ammonia-oxidizing archaeon obtained from sand of an eelgrass zone. Microb Environ 26:23–29
- Mechichi T, Stackebrandt E, Gad'on N, Fuchs G (2002) Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. Arch Microbiol 178:26–35
- Midgley DJ, Hendry P, Pinetown KL, Fuentes D, Gong S, Mitchell DL, Faiz M (2010) Characterisation of a microbial community associated with a deep, coal seam methane reservoir in the Gippsland Basin, Australia. Int J Coal Geol 82:232–239
- Mochimaru H, Tamaki H, Hanada S, Imachi H, Nakamura K, Sakata S, Kamagata Y (2009) *Methanobolus profundus* sp. nov., a methylotrophic methanogen isolated from deep subsurface sediments in a natural gas field. Int J Syst Evol Microbiol 59:714–718
- Morasch B, Schink B, Tebbe CC, Meckenstock RU (2004) Degradation of o-xylene and m-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. Arch Microbiol 181:407–417
- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
- Oremland RS, Polcin S (1982) Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl Environ Microbiol 44:1270–1276
- Paarup M, Friedrich MW, Tindall BJ, Finster K (2006) Characterization of the psychrotolerant acetogen strain SyrA5 and the emended description of the species *Acetobacterium carbinolicum*. Anton Leeuw 89:55–69
- Penner T, Foght J, Budwill K (2010) Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. Int J Coal Geol 82:81–93
- Pfeiffer RS, Ulrich GA, Finkelstein M (2010) Chemical amendments for the stimulation of biogenic gas generation in deposits of carbonaceous material. US Patent No. 7696132
- Qiu YL, Sekiguchi Y, Imachi H, Kamagata Y, Tseng IC, Cheng SS, Ohashi A, Harada H (2004) Identification and isolation of anaerobic, syntrophic phthalate isomer-degrading microbes from methanogenic sludges treating wastewater from terephthalate manufacturing. Appl Environ Microbiol 70:1617–1626
- Ramamoorthy S, Sass H, Langner H, Schumann P, Kroppenstedt R, Spring S, Overmann J, Rosenzweig R (2006) *Desulfosporosinus lacus* sp. nov., a sulfate-reducing bacterium isolated from pristine

- freshwater lake sediments. *Int J Syst Evol Microbiol* 56:2729–2736
- Raskin L, Stromley J, Rittmann B, Stahl D (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl Environ Microbiol* 60:1232–1240
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ (2009) Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541
- Scott AR, Kaiser W, Ayers WB (1994) Thermogenic and secondary biogenic gases, San Juan Basin, Colorado and New Mexico—implications for coal bed gas producibility. *Bull Am Assoc Petrol Geol* 78:1186–1209
- Shimizu S, Akiyama M, Naganuma T, Fujioka M, Nako M, Ishijima Y (2007) Molecular characterization of microbial communities in deep coal seam groundwater of northern Japan. *Geobiology* 5:423–433
- Shimizu S, Upadhye R, Ishijima Y, Naganuma T (2011) *Methanosarcina horonobensis* sp. nov., a methanogenic archaeon isolated from a deep subsurface Miocene formation. *Int J Syst Evol Microbiol* 61:2503–2507
- Shinoda Y, Sakai Y, Uenishi H, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Kato N (2004) Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. *Appl Environ Microbiol* 70:1385–1392
- Simankova MV, Kotsyurbenko OR, Lueders T, Nozhevnikova AN, Wagner B, Conrad R, Friedrich MW (2003) Isolation and characterization of new strains of methanogens from cold terrestrial habitats. *Syst Appl Microbiol* 26:312–318
- Smith CJ, Nedwell DB, Dong LF, Osborn AM (2006) Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ Microbiol* 8:804–815
- Steven B, Briggs G, McKay CP, Pollard WH, Greer CW, Whyte LG (2007) Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. *FEMS Microbiol Ecol* 59:513–523
- Strapoć D, Mastalerz M, Eble C, Schimmelmann A (2007) Characterization of the origin of coal bed gases in southeastern Illinois Basin by compound-specific carbon and hydrogen stable isotope ratios. *Org Geochem* 38:267–287
- Strapoć D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, Lin YS, Ertefai TF, Schubotz F, Hinrichs KU, Mastalerz M, Schimmelmann A (2008) Methane-producing microbial community in a coal bed of the Illinois Basin. *Appl Environ Microbiol* 74:2424–2432
- Strapoć D, Ashby M, Wood L, Levinson R, Huizinga B (2010) Significant contribution of methyl/methanol-utilising methanogenic pathway in a subsurface biogas environment. In: Skovhus T, Whitby C (eds) *Applied microbiology and molecular biology in oilfield systems*. Springer, pp 211–216
- Strapoć D, Mastalerz M, Dawson K, Macalady J, Callaghan AV, Wawrik B, Turich C, Ashby M (2011) Biogeochemistry of microbial coal-bed methane. *Annu Rev Earth Pl Sc* 39:617–656
- Su X, Zhang L, Zhang R (2003) The abnormal pressure regime of the Pennsylvanian No. 8 coal bed methane reservoir in Liulin-Wupu District, Eastern Ordos Basin, China. *Int J Coal Geol* 53:227–239
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Wu SY, Lai MC (2011) Methanogenic archaea isolated from Taiwan's Chelungpu Fault. *Appl Environ Microbiol* 77:830–838
- Zhang G, Jiang N, Liu X, Dong X (2008) Methanogenesis from methanol at low temperatures by a novel psychrophilic methanogen, “*Methanolobus psychrophilus*” sp. nov., prevalent in Zoige Wetland of the Tibetan Plateau. *Appl Environ Microbiol* 74:6114–6120
- Zhilina T, Kevbrin V, Tourova T, Lysenko A, Kostrikina N, Zavarzin G (2005) *Clostridium alkalicellum* sp. nov., an obligately alkaliphilic cellulolytic bacterium from a soda lake in the Baikal region. *Microbiology* 74:557–566