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# Microbial diversity and biogenic methane potential of a thermogenic-gas coal mine

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## Abstract

The microbial communities and biogenic methane potential of a gas coal mine were investigated by cultivation-independent and cultivation-dependent approaches. Stable carbon isotopic analysis indicated that *in situ* methane in the coal mine was dominantly of a thermogenic origin. However, a high level of diversity of bacteria and methanogens that were present in the coal mine was revealed by 454 pyrosequencing, and included various fermentative bacteria in the phyla of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, and acetotrophic, hydrogenotrophic, and methylotrophic methanogens. Methane was produced in enrichments of mine water samples supplemented with acetate under laboratory conditions. The microbial flora obtained from the enrichments could stimulate methane formation from coal samples. 16S rRNA gene clone library analysis

indicated that the microbial community from coal cultivation samples supplemented with the enriched microbial consortium was dominated by the anaerobic fermentative Clostridiales and facultative acetoclastic *Methanosarcina*. This study suggests that the biogenic methane potential in the thermogenic-gas coal mine could be stimulated by the indigenous microorganisms.

Key words: biogenic methane, acetotrophic methanogens, 454 pyrosequencing, real-time PCR

## 1. Introduction

With constant industrial development, coal mining activity continues to increase in many countries. However, the coal recovery is low for the majority of coal mines. For example, it is about 30% for the majority of coal mines in China (Cui, 2006). Thus, the recovery and utilization of coal mines has been an issue in recent years. The large amount of coal in abandoned coal mines is a very attractive source for methane generation (Beckmann et al., 2011b). In fact, coal mine methane has been of great interest for both energy production and greenhouse gas reduction in recent years (Karacan et al., 2011). Additionally, it is a pressing concern for governments in terms of reducing coal mine gas explosions.

Methane emission from a coal seam can be generated from thermogenic, biogenic, or mixed pathways. Thermogenic methane is primarily generated through the thermal decomposition of mature organic matter. Biogenic methane includes primary and secondary biogenic gas, which is the result of anaerobic microbial degradation of the organic matter in

coal at low temperatures (Ni et al., 2012). Mixed genesis methane refers to a mixture of methane genesis types with different geochemical characteristics from both biogenic and thermogenic origins.

There has been considerable interest in biogenic methane in recent years (Flores et al., 2008; Green et al., 2008; Papendick et al., 2011; Strąpoć et al., 2010), providing an opportunity for energy production via methane regeneration from coal mines. In general, biogenic methane is produced from acetate, hydrogen, or methyl-bearing substrates as precursors, which are respectively coupled with the acetotrophic, hydrogenotrophic, and methylotrophic methanogens. Acetotrophic and hydrogenotrophic methanogenesis are the predominant pathways identified in numerous microbial studies on biogenic methane formation of coal beds, such as abandoned coal mines in Germany (Beckmann et al., 2011b), the coal bed methane reservoir in the Powder River Basin (Green et al., 2008), and the Queensland coal seams in the Surat Basin (Papendick et al., 2011). Very few studies have focused on the methylotrophic methanogenesis with the exception of the Eastern Ordos Basin in China from our previous work (Guo et al., 2012) and a biogenic coal bed methane field in Alaska (Strąpoć et al., 2010).

The chemical composition of coal mine gas ($C_1$–$C_3$ gaseous alkanes), stable carbon and hydrogen isotope ratios of methane ($\delta^{13}C_{-CH4}$, $\delta^{13}C_{-CO2}$, and $\delta\ D_{-CH4}$), and isotopic fractionation between $CO_2$ and $CH_4$ ($\Delta^{13}C_{CO2\text{-}CH4}$) are routinely used to differentiate the various origins of coal mine methane (Conrad, 2005). Using the stable isotope method, the origins of methane from different coal seams are typically distinguished. It has been shown

that biogenic methane reserves account for about 20% of gas reserves worldwide (Rice & Claypool, 1981). For example, the coal bed methane from the Powder River Basin (Flores et al., 2008) and eastern Australia (Faiz & Hendry, 2006) with the $\delta^{13}C_{-CH4}$ value < –55‰ and $\delta D_{-CH4}$ < –300‰ suggested the biogenic origin. Mixed methane of both thermogenic and biogenic origin is also widely observed, e.g., coal mines in Ruhr Basin in Germany (Krüger et al., 2008) and coal seams in the San Juan Basin in the United States (Wawrik et al., 2012), and the Eastern Ordos Basin in China (Guo et al., 2012). However, the presence of thermogenic gas does not indicate whether or not there is a potential for biogenic methanogenesis in coal seams. Little is known about microbial communities and the biogenic potential in thermogenic gas coal mines (Kimura et al., 2010). Furthermore, the biogenic methane potential of thermogenic gas coal mines has never been evaluated.

In this study, coal and water samples were collected from a thermogenic-gas coal mine that was confirmed by the stable isotopic signatures. Molecular techniques combined with anaerobic cultivation were used to determine the potential for biogenic methane generation of the coal mine. We discuss the members of the microbial consortium that were mainly present in coal seams and whether biogenic methane could be generated from similar coal mines in the future.

## 2. Materials and methods

### 2.1 Study area and sample collection

Samples were collected from a coal mine located in Hubei, China (Fig. 1). The coal seam

is Upper Triassic in age, belonging to the Jiuligang Formation in the Jingmen-Dangyang Basin. It is a sedimentary basin that evolved from a lake. The Jiuligang Formation is the coal-bearing strata characterized by a thin substrate of coal-bearing sediment and is composed of siltstone, sandy shale, mudstone, carbonaceous mudstone, and thin coal seams (Fig. 2). When compared to previous studies of coal mines in Hubei (Wei et al., 2013), the sampling mine in the present study is a representative gassy coal mine. The depth of the samples was 158 ± 5 m, and the average thickness of the coal seam was 0.8 m. The methane concentration in ventilation air varies from 0.3% to 1.7% over a year. High methane content seasons are spring and autumn. The gas samples were collected at three different newly exposed mining faces in the working area by injection into sealed 500 mL aluminum foil sampling bags. Coal samples were also collected from the three newly exposed mining faces at the same work site. For each coal sample, 4–5 kg of fresh working-face coal was obtained and immediately put in sterilized glass bottles. An uninterrupted drainage system was kept to drain mine water for the underground mine. Water samples were pumped from the drainage pipes without air and other contaminants. Three high-density polyethylene bottles of 10 L were filled with mine water with no headspace and tightly sealed with butyl rubber stoppers. The coal seam temperature was 33 ± 5°C. Water temperature, pH, conductivity, and dissolved oxygen (DO) were measured with a portable detector (HQ 40d, Hach, Loveland, CO, USA). The *in situ* concentrations of $CH_4$ and $CO_2$ were measured with portable gas detectors GJC4/100 and GRG5H (China Coal Industrial Equipment Corp, Ltd, Beijing, China), respectively. The stable carbon isotopic compositions of $CH_4$ and $CO_2$ in coal mine gas were measured by

Trace GC Ultra (Thermo Electron Corporation, Austin, TX, USA) and Thermo Quest Delta plus XL isotope ratio mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Coal and water samples were kept on ice in the field and then transported to the lab. Coal properties were analyzed at the National Coal Quality Supervision and Inspection Center in China. Analysis of the chemical and physical properties of the water was carried out by Pony Testing International Group in Beijing, China.

**2.2 Detection of biogenic methane potential of the coal mine**

Coal samples were ground to coal powder and screened through a 100-mesh sieve (less than 150 μm) in an anaerobic glove box (Xingmiao YQX-11, Shanghai, China). Mine water samples (1 L) were filtered through sterile 0.22 μm pore-size membrane filters (Whatman Japan KK, Tokyo, Japan). Coal powder (0.3 g) and membranes containing the filtered microorganisms from water were respectively placed into 140 mL serum bottles containing 30 mL anaerobic medium. The anaerobic medium was prepared as previously described (Strapoc et al., 2008). $Na_2S$ and cysteine were supplemented to maintain reducing conditions. To study the methanogenic pathway, available substrates were provided for methanogens, including sodium formate (50 mM), acetate (50 mM), methanol (50 mM), trimethylamine (50 mM), $H_2$, and $CO_2$ (4:1, V/V; $10^5$ Pa). To inhibit biomethanogenesis, 10 mM 2-bromoethanesulfonate (BES) was added as a control. All of the serum bottles were filled with high purity nitrogen, except for $H_2$-$CO_2$ bottles, and incubated at 35°C. Cultivations were carried out in triplicate. Methane generation in the headspace of the serum bottles was

detected every 5 days using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector. The limit of detection for $CH_4$ was 10 ppm. However, there was not obvious methane generation from the enrichments using coal samples. Thus, the 30 mL culture solution from the water sample enrichments that produced methane was centrifuged at 5,000 rpm for 10 min to collect the microbial cells. The cells were washed with PBS buffer to remove the medium and substrates, and added to 30 mL of the medium containing 0.3 g of coal.

### 2.3 Pyrosequencing of original samples

#### 2.3.1 DNA extraction, PCR, sequencing

The archaea and bacteria in the original coal and mine water samples were investigated by 454 pyrosequencing. The coal genomic DNA sample was a mixture of three independent coal samples obtained from the three newly exposed mine faces in the coal mine. The coal samples were pretreated due to the difficulty of extracting DNA from coal samples. About 50-g coal was macerated in 100 mL of 0.05 M phosphate buffer with 0.2% Tween 80. The mixed samples were incubated at 37°C with shaking at 150 rpm for 1 h, sonicated for 30 min with a 10-s interval after each 5-min sonication (Ahmed & Smith, 2001), and centrifuged. The microbial cells obtained were collected using a Whatman filter (Whatman Japan KK). Genomic DNA was extracted from the filters using a FastDNA SPIN kit for soil (Bio101 Systems, Carlsbad, CA, USA) according to the manufacturer's instructions. For mine water samples, l L of water from each sampling site was filtered through a 0.22-μm membrane filter.

Genomic DNA was extracted from the membrane filters containing the filtered microorganisms using a FastDNA SPIN kit in triplicate.

For 454 pyrosequencing, the 508-bp and 571-bp DNA fragments of the 16S rRNA gene containing the V1-V3 and V3-V5 variable regions were amplified for bacteria and archaea, respectively. Primer sets BAC-27F/533R (Liu et al., 2012a) and AR-344F/915R (Gantner et al., 2011) were used for bacterial and archaeal amplification, respectively. PCR was performed at 95°C for 4 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension of 10 min at 72°C. PCR was performed in triplicate for the original samples. The PCR products were purified with a DNA Purification Kit (Fermentas, Lithuania) and then sequenced using the pyrosequencing technology (Roche Diagnostics, Indianapolis, IN, USA).

**2.3.2 Phylogenetic and statistical analysis**

Raw sequences were processed by quality control with Mothur (http://www.mothur.org/wiki) (Schloss et al., 2009). Primers and ambiguous bases were deleted. Sequences shorter than 200 bp were also removed by Mothur. PHYLIP (version 3.68) was used to calculate distance matrices. Chimeric sequences were detected using UCHIME, and removed (Edgar et al., 2011). For the taxonomic analysis, identical sequences were grouped and representative sequences were aligned against the Silva database (http://www.arbsilva.de) (Pruesse et al., 2007). The statistical analysis of sequences from each sample was performed in Mothur. Operational taxonomic units (OTUs) were defined at 97%

sequence similarity based on the matrices. The abundance-based coverage estimator (ACE), bias-corrected Chao1 richness estimator, and Shannon diversity indices, as well as rarefaction analysis, were also defined at 97% sequence similarity. The 16S rRNA gene sequences derived from 454 pyrosequencing were submitted to the NCBI Sequence Read Archive under accession number SRA 065105.

### 2.4 Clone library analysis of the microbial community in enrichment samples

#### 2.4.1 Library construction and sequencing

Genomic DNA from the coal cultivations supplemented with the water-enriched microbial consortium was extracted. The 16s rRNA genes of bacteria and archaea were used for microbial community analysis. The universal bacterial primer set BAC-27F/1492R and archaeal primer set AR-109F/915R were used for amplification. PCR products were purified with a Purification Kit (Fermentas) and ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA). The vector was then transformed into *Escherichia coli* DH5α competent cells (Promega). Recombinant plasmids were checked by PCR with the M13 primer and then sequenced with an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

#### 2.4.2 Phylogenetic and statistical analysis

The chimeric sequences were checked by UCHIME. The OTUs were defined at 97% sequence similarity with Mothur software. The representative sequences for each OTU were

compared with the NCBI nucleotide sequence database (http: //www.ncbi.nlm. nih.gov/BLAST/). For statistical analysis, the ACE, Chao 1, and Shannon diversity indices were also used in Mothur software. The bacterial and archaeal 16s rRNA gene sequences obtained have been deposited in GenBank under accession number KC215425-KC215437 and KC215420-KC215421, respectively.

### 2.5 Real-time PCR for archaea, bacteria, and methanogens

#### 2.5.1 Real-time PCR amplification

The abundance of bacteria, archaea, and methanogens in the original and enriched samples was determined by real-time PCR, which was carried out as described previously (Denman & McSweeney, 2005). Real-time PCR was performed using the SYBR Green real-time PCR Master Mix (Fermentas, Hanover, MD, USA) on an ABI 7300 Sequence Detection System (Applied Biosystems). Primer sets of BAC-338F/518R (Lane, 1991), AR-519F/519R (Liu et al., 2012b), and MLf/r (Juottonen et al., 2006) were used to detect bacteria, archaea, and methanogens, respectively. Real-time PCR was carried out in 25-μL reactions containing 12.5 μL of 2 × SYBR Green PCR Master Mix, 1 μL of each primer (10 nM), 1 μL of sample DNA, and 9.5 μL of sterilized ultrapure $H_2O$. The PCR was initiated at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Fluorescence signals were collected at 72°C during the elongation step. Each DNA template process was performed in triplicate.

### 2.5.2 Statistical analysis

Plasmids containing the bacterial 16S rRNA gene of *Desulfosporosinus* sp. (KC215425), archaeal 16S rRNA gene of *Methanosarcina* sp. (KC215420), and the *mcr*A gene of *Methanosarcina* sp. (KC244184) were used to make standard curves for bacteria, archaea, and methanogens, respectively. Plasmids were quantified with a NanoDrop ND-1000 UV-Vis Spectrophotometer. Ten-fold serial dilutions of the plasmids ranging from $10^0$ to $10^{10}$ copies/μL were prepared to generate the standard curves and determine detection limits. Standard curves were acquired using the 7300 Real-Time PCR System Sequence Detection Software ver. 1.3. The slope of the standard curve was appraised to determine the PCR amplification efficiency (E), which was estimated by the formula $E = 10^{-1/slope} - 1$ (Klein et al., 1999). Copy numbers and cell numbers were calculated according to methods described by Zhang (Zhang et al., 2008) and Klappenbach (Klappenbach et al., 2001). For each sample, the mean value and standard deviation were determined from triplicate experiments.

The statistical analysis of real-time PCR data was obtained after amplification. Standard curves based on cycle threshold (Ct) values and 10-fold serial dilutions of plasmids (log $C_0$) were created. The amplification efficiency (E) between 0.938 and 0.995 represented valid PCR amplification reactions, because the accepted amplification efficiency range is 0.9–1.1. Detection limits for bacteria, archaea, and methanogens were defined as 1–10, 10–100, and 10–100 copies/μL, respectively. Negative controls were below the detection limits.

## 3. Results

### 3.1 Gas concentrations and stable carbon compositions

The concentrations and stable carbon compositions of methane and carbon dioxide are listed in Table 1. Hydrocarbons with two or more carbon atoms, such as ethane, ethylene, and propane, were below the detection limit of 0.01%. The $\delta^{13}C$-values for methane and carbon dioxide were similar among the samples collected from the three newly exposed mining faces. The $\delta^{13}C_{CH4}$ was between -20.97‰ and -21.47‰. The $\delta^{13}C_{CO2}$ varied from -15.47‰ to -14.74‰. Isotopic fractionation between $CO_2$ and $CH_4$ ($\Delta^{13}C_{CO2\text{-}CH4}$) varied from 6.07‰ to 6.51‰. The stable isotope data fall in the range typically for a thermogenic source of methane.

### 3.2 Properties of coal samples

Coal from the gassy coal mine was classified as anthracite based on the Chinese classification (GB5751-86) (Chen, 2000) (Table 2). Ultimate analysis revealed that the coal contained high carbon and sulfur content, and lower hydrogen and oxygen content. The coal macerals were composed of vitrinite, liptinite, and mineral matter. The vitrinite contents were the most abundant whereas the inertinite was below the detection limit (0.1%). Coal macerals can be related to the coalification level (coal rank). As the high rank coal, anthracite had high reflectance ($R_{o,\,max}$ 3.0%).

### 3.3 Properties of mine water

Mine water was classified as $SO_4^{2-}$ and $Na^+$ type based on the ionic characteristic according to the Chinese classification of coal mine water (GB/T 19223-2003) (Table 3). It was typically alkaline with a pH of 8.23. The DO in the mine water (1.21 mg/L) was relatively low. Total organic carbon (TOC), dissolved organic carbon (DOC), and total nitrogen were also at low levels, especially for nitrate and nitrite, which were below the detection limit. However, the conductivity, total dissolved solids (TDS), dissolved inorganic carbon (DIC), sodium, and sulfate levels were at high levels based on the Chinese classification of mine water.

### 3.4 Detection of biogenic methane potential for coal and mine water samples

Biogenic methane formation in the laboratory was observed by enrichments supplemented with available substrates for methanogens. Original coal and water samples were incubated in liquid medium at 35°C for 70 days. Biogenic methane generation by the coal samples was insignificant. However, water samples supplemented with acetate displayed significant methane formation (157.43 ± 4.63 μmol), whereas the cultivations supplemented with other substrates did not generate detectable methane (Fig. 3A). The coal cultivation exhibited insignificant biogenic methane formation, suggesting that the microbial population of the coal was not efficient enough in converting coal to methane. Therefore, the microbial biomass enriched from the mine water was added to test the bioavailability of the coal. In this set-up, coal provided the organic carbon source. The acetate was monitored as potential intermediate

using gas chromatography every 5 days in the subsequent cultivations of coal powder inoculated with the microbial consortium obtained from the enrichments of the water samples.

Cultures of coal powder inoculated with the microbial consortium generated detectable biogenic methane after incubation for 60 days at 35°C, while the negative controls did not produce methane (Fig. 3B). The acetate accumulated rapidly during the first few days, and then gradually decreased to a low level (Fig. 3C). With the consumption of acetate, the biogenic methane increased and then remained stable.

### 3.5 Microbial abundance determined by real-time PCR

To acquire a better understanding of the correlation between microbial abundance and biogenic methane potential, real-time PCR was performed. The abundance of total bacteria, archaea, and methanogens is shown in Fig. 4. In the original coal samples, total bacteria and archaea were up to $10^5$ and $10^4$ cells/g, respectively, however, in mine water, they were at $10^6$ and $10^5$ cells/mL, respectively. Methanogens, based on real time PCR targeting the *mcrA* gene, showed that methanogens were present at $10^4$ cells/g and $10^5$ cells/mL in coal and mine water, respectively. For the coal samples supplemented with acetate-enriched microbial consortia, total bacteria and archaea were at $10^8$ cells/g and $10^7$ cells/g, respectively (Table S3). In comparison to the original coal sample, the abundance of bacteria and archaea was significantly higher. The methanogen population accounted for more than 90% of the archaeal community for the coal samples supplemented with acetate-enriched microbial consortia.

According to the aforementioned results about enrichments, original coal samples did not generate biogenic methane. However, coal enrichments with the enriched microbial consortium from water samples did produced methane. Possibly, the microbial concentration may play an important role for methane formation. The methanogens in the cultivation of original coal samples was $10^4$ cells/mL, much lower than that ($10^7$ cells/mL) in the cultivation that was inoculated with the microbial consortium. This greatly increased the microbial abundance and concentration, whereas anaerobic bacteria and methanogens were at levels to $10^3$ times higher than the original samples (Table S3). Thus, coal bioavailability may be accelerated, which provided the available substrates to fuel methanogens, consequently resulting in methane formation.

### 3.6 Investigation of the microbial community by pyrosequencing

In order to understand the correlation between coal seam microorganisms and biogenic methane potential, a deep investigation of microbial communities in coal and mine water samples was performed by pyrosequencing. A total of 101,858 high quality sequences were obtained by pyrosequencing. The average length of sequences for bacteria and archaea was 477 bp and 496 bp, respectively. OTUs (Operational Taxonomic Units) (Wooley et al., 2012), ACE (Abundance Coverage-based Estimator) (Chao et al., 2000), Chao1, Shannon, and the coverage were defined at 97% similarity (Table S1). 3233 (bacteria in coal), 2,065 (bacteria in mine water), 784 (archaea in coal), and 548 (archaea in mine water) OTUs were obtained. However, the full diversity was not approached (Fig. S1). Total OTUs estimated by ACE and

Chao1 were about 1.4–1.8 times of the identified OTUs. The Shannon index indicated high bacterial diversity (from 5.36 to 5.89). Coverage for bacteria and archaea ranged from 0.96 to 0.98, suggesting a sufficient sequencing depth.

### 3.6.1 Diversity of bacterial 16S rDNA

For bacteria, a total of 27 and 26 phyla, including the candidate divisions, were obtained from coal and mine water samples, respectively (Fig. 5). *Proteobacteria* was the most abundant phyla, accounting for 64.75% and 75.41% of total bacteria in coal and mine water, respectively. Other bacteria identified in coal were members of *Actinobacteria* (16.5%), *Firmicutes* (5.86%), *Bacteroidetes* (5.66%) and *Chloroflexi* (1.39%). In contrast, bacteria in the mine water were affiliated with the phyla of candidate division OD1 (8.34%), *Bacteroidetes* (4.03%), candidate division OP11 (2.48%), *Verrucomicrobia* (1.64%), and *Firmicutes* (1.6%).

The abundant bacteria and their closest genera in coal and mine water are listed in Table 4. In coal, they were distributed in the phyla of *Actinobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Deltaproteobacteria*, *Firmicutes*, and *Gammaproteobacteria*. A high proportion of *Actinobacteria* consisted of the genera of Candidatus *Microthrix* and *Tetrasphaera*. *Flavobacterium* was the only *Bacteroidetes* genus detected in coal. *Betaproteobacteria* contained the genera of *Acidovorax*, *Comamonas*, *Dechloromonas*, *Ferribacterium*, and *Propionivibrio*. The genus *Bdellovibrio* belongs to *Deltaproteobacteria*. The most abundant genus of *Gammaproteobacteria* was *Pseudomonas*, followed by

*Rheinheimera*. *Firmicutes* bacteria included the genus of *Desulfosporosinus*.

In mine water, the abundant bacteria were distributed in three phyla, *Bacteroidetes*, *Betaproteobacteria*, and *Gammaproteobacteria*. *Flavobacterium* belongs to the *Bacteroidetes*. The dominant genera of *Betaproteobacteria* were *Hydrogenophaga*, *Polaromonas*, and *Rhodocyclus*. *Gammaproteobacteria* included the genera of *Methylosoma*, *Legionella*, *Pseudomonas*, and *Desulfobacca*.

### 3.6.2 Diversity of archaeal 16S rDNA

At the phylum level, archaea were dominated by *Euryarchaeota*, comprising 63.52% and 99.3% of the population in coal and mine water, respectively. *Crenarchaeota* accounted for 36.27% and 0.68% of the population in coal and mine water, respectively. At the class level (Fig. 6), 8 classes were separately observed for coal and mine water samples. In coal, archaea were dominated by *Methanomicrobia* (40.96%). In contrast, *Halobacteria* (74.73%) was the most abundant class in mine water samples. Methanogens including the *Methanomicrobia* and the *Methanobacteria* accounted for 17.93% of the population.

The closest genera of abundant OTUs are shown in Table 4. The archaea of *Halobacteria* were observed in coal and mine water samples. A diversity of methanogens was observed in coal and mine water samples. In coal, acetotrophic *Methanosaeta* was the dominant archaea, followed by the hydrogenotrophic methanogens including *Methanobrevibacter*, *Methanobacterium*, *Methanoregula*, *Methanospirillum*, and *Methanosphaerula*, and the methylotrophic *Methanosphaera*. Methanogens in mine water contained the acetotrophic

*Methanosaeta*, hydrogenotrophic *Methanobacterium*, *Methanosphaerula*, *Methanoregula*, *Rice Cluster I*, *Methanospirillum*, and *Methanobrevibacter*, and methylotrophic *Methanolobus* and *Methanomethylovorans*. *Methanosarcina*, which has a wide substrate range, was also observed in mine water.

### 3.7 Microbes associated with methane generation in coal cultivations

To get a deep understanding of the microbial consortium that contributed to methane formation, the microbial composition of coal cultivation samples supplement with acetate-enriched microbial consortium was analyzed based on clone libraries. A total of 41 and 31 correct-sized clones were obtained and classified into 13 and 2 OTUs for bacteria and archaea, respectively. Diversity estimators including Chao 1, ACE, the Shannon index, and coverage defined at 97% similarity are listed in Table S2. For bacteria, the estimated OTUs (Chao 1 and ACE) were higher than the observed OTUs. However, the coverage (78.05 %) confirmed that the core bacterial community was retrieved. For archaea, the OTUs were equal to Chao 1 and ACE. Moreover, the coverage (100%) and Shannon diversity index (0.38) indicated sufficient sequencing.

It should be mentioned that the diverse bacteria and methanogens associated with the original coal and mine water were not predominant after anaerobic enrichments. In the methanogenic community after enrichments, the emerging dominants involved in the conversion of coal to methane were identified. The identified microbes from coal cultivations supplemented with acetate-enriched microbial consortium are listed in Table 5. Bacteria were

classified into *Firmicutes* and *Deltaproteobacteria*. All the bacteria of *Firmicutes* belong to the order of *Clostridiales*, which included the genera of *Desulfosporosinus*, *Clostridium*, *Desulfotomaculum*, *Oscillibacter*, *Sporotomaculum*, and *Sporobacter*. The *Deltaproteobacteria* was affiliated with *Desulfovibrio*. In the anaerobic cultivation, methanogens were sensitive to the incubation methods and medium. For example, the reductants, salts, and temperature may inhibit some methanogens, resulting that some methanogens were enriched, while others cannot survive. Therefore, for archaea, only 2 OTUs affiliated with the species of *Methanosarcina barkeri* and *Methanosarcina horonobensis* (Fig. S2) were detected.

## 4. Discussion

In this study, geochemical and microbiological analyses were performed to evaluate the biogenic methane potential of a thermogenic-gas coal mine. Although the stable carbon isotopic analysis implied that coal mine methane was mainly from a thermogenic origin, 454 pyrosequencing of indigenous microbial communities and enrichment cultivations revealed the presence of various anaerobic fermentative bacteria and methanogens in the coal seam. The thermogenic methane could be a remainder of the geological processes in the coal mine, and the formation of methane in microbial enrichments indicates that there is a potential for biogenic methane from coal under certain conditions: the suitable temperature and nutrition, also including anaerobic conditions, contact of coal with mine water, etc.

### 4.1 Thermogenic gas determined based on stable carbon composition

The analysis of stable carbon composition indicated that the methane in the coal mine was mainly thermogenic based on values of $\delta^{13}C_{CH4}$, $\delta^{13}C_{CO2}$, $\Delta^{13}C_{CO2\text{-}CH4}$, and $R_{o\ max}$ of coal. The $\delta^{13}C_{CH4}$ ranged from -20.97‰ to -21.47‰, which was within the theoretically anticipated thermogenic methane range of -50‰ - -20‰ (Whiticar, 1999). For the $\delta^{13}C_{CO2}$, the values ranged from -15.47‰ to -14.74‰ and was also considered a thermogenic gas characteristic because it was within the theoretical range of -30‰ - -10‰ for a thermogenic origin (Scott et al., 1994). Similarly, the isotopic fractionation between $CO_2$ and $CH_4$ ($\Delta^{13}C_{CO2\text{-}CH4}$) varied from 6.07‰ to 6.51‰ and exhibited obvious thermogenic methane characteristics. For example, the isotopic fractionation ranging from 3‰ to 15‰ supports the contention that thermogenic methane exists in the coal bed methane reservoir of the Qinshui Basin in China (Strąpoć et al., 2006). Furthermore, the methane from the present high rank coal with the $R_{o\ max}$ value of 3.0 ± 0.17 was typically classified as thermogenic gas (Song et al., 2012). The thermogenic methane would be released and exhausted with the accompanying coal mining activities.

### 4.2 Microbial community of original samples in the coal seam

The indigenous microbial communities included fermentative bacteria and methanogens were identified using 454 pyrosequencing. This is a significant and interesting result because the stable carbon composition analysis indicated that the methane was produced with a thermogenic origin in the coal mine. In coal samples, the abundant bacteria

distributed among the phyla of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were reported to be involved in coal degradation and fueling methanogens to generate methane (Strąpoć et al., 2011) (Fig. 5). *Chloroflexi* are often observed in coal samples, which are typically associated with coal decomposition (Li et al., 2014; Watts et al., 2005). At the genus level, *Candidatus Microthrix* and *Tetrasphaera* from *Actinobacteria* are found in the activated sludge or waste water, which have been reported to play an important role in the degradation of organic compounds (Hanada, 2002; Nielsen et al., 2002). *Flavobacterium* belonging to *Bacteroidetes* has been described as a degrader of pentachlorophenol (Saber & Crawford, 1985). *Desulfosporosinus* from *Firmicutes* is a moderately acidophilic sulfate-reducing bacterium that can be isolated from acid mining drainage (Alazard et al., 2010). The predominant *Proteobacteria* included the genera of *Pseudomonas*, *Rheinheimera*, *Comamonas*, *Dechloromonas*, *Acidovorax*, *Ferribacterium*, and *Propionivibrio*. *Pseudomonas*, the most abundant *Proteobacteria*, has been detected in coal seams in western Canada (Penner et al., 2010) and the Ordos Basin of China (Tang et al., 2012) and is a hydrocarbon-degrading bacterium (Ma et al., 2006). Some stains can produce surfactants, which could help release organics from coal (Singh & Tripathi, 2013). *Rheinheimera*, which is associated with oil degradation, has been observed in a petroleum reservoir (Wang et al., 2008). *Comamonas* and *Dechloromonas* have been detected in coal bed reservoirs in India and are associated with the nitrate cycle and polyaromatic hydrocarbon degradation (Singh et al., 2012). *Acidovorax* has also been identified as a denitrifying bacteria involved in nitrate-reduction (Heylen et al., 2008). *Ferribacterium* isolated from mining-impacted

freshwater lake sediment is capable of oxidating acetate and other organic acids together with the reduction of ferric iron (Cummings et al., 1999). *Propionivibrio* is described as an anaerobic fermentative bacteria that can degrade hydroaromatic compounds (Brune et al., 2002). The activities of the aforementioned bacteria might be related to methanogenesis from coal. It should be emphasized that most of abundant bacteria detected in coal were aerobic, *e.g*. *Tetrasphaera*, *Flavobacterium*, *Pseudomonas*, *Rheinheimera*, *Comamonas*, *Dechloromonas*, *Acidovorax*, and *Ferribacterium*. These organisms would not survive under strictly anaerobic environment. The anaerobic bacteria such as *Desulfosporosinus* accounted a small proportion in coal. However, such bacteria might be enriched during anaerobic cultivation.

Based on the methanogen DNA, the predominant methanogens identified in coal were *Methanosaeta*, which was the acetotrophic methanogens. In addition, the hydrogenotrophic *Methanobacterium*, *Methanobrevibacter*, *Methanoregula*, *Methanospirillum*, and *Methanosphaerula*, and the methylotrophic *Methanosphaera* were also identified. The presence of methanogen DNA indicates the methanogenic potential via a variety of pathways under the right conditions, *e.g.* suitable temperature, anaerobic conditions.

In mine water, the abundant bacterial genera were distributed in the phyla of *Bacteroidetes* and *Proteobacteria* (Fig. 5). These two phyla are widely detected in anaerobic environments and may play an important role in fermentation, hydrogen, and sulfur metabolism (Wrighton et al., 2012), which were associated with the methanogenesis. The aerobic bacteria identified in mine water including *Flavobacterium*, *Hydrogenophaga*,

*Methylosoma*, *Polaromonas* and *Pseudomonas*. These bacteria may be dead in anaerobic conditions. *Flavobacterium* belonging to the *Bacteroidetes* was involved in anaerobic degradation of polyaromatic hydrocarbons (Rahman et al., 2002). The dominant *Proteobacteria* included the genera of *Hydrogenophaga*, *Methylosoma*, *Polaromonas*, *Pseudomonas*, and *Desulfobacca*. *Hydrogenophaga* was the predominant genus in mine water, and it has been detected in abandoned coal mines (Beckmann et al., 2011b) and is capable of utilizing biphenyl (Lambo & Patel, 2006). *Methylosoma* is a methanotroph that was previously detected in alkaline soil from a Chinese coal mine (Han et al., 2009). The methanogenic and methanotrophic pathways may be present in mine water of the coal mine, which involved in the carbon metabolism. *Polaromonas* has been identified in naphthalene-contaminated sediment and has the ability to degrade naphthalene (Jeon et al., 2004). *Desulfobacca* (Göker et al., 2011), the anaerobic bacterium from the *Syntrophaceae*, has been observed in sludge and is known to be a sulfate-reducing. Although *Desulfobacca* might compete with methanogens, *Desulfobacca* associated with sulfate metabolic and decrease the oxidation reduction potential. The low oxidation reduction potential was a favorable factor for the survival of methanogens. For the archaea identified in the mine water, a high proportion of *Halobacteriaceae* was observed, which may be a result of the high salinity of the water (Fig. 6). *Methanomicrobia* and *Methanobacteria* were also observed in mine water. However, the *Halobacteriaceae* would not survive during anaerobic conditions and methanogens would be predominant. The presence of diverse methanogens would benefit methane generation in the coal mine.

### 4.3 Microorganisms associated with methane formation after enrichments

Although there was a relative abundance of diverse organisms in the original coal and mine water, they were inactive after anaerobic cultivation. In the anaerobic methanogenic consortium, only a small subpopulation (including bacteria and methanogens) was selected and became dominant due to the selective pressures including the medium, temperature, and pH conditions (Faiz & Hendry, 2006). The enriched organisms may be involved in the conversion of coal to methane. In the microbial consortium of the enrichments, *Clostridia* were the dominant bacteria from the phylum of *Firmicutes* (Table 5) and contained a lineage of anaerobic fermentative bacteria, which could produce various organic acids and degrade aromatic compounds (Brenner et al., 2005; Lee et al., 2008). *Desulfosporosinus* was the most abundant bacteria in *Clostridia* and was detected in the original coal and mine water samples. It is a sulfate-reducing bacterium that can reduce sulfate to sulfide and incompletely oxidize organic substrates such as lactate, pyruvate, and ethanol to acetate (Alazard et al., 2010). *Desulfotomaculum* from *Clostridia* was a sulfate-reducing bacterium that had also been detected in the original water sample. It accounted for only 0.07% of the population and can incompletely oxidize pyruvate and butyrate to acetate and CO (Fardeau et al., 1995). *Clostridium clariflavum*, *Clostridium thermocellum*, and *Clostridium hydroxybenzoicum* are anaerobic fermentative bacteria that can produce $H_2$, acetate, ethanol, lactate, etc. (Islam et al., 2006; Shiratori et al., 2009; Zhang et al., 1994). *Oscillibacter* and *Sporobacter* are routinely found in the animal alimentary canal, anaerobic sewage sludge, and anaerobic digesters. They can degrade macromolecules, organic acids, or methoxylated aromatic compounds to simple

sugars, acids, or alcohols, such as ammonia, valerate, propionate, and acetate (Grech-Mora et al., 1996; Iino et al., 2007). The identified genus of *Sporotomaculum* contains many syntrophic bacteria. *Sporotomaculum syntrophicum* is a syntrophic benzoate-degrading bacterium isolated from methanogenic sludge (Qiu et al., 2003). The *Desulfovibrio* from *Deltaproteobacteria* is a sulfate-reducing bacterium and it is known to be syntrophic with *Methanosarcina* (McInerney & Bryant, 1981). *Desulfovibrio carbinoliphilus* is a benzyl alcohol-oxidizing bacterium and can oxidize lactate to produce acetate (Allen et al., 2008).

Of the methanogens identified in this study, the closest species were affiliated with *Methanosarcina barkeri* and *Methanosarcina horonobensis* (Table 5). *Methanosarcina* is a facultative acetoclastic methanogen that can utilize a wide variety of substrates, including acetate, hydrogen, and carbon dioxide. Interestingly, the *Methanosarcina* identified in the present study were seemingly better adapted for the presence of acetate. In accordance with previous studies, *Methanosarcina* is able to utilize acetate to generate methane (Joulian et al., 1998; Shimizu et al., 2011). Moreover, it has been detected in other coal seams such as abandoned coal mines in Germany (Beckmann et al., 2011b; Krüger et al., 2008) and coal bed methane reservoir in the Powder River Basin (Green et al., 2008).

Previous studies have demonstrated that the *Firmicutes* and *Deltaproteobacteria* include numerous syntrophic bacteria, *e.g. Desulfotomaculum*, *Clostridium*, and *Desulfovibrio*, which are able to degrade organic matter to acetate and grow in syntrophic association with methanogens (Hattori, 2008; Midgley et al., 2010). In the methanogenic consortium, the syntrophic bacteria could initiate the decomposition of coal and produce soluble

intermediates that could be utilized by methanogens. In a recent study of enrichments of coal samples, the methanogenic consortium of coal samples was shown to use volatile matter in coal to produce methane (Xiao et al., 2013). In the present study, acetate may be one of the intermediates decomposed from coal (Fig. 3C). Acetate accumulated in early cultivation stage of coal with enriched microorganisms, then decreased with a concomitant increase in methane, indicating that acetate is an important intermediate. In comparison to the controls of cultivation with coal and BES, the acetate was accumulated and not consumed by methanogens since BES can inhibit the activity of methanogens. For the negative control of sterile water, coal was not added and the yield of acetate was far lower than the cultivation with coal. Therefore, the most part of acetate in the cultivation was provided by coal through bacterial anaerobic fermentation. Methane generation was related to acetate production and consumption. With the consumption of acetate, the production of methane increased and then remained stable. However, more tracking studies will need to be carried out in subsequent studies, *e.g.* isotopic methods will be used to detect intermediate products and verify the methanogenic pathway that is utilized during coal degradation.

**4.4 Analysis of biogenic methane potential of the coal mine**

The anaerobic enrichments in the laboratory indicated that biogenic methane could be produced from water and coal samples. Based on the DNA analysis, the coal mine would present biogenic methane potential, if the environment was modified to promote the anaerobic pathway of converting a fraction of the coal to methane. The tested mine water was

alkaline and had a low DO value. The strictly anaerobic condition could be further formed by geological activity or human interference, which was a favorable factor for the growth of anaerobic microorganisms. The available organic materials from TOC in water also supported bacterial growth. It should be mentioned that there was high sulfate in mine water. The sulfate-reducing bacteria and methanogens identified in the environment were competitive relationship. The substrate competition will occur during these organisms. In a closed environment, the sulfate-reducing bacteria initially activated and consumed the most of the sulfate combined with the decrease of oxidation reduction potential. Methanogens could coexistence with sulfate-reducing bacteria. The low oxidation reduction potential was available for methanogens and would promote methanogenesis.

A comparison of original coal, mine water, and the enrichments samples indicated that there is an overlap of the microbial composition, which suggests that this thermogenic-gas coal mine has methane generation potential (See Fig. 7). In the enrichment samples, it was worth noting that the microorganisms associated with methane generation were also present in the original coal and mine water samples, *e.g. Clostridium*, *Desulfosporosinus*, *Desulfotomaculum*, *Sporotomaculum*, *Desulfovibrio*, and *Methanosarcina*. The shared microorganisms of the original coal and water samples also supported methane generation, including the methanogens and the bacteria that contributed to coal biodegradation. Similar methanogenic consortia, such as *Clostridium*, *Pseudomonas*, *Desulfovibrio*, *Desulfotomaculum*, *Methanosarcina*, and *Methanossaeta*, have also been detected in other coal seams (Beckmann et al., 2011a; Green et al., 2008; Kimura et al., 2010).

In addition, the coal in the present study was identified as anthracite and was characterized by highly aromatic structures. Its complex composition and compact internal structure may be obstacles for microbial degradation (Jones et al., 2008; Papendick et al., 2011). In this study, we showed that even a high rank coal could be converted to biogenic methane under the right conditions. The present results were consistent with a previous study on biogenic methane from nonproductive coal samples (Jones et al., 2010), where biogenic methane could be generated by the addition of nutrients or a microbial consortium. Moreover, the field tests have revealed that the introduction of acetate into coal bed may active the *in situ* microbial community and lead to new methane formation (Ulrich & Bower, 2008). It should be mentioned that the introduction of microbial consortia or nutrients could happen naturally. The groundwater recharging events or meteoric water infiltration could introduce nutrients and microbes into the coal seam, played a significant role in methane generation (Flores et al., 2008; Rice et al., 2008). Therefore, although thermogenic methane was predominant in the present coal mine, the methanogens and other associated bacteria could be introduced and activated, resulting in the generation of biogenic methane by coal bioconversion over time.

## 5. Conclusion

To the best of our knowledge, this is the first report of the microbial analysis regarding the biogenic methane potential of a thermogenic-gas coal mine. The results indicated that: (1) a high level of diversity of syntrophic bacteria and methanogenic archaea existed in the coal

seam of the coal mine; (2) biogenic methane regeneration from coal could be realized by the addition of an enriched indigenous microbial consortia; (3) acetotrophic methanogenesis was predominant based on enrichments and clone library analysis; and (4) The potential of biogenic methane from coal may occur when the *in situ* environment was modified to promote the anaerobic pathway, *e.g.* anaerobic condition and available organic carbon. These results suggest that the potential for biogenic methane production from high rank coal might exist in the coal mine.

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**Tables**

Table 1 Concentration and stable carbon isotope analyses of methane and carbon dioxide in the high gas content mine

| | Mine face 1 | Mine face 2 | Mine face 3 |
|---|---|---|---|
| $CH_4$ (v/v %) | 0.75 ± 0.12 | 0.93 ± 0.17 | 0.82 ± 0.09 |
| $\delta^{13}CH_4$ (‰ PDVB) | -20.97±1.28 | -21.47±1.32 | -21.25±1.07 |
| $CO_2$ (v/v %) | 0.15±0.08 | 0.14±0.06 | 0.17 ±0.04 |
| $\delta^{13}CO_2$ (‰ VPDB) | -14.90±1.02 | -15.06±1.47 | -14.74±1.25 |
| $\Delta^{13}C_{(CO2\text{-}CH4)}$ | 6.07±2.30 | 6.41±2.79 | 6.51±2.32 |

Table 2 Properties of coal samples in the gassy coal mine

| Coal properties | | Yield % |
|---|---|---|
| Proximate analyses (air dried) % | | |
| | Moisture | 2.4 |
| | Ash | 7.3 |
| | Volatile matter | 8.5 |
| | Fixed carbon | 81.8 |
| Ultimate analysis vol. (dry ash free)% | | |
| | Carbon | 91.9 |
| | Hydrogen | 3.6 |
| | Nitrogen | 1.3 |
| | Sulfur | 1.8 |
| | Oxygen | 1.4 |
| Petrographic analysis vol.% | | |
| | Vitrinite | 62.4 |
| | Liptinite | 19.7 |
| | Inertinite | - |
| | Mineral matter | 17.9 |
| Reflectance ($R_o$, max)% | | 3.0±0.17 |
| Classification | | Anthracite |

Table 3 Properties of mine water in the gassy coal mine

| Water properties | Content |
|---|---|
| Temperature | 27$^{o}$C |
| pH | 8.23 |
| $SO_4^{2-}$ (mg/L) | 3740 |
| $Cl^{-}$ ( mg/L ) | 9.48 |
| $NO^{3-}$ ( mg/L ) | - |
| $NO^{2-}$ ( mg/L ) | - |
| $NH^{4+}$ ( mg/L ) | 2.48 |
| $Na^{+}$ ( mg/L) | 860 |
| $K^{+}$ ( mg/L) | 9.68 |
| $Ca^{2+}$ ( mg/L ) | 118 |
| $Mg^{2+}$ ( mg/L ) | 41.8 |
| DO (mg/L) | 1.21 |
| Conductivity (us/cm) | 6980 |
| TOC (mg/L) | 1.6 |
| TDS (mg/L) | 5560 |
| DOC (mg/L) | 0.7 |
| DIC (mg/L) | 282 |

The detection limit for $NO^{3-}$, $NO^{2-}$, TOC, and DOC was 0.01, 0.01, 0.5, and 0.5, respectively.

Table 4 The identified genera in coal and mine water samples by 454 pyrosequencing.

| Genus | sample | | Genus | sample | | Genus | sample | |
|---|---|---|---|---|---|---|---|---|
| | Coal (%) | Water (%) | | Coal (%) | Water (%) | | Coal (%) | Water (%) |
| **Archaea** | | | | | | | | |
| *Methanobacterium* | 1.61 | 2.98 | *Methanoregula* | 0.85 | 0.11 | *Methanosphaerula* | 0.14 | 0.13 |
| *Methanobrevibacter* | 14.8 | 0.01 | *Methanosaeta* | 39.24 | 0.95 | *Methanospirillum* | 0.63 | 0.05 |
| *Methanolobus* | 0 | 1 | *Methanosarcina* | 0 | 2.17 | uncultured *Halobacteria* | 5.08 | 74.73 |
| *Methanomethylovorans* | 0 | 0.16 | *Methanosphaera* | 0.88 | 0 | | | |
| **Bacteria** | | | | | | | | |
| *Achromobacter* | 0.15 | 0 | *Desulfovibrio* | 0.01 | 0.1 | *Nocardioides* | 0.79 | 0.05 |
| *Acidovorax* | 2.01 | 0.22 | *Devosia* | 0.09 | 0.2 | *Bdellovibrio* | 1.05 | 0 |
| *Acinetobacter* | 0.79 | 0.19 | *Diaphorobacter* | 0.17 | 0 | *Ottowia* | 0.29 | 0.01 |
| *Actinobaculum* | 0.3 | 0 | *Elusimicrobium* | 0 | 0.21 | *Paracoccus* | 0.31 | 0.23 |
| *Aeromonas* | 0.28 | 0.15 | *Erysipelothrix* | 0.02 | 0.14 | *Phenylobacterium* | 0.24 | 0.05 |
| *Anaerolinea* | 0.32 | 0.28 | *Exiguobacterium* | 0.05 | 0.31 | *Planctomyces* | 0.32 | 0.04 |
| *Aquabacterium* | 0.74 | 0.06 | *Ferribacterium* | 5.56 | 0.05 | *Planomicrobium* | 0.13 | 0.15 |
| *Aquimonas* | 0.01 | 0.23 | *Ferruginibacter* | 0.69 | 0 | *Polaromonas* | 0 | 2.11 |
| *Arcobacter* | 0.36 | 0.14 | *Flavobacterium* | 0.45 | 2.97 | *Propionibacterium* | 0.13 | 0 |
| *Arthrobacter* | 0.06 | 0.14 | *Flexibacter* | 0.34 | 0.01 | *Propionivibrio* | 2.13 | 0 |
| *Azoarcus* | 0 | 0.1 | *Fodinibacter* | 0.29 | 0 | *Pseudomonas* | 16.52 | 2.05 |
| *Azonexus* | 0 | 0.12 | *Fodinicola* | 0.46 | 0 | *Pseudoxanthobacter* | 0.16 | 0.13 |
| *Brevundimonas* | 0.23 | 0.19 | *Gemmatimonas* | 0.13 | 0 | *Ralstonia* | 0.19 | 0.04 |
| *Burkholderia* | 0.1 | 0.31 | *Geothermobacter* | 0 | 0.73 | *Rheinheimera* | 14.57 | 0.02 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Caldilinea* | 0.69 | 0.01 | *Hydrogenophaga* | 0.71 | 21 | *Rhizobium* | 0.41 | 0.26 |
| *Candidatus Microthrix* | 8.07 | 0 | *Jiangella* | 0.43 | 0 | *Rhodobacter* | 0.35 | 0.31 |
| *Candidatus Captivus* | 0 | 0.45 | *Lachnospiraceae* | 0.22 | 0.17 | *Rhodocyclus* | 0.17 | 1.01 |
| *Cellvibrio* | 0.02 | 0.27 | *Legionella* | 0.04 | 1.2 | *Saprospira* | 0.57 | 0.01 |
| *Chitinimonas* | 0.72 | 0 | *Limnobacter* | 0.01 | 0.79 | *Sediminibacterium* | 0.46 | 0 |
| *Chitinophagaceae* | 0.3 | 0.01 | *Methylobacter* | 0 | 0.24 | *Simplicispira* | 0.06 | 0.67 |
| *Chryseobacterium* | 0.1 | 0.01 | *Methylobacterium* | 0.3 | 0 | *Sporotomaculum* | 0 | 0.05 |
| *Clostridium* | 0.84 | 0.28 | *Methylophaga* | 0 | 0.11 | *Tetrasphaera* | 2.55 | 0 |
| *Comamonas* | 1.44 | 0.03 | *Methylophilus* | 0.33 | 0.86 | *Thauera* | 0.69 | 0.01 |
| *Coxiella* | 0 | 0.18 | *Methylosoma* | 0.03 | 4.77 | *Thiobacillus* | 0.01 | 0.18 |
| *Dechloromonas* | 2.49 | 0.01 | *Methylotenera* | 0.02 | 0.86 | *Trichococcus* | 0.83 | 0.08 |
| *Delftia* | 0.23 | 0.33 | *Methyloversatilis* | 0.64 | 0.15 | *Undibacterium* | 0.67 | 0 |
| *Desulfobacca* | 0 | 1.17 | *Microbacterium* | 0.38 | 0.04 | *Zoogloea* | 0.57 | 0 |
| *Desulfosporosinus* | 2.45 | 0.13 | *Nannocystis* | 0.44 | 0 | | | |
| *Desulfotomaculum* | 0 | 0.07 | *Nitrosomonas* | 0.37 | 0.03 | | | |

Genera with proportion more than 0.1% are shown.

Table 5 Archaeal and bacterial 16S rRNA gene sequences from coal cultivations supplemented with an enriched microbial consortium

| OUT | GenBank accession no. | No. of clones | Phylogenetic group | Closest cultivated species | Identity (%) |
|---|---|---|---|---|---|
| **Archaea** | | **31** | | | |
| OTU1 | KC215420 | 25 | *Methanosarcinales* | *Methanosarcina barkeri* | 99 |
| OTU2 | KC215421 | 6 | *Methanosarcinales* | *Methanosarcina horonobensis* | 99 |
| **Bacteria** | | **41** | | | |
| OTU1 | KC215425 | 21 | *Clostridiales* | *Desulfosporosinus acidophilus* | 98 |
| OTU2 | KC215426 | 3 | *Clostridiales* | *Clostridium thermocellum* | 89 |
| OTU3 | KC215427 | 2 | *Clostridiales* | *Desulfosporosinus acidiphilus* | 100 |
| OTU4 | KC215428 | 4 | *Clostridiales* | *Clostridium clariflavum* | 97 |
| OTU5 | KC215429 | 2 | *Desulfovibrionales* | *Desulfovibrio carbinoliphilus* | 97 |
| OTU6 | KC215430 | 1 | *Clostridiales* | *Desulfotomaculum thermosapovorans* | 93 |
| OTU7 | KC215431 | 1 | *Clostridiales* | *Sporotomaculum syntrophicum* | 92 |
| OTU8 | KC215432 | 1 | *Clostridiales* | *Desulfotomaculum thermosapovorans* | 95 |
| OTU9 | KC215433 | 2 | *Clostridiales* | *Oscillibacter valericigenes* | 98 |
| OTU10 | KC215434 | 1 | *Clostridiales* | *Clostridium* sp. | 92 |
| OTU11 | KC215435 | 1 | *Clostridiales* | *Desulfosporosinus orientis* | 98 |
| OTU12 | KC215436 | 1 | *Clostridiales* | *Sporobacter termitidis* | 93 |
| OTU13 | KC215437 | 1 | *Clostridiales* | *Clostridium hydroxybenzoicum* | 94 |

OTUs were defined at 97% sequence similarity.

**Figure Legends**

**Fig. 1** Location of the thermogenic gas mine used for sample collection.

**Fig. 2** The stratigraphic column of the Jiuligang Formation in Jingmen-Dangyang basin. Coal and fluvial channel sandstone beds are in the Upper Triassic, Jiuligang Formation shown in the figure, modified from Chen et al. (Chen et al., 2011).

**Fig. 3** Methane generation from original coal and water samples (Fig. 3A). Methane (Fig. 3B) and acetate (Fig. 3C) formation from coal samples supplemented with the enriched native microbial consortium at 35°C. Methane and acetate that accumulated in the headspaces were measured in triplicate. Error bars represent the standard deviation.

**Fig. 4** Microbial concentrations in original and enriched samples were determined by real-time PCR. The enriched samples refer to the coal samples supplemented with the enriched native microbial consortium. Each DNA template was performed in triplicate. Error bars represent the standard deviation.

**Fig. 5** Diversity of bacterial 16S rDNA identified at the phylum level by 454 pyrosequencing. Abundance of sequences of less than 1% was classified as “others”.

**Fig. 6** Diversity of archaeal 16S rDNA identified at the class level by 454 pyrosequencing.

**Fig. 7** Venn diagram groupings of representative bacterial and archaeal genera (based on 97% sequence similarity) shared between the original and enrichment samples.

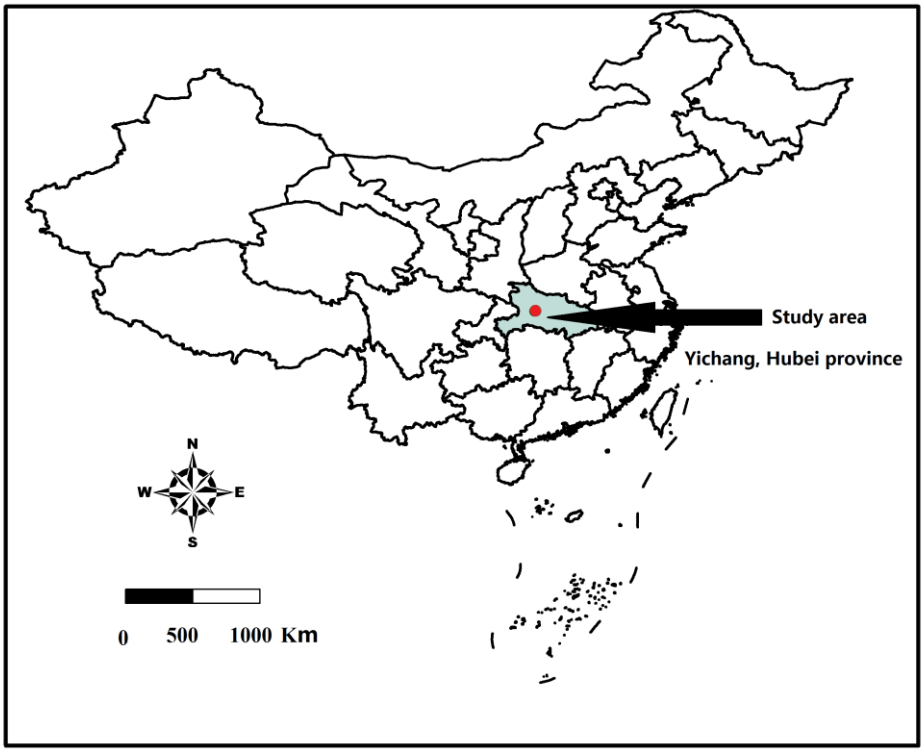



Figure 1

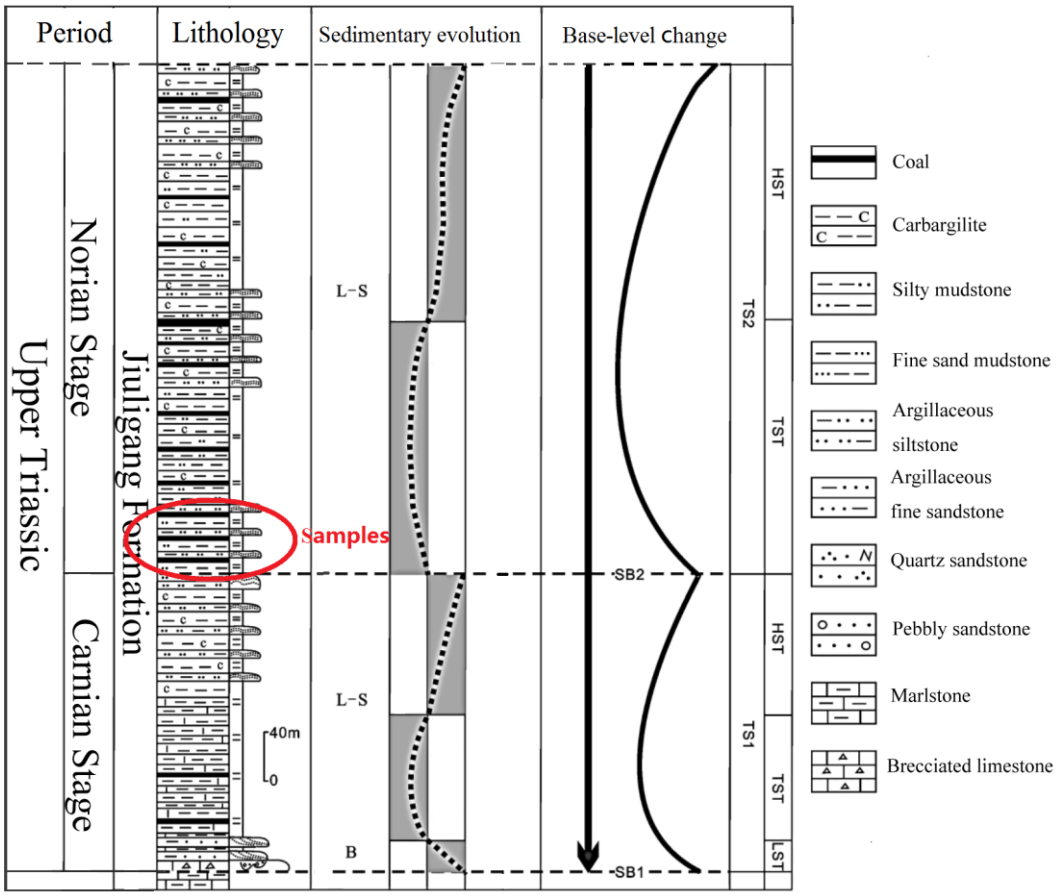



Figure 2

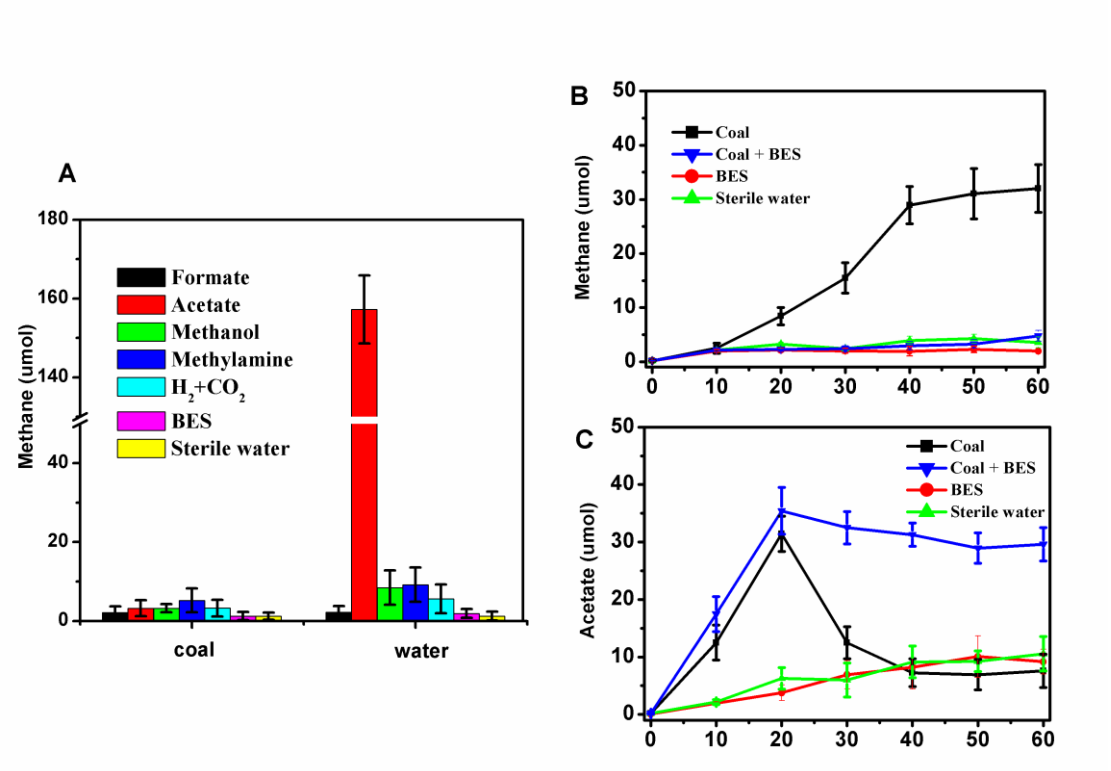



Figure 3

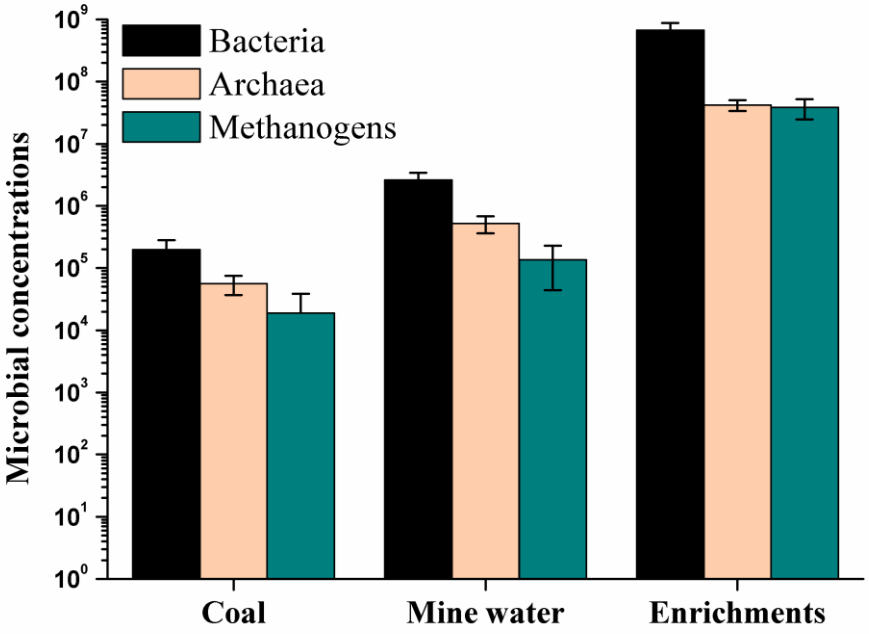



Figure 4

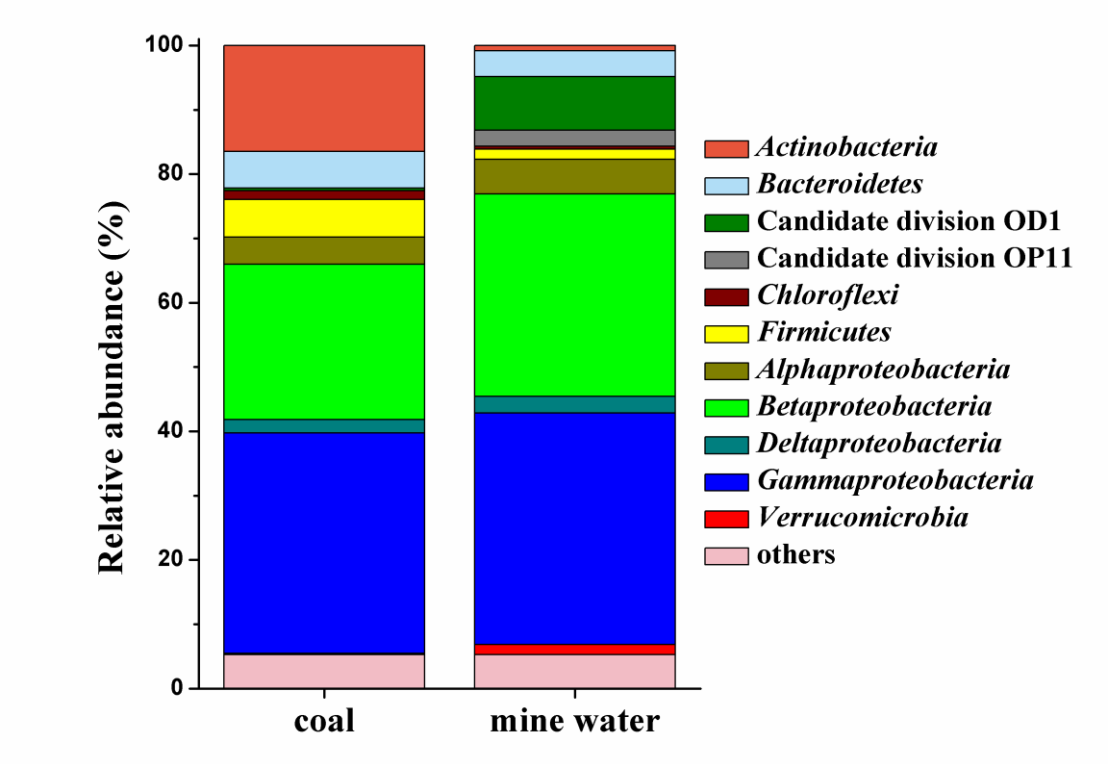



Figure 5

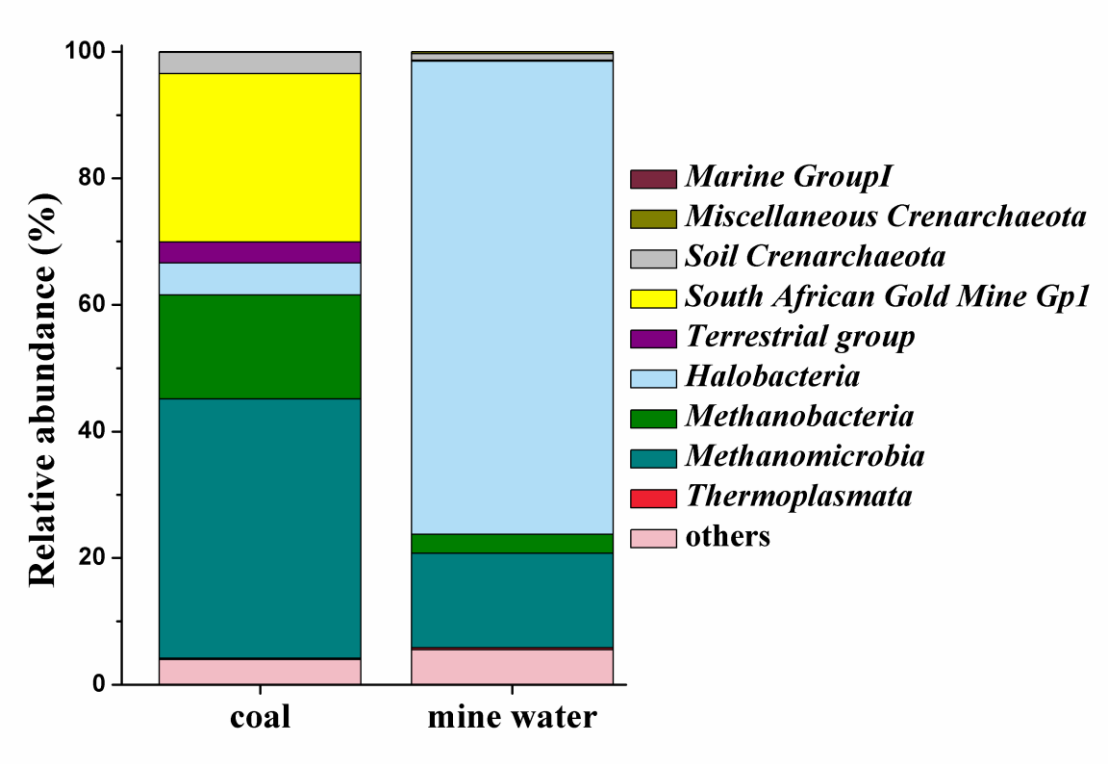



Figure 6

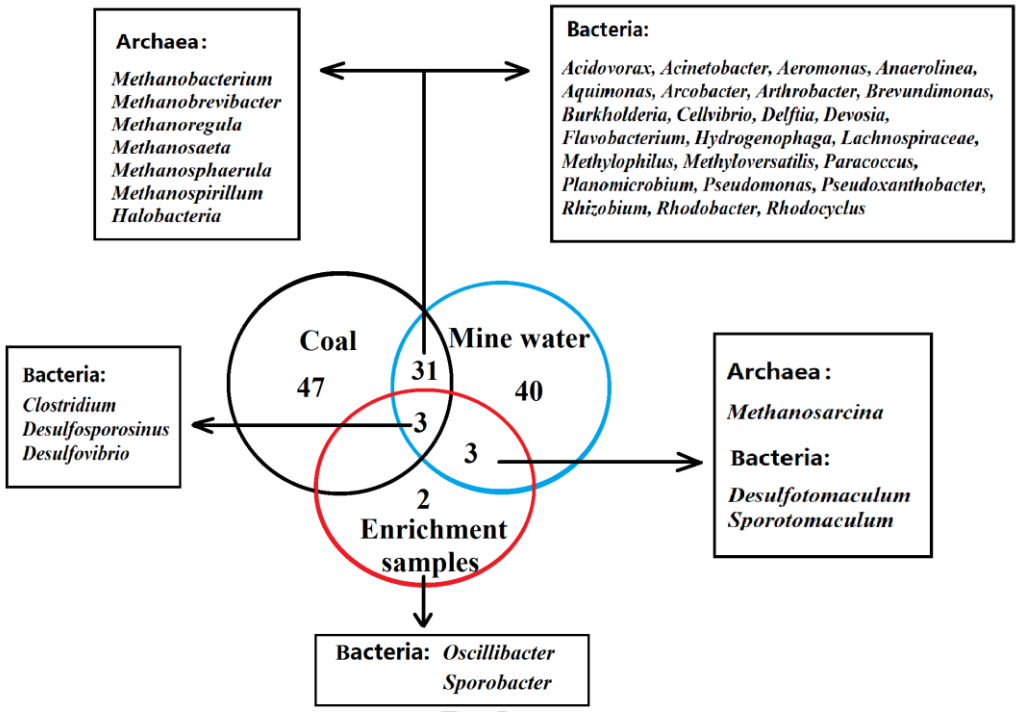
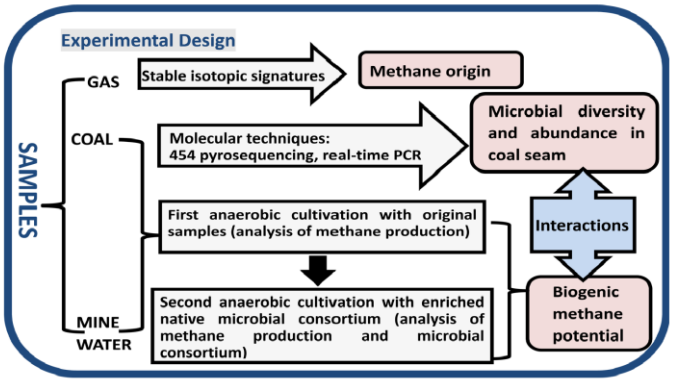
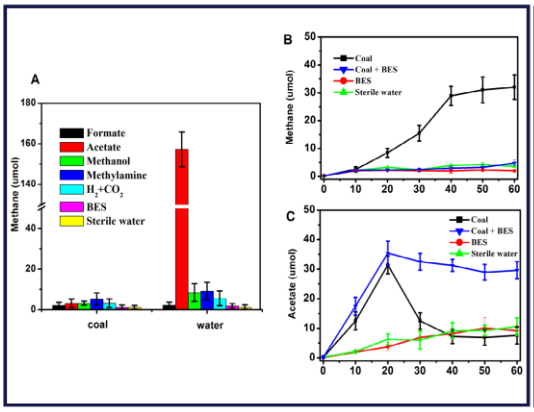


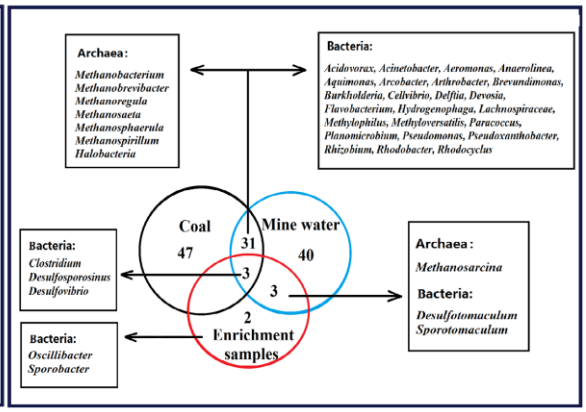

Figure 7




**Experimental design**




**Methane production**




**Microbial communities**

Graphical abstract

**Highlights**

1. There existed high diversity of methanogenic archaea and numerous bacteria capable of degrading complex organics in the thermogenic-gas coal mine.
2. Acetotrophic methanogenesis was predominant in this coal mine.
3. Biogenic methane formation from coal could be realized by adding enriched native microbial consortia.