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Microbial communities responsible for fixation of CO_2 revealed by using *mcrA, cbbM, cbbL, fthfs, fefe-hydrogenase* genes as molecular biomarkers in petroleum reservoirs of different temperatures

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Abstract

Sequestration of CO_2 in oil reservoir is one of the feasible options for mitigating atmospheric CO_2 building up. The in situ bioconversion of sequestrated CO_2 to methane by microorganisms inhabiting oil reservoirs is feasible. To evaluate the potential of in situ microbial fixation and conversion of CO_2 into CH_4 in oil reservoirs, a comprehensive molecular survey was performed to reveal microbial communities inhabiting four oil reservoirs with different temperatures by analysis of functional genes involved in the biochemical pathways of CO_2 fixation and CH_4 synthesis (*cbbM*, *cbbL*, *fthfs, [FeFe]-hydrogenase* encoding gene, and *mcrA*). A rich diversity of these functional genes was found in all the samples with both high and low temperatures and they were affiliated to members of the *Proteobacteria* (*cbbL* and *cbbM*, *fthfs*), *Firmicutes* and *Actinobacteria* (*fthfs*), uncultured bacteria (*[FeFe]*-hydrogenase), and *Methanomirobiales*, *Methanobacteriales* and *Methanosarcinales* (*mcrA*). The predominant methanogens were all identified to be hydrogenotrophic CO_2-reducing physiological types. These results showed that functional microbial communities capable of microbial fixation and bioconversion of CO_2 into methane inhabit widely in oil reservoirs, which is helpful to microbial recycling of sequestrated CO_2 to further new energy in oil reservoirs.

1 Introduction

Storage of CO_2 in deep geological formations, such as oil reservoirs, is one of the feasible solutions to reduce CO_2 emissions and further build up in the atmosphere. It is of great scientific interest and significance to understand the fate of CO_2 in the subsurface environment, which has received increasing attention due to the fact that abundant microorganisms inhabit these formations, and microbial fixation and conversion of the sequestered CO_2 into CH_4 are highly feasible.

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As to the microbial involvement in CO_2 fixation, six autotrophic CO_2 fixation biochemical pathways have been documented, of which the Calvin–Benson–Bassham (CBB) cycle plays an important role in autotrophic CO_2 fixation in nature (Berg, 2011). The CBB cycle was known to occur in *Proteobacteria*, in some members of *Firmicutes*, *Actinobacteria* and *Chloroflexi* as well as in plants, algae and cyanobacteria (Caldwell et al., 2007; Zakharchuk et al., 2003; Lee et al., 2009; Berg et al., 2005; Ivanovsky et al., 1999). Another important pathway of CO_2 fixation is the reductive acetyl-CoA pathway that has been documented to occur in acetogenic prokaryotes, ammonium-oxidizing *Planctomycetes* (Strous et al., 2006), sulfidogenic bacteria (Schauder et al., 1988), and in autotrophic archaea affiliated with the order *Archaeoglobales* (Vorholt et al., 1997; Vornolt et al., 1995). This pathway is also utilized by acetogenic prokaryotes for energy conservation (Biegel and Muller, 2010; Ragsdale and Pierce, 2008; Thauer et al., 2008). Petroleum reservoirs are known to harbor diverse microorganisms including bacteria such as *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Chloroflexi* and archaea such as methanogens and *Archaeoglobales* (Wang et al., 2011; Magot et al., 2000; Li et al., 2010, 2011; Mbadinga et al., 2012) and they are expected to use effective means for CO_2 fixation and bioconversion.

To investigate whether oil reservoirs have the potential capability of CO_2 fixation and conversion of CO_2 into CH_4, and to have a better knowledge on microorganisms involved in this process, studies from a viewpoint of functional genes are necessary. Functional genes involved in CO_2 fixation and conversion into CH_4 have been shown to be valuable biomarkers for detecting members in the microbial communities in both environments and enrichment cultures. The genes *cbbL* and *cbbM* respectively encoding the key enzymes ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I and II of the CBB cycle for CO_2 fixation have been applied to study microbial communities from hydrothermal vents of the Logatchev field (Hugler et al., 2010). The gene *fthfs* encoding formyltetrahydrofolate synthetase, a key enzyme in the reductive acetyl-CoA pathway, has been used to investigate the diversity of homoacetogenic bacteria in thermophilic and mesophilic

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anaerobic sludge (Ryan et al., 2008). Methyl-coenzyme M reductase (*mcr*) is vital for CH_4 formation, and the α-subunit of MCR (*mcrA* gene) is commonly used for the detection of specific groups of methanogens (Juottonen et al., 2006). In addition, H_2 should be supplied in the process of CO_2 bioconversion into CH_4. However, H_2-producing bacteria are polyphyletic. *[Fe-Fe]*-hydrogenases are known to catalyze H_2 production in fermentative microorganisms. Thus, gene encoding for *[Fe-Fe]*-hydrogenases represent a useful marker gene for the detection of H_2-producing anaerobes (Schmidt et al., 2010).

Microbial fixation of CO_2 and conversion of CO_2 to CH_4 in oil reservoirs is becoming increasingly recognized. Although the potential of in situ microbial conversion of sequestered CO_2 into CH_4 by hydrogenotrophic methanogens has been observed in a laboratory test by Sugai et al. (2012) and some studies on the effects and feasibility of CO_2 injection and storage in a deep saline aquifer was examined (Wandrey et al., 2011), information on the functional microbial communities involved in microbial fixation and conversion of CO_2 into CH_4 in different oil reservoirs is still very limited. The objective of this study was to evaluate the potential of in situ microbial fixation and conversion of CO_2 into CH_4 in subsurface oil reservoirs through analysis of functional genes (*cbbM*, *cbbL*, *fthfs*, *[FeFe]-hydrogenase* encoding gene and *mcrA*) by characterization of the functional microbial communities involved in this process which inhabited in the production waters.

2 Materials and methods

2.1 Sampling sites and production water samples

Production water samples were collected from four oil fields in China with different temperatures, including Kelamayi Oilfield (XJ6, 21 °C; XJ7: 32 °C), Huabei Oilfield (45 °C), and Daqing Oilfield (90 °C). Five liters of production water samples from each production oil well were collected directly from the production valve of the pipeline at

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the well head into sterile bottles after initial flushing for 10–15 min. The sterilized bottles were completely filled with oil/water mixture, tightly sealed and immediately transported back to laboratory for treatment. The physicochemical properties of these production waters from these oilfields are listed in Table 1. Aliquot, after separation oil from the oil/water mixture, was used to concentrate the microbial cells directly through filtration (membrane filter 0.2 µm-pore-size).

2.2 DNA extractions

Microbial biomass in the oil/water samples was concentrated onto membrane filters as described above and previously by Wang et al. (2012). Total genomic DNA was extracted from 2.0 L of production water samples using AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., CA, USA) according to the manufacturer's DNA Miniprep spin protocol after concentration onto membrane filters. The genomic DNAs obtained were purified with a DNA purification kit (U-gene, China) according to the manufacturer's instructions. The extracted DNAs were stored at −20 °C until PCR amplification of functional genes.

2.3 PCR amplifications

Amplifications of the *cbbL* gene fragment (771 bp) and the *cbbM* gene fragment (328 bp) were carried out under the conditions according to Campbell et al. (2004). For amplification of a portion (1102 bp) of the *fthfs* gene, the PCR conditions used were those described previously by Leaphart and Lovelle (2001). For amplification of a fragment (620 bp) of *[Fe-Fe]*-hydrogenase encoding gene, the primer set HydH1f/HydH3r was applied using the conditions described by Schmidt et al. (2010). A fragment (470 bp) of the *mcrA* genes was amplified using the primer set MLf/MLr (Luton et al., 2002), applying the conditions as reported previously (Galand et al., 2005). The primer information used for PCR is summarized in Table 2. Functional gene fragments were all amplified in five parallel PCR reactions in a Peltier thermal cycler

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(Bio-Rad, USA), which was subsequently pooled for cloning exercises and construction of the respective gene libraries.

2.4 Construction of functional genes clone libraries

The amplified and pooled PCR products were gel-purified using the Gel Extraction Kit (U-gene, China) and then cloned into *Escherichia coli* using a pMD19®-T simple vector kit (Takara, Japan) following the instructions of the manufacturer. For each gene clone library, the white colonies obtained were randomly picked and cultured overnight at 37 °C in 0.8 mL Luria broth (LB) medium supplemented with ampicillin (50 $mg\,mL^{-1}$). The inserted DNAs were amplified by using M13-47 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and RV-M (5'-GAGCGGATAACAATTTCACA CAGG-3') primers targeting the flanking vector sequence, followed by agarose gel electrophoresis with ethidium bromide staining (Guan et al., 2013).

2.5 Sequencing and phylogenetic analyses

Sequencing was performed with an ABI 377 automated sequencer. After sequencing, reads were first trimmed for vector before subsequent analyses. Bellerophon was used to check for putative chimeric sequences (Huber et al., 2004). Sequences with more than 97 % similarity were assembled into the same operational taxonomic units (OTUs) using FastGroupII (Yu et al., 2006), and one representative sequence was chosen from each OTU to compare with sequences in the BLAST network service (Altschul et al., 1997). Phylogenetic trees were generated using MEGA5 software (Tamura et al., 2011). The topology of the tree was obtained by the neighbor-joining method (Saitou and Nei, 1987) and 1000 bootstrap replicates were applied to estimate the support for the nodes in the tree.

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2.6 Nucleotide sequence accession numbers

Gene sequences data reported here are available in GenBank sequence database under the accession numbers KF836768-KF836781, KF836820-KF836834, KF836850-KF836886, KF836835-KF836849, and KF836785-KF836819 for *cbbM*, *cbbL*, *mcrA*, *fthfs*, and *[Fe-Fe]*-hydrogenase, respectively.

3 Results

3.1 CO_2 fixation

The *cbbL* gene types were detected positively in all samples from the four oil reservoirs with different temperature as shown in Fig. 1, implying that microorganisms inhabited different oilfields universally and could fix CO_2 through the Calvin cycle. The *cbbL* gene clone libraries from sample XJ6 resulted in two OTUs represented by *cbbL*-XJ6-38 as one (62.9 % of the XJ6 *cbbL* clones) sharing 95 % similarity with *Thiobacillus thioparus* in GenBank, a member of *Beta-Proteobacteria*; the other OTU represented by *cbbL*-XJ6-32 (37.1 % of the XJ6 *cbbL* clones) sharing 93 % similarity with *Allochromatium minutissimum*, a member of *Gamma-Proteobacteria*. The obtained clones in XJ7 sample were divided into 5 OTUs, among which OTUs represented by *cbbL*-XJ7-15, *cbbL*-XJ7-6 and *cbbL*-XJ7-26 (totally 93.3 % of the XJ7 *cbbL* clones) are closely related to *Thiobacillus thioparus* of *Beta-Proteobacteria.* In the three OTUs in HB sample, one OTU represented by *cbbL*-HB-12 (1.4 %) shared 87 % similarity with *Rhodovulum adriaticum* of *Alpha-Proteobacteria*, OTU *cbbL*-HB-9 (55.1 %) and *cbbL*-HB-22 (43.5 %) shared 86 and 85 % similarity, respectively, with *Caenispirillum salinarum*, a member of *Alpha-Proteobacteria*. Five OTUs from DQ sample were divided in the *cbbL* gene clone libraries constructed and the sequence of *cbbL*-DQ-29 (14.3 %) shared high identity with *Acidithiobacillus ferrooxidans* ATCC 53993 of *Gamma-Proteobacteria*. One OTU represented by *cbbL*-DQ-34 (7.1 %)

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showed the highest identity with *Rhodobacter capsulatus* of *Alpha-Proteobacteria*. While, 20 in 28 (71.4 %) *cbbL* gene sequences in *cbbL*-DQ-30 OTU showed 91 % similarity to *Rhodospirillum photometricum* in GenBank of *Alpha-Proteobacteria*. As shown above, all the *cbbL* gene sequences obtained in these four samples related to members within *Proteobacteria*.

Similarly, the *cbbM* gene types were also detected with relatively higher diversity in these four samples as shown in Fig. 2, suggesting that *cbbM* gene could be found universally in microorganisms of oil reservoirs. The 53 clones in XJ6 sample were divided into 3 OTUs in which 38 clone (represented by *cbbM*-XJ6-34, 71.7 %) sequences are closely related to *Rhodovulum sulfidophilum*, a member of *Alpha-Proteobacteria* while 8 clones represented by *cbbM*-XJ6-37 showed highest similarity with *Thiobacillus thiophilus* of *Beta-Proteobacteria*. 7 clones represented by *cbbM*-XJ6-20 shared 90 % similarity with *Acidithiobacillus ferrivorans*, a member of *Gamma-Proteobacteria*. As for XJ7 sample, 6 OTUs were distributed among the whole phylogenetic tree and none of them were closely related to any sequences in GenBank, indicating that they are most likely new members of the uncultured microorganisms. The only OTU represented by *cbbM*-HB-18 was similar to the known sequences of *Rhodopseudomonas palustris*, a member of *Alpha-Proteobacteria*, with 93 % similarity. DQ sample was comprised of 5 OTUs, in which, sequences represented by *cbbM*-DQ-21 (26.7 %) shared similarities with *Rhodopseudomonas palustris* within *Alpha-Proteobacteria*. Also, the OTU represented by *cbbM*-DQ-23 (26.7 %) was related to *Magnetospirillum* and OTU *cbbM*-DQ-27 (13.4 %) to *Phaeospirillum fulvum* within *Alpha-Proteobacteria* with 90 % similarity. The OTU represented by *cbbM*-DQ-22 (20 %) were closely related to *Magnetospira thiophila* (91 %), *Alpha-Proteobacteria*. All these results suggested that most of the *cbbM* gene sequences obtained in these four samples were related to members within *Proteobacteria*.

3.2 Acetogenesis

The *fthfs* gene sequences were also detected in all these four samples and the obtained clones were classified into 5, 3, 3 and 4 OTUs in XJ6, XJ7, DQ and HB samples, respectively (Fig. 3). One OTU (*fthfs*-XJ6-50, 46.2 %) of sample XJ6 was most similar to *Acetobacterium carbinolicum* (99 %), a member of the genus *Acetobacterium* within *Firmicutes*. The OTU represented by *fthfs*-XJ7-2 (68.8 %) shared 84 % similarities with *Blastomonas* sp., *Alpha-Proteobacteria*. While sequences of other clones screened in XJ7 sample shared low similarity with the known sequences in GenBank. The sequenced clones obtained in DQ sample were assembled into 3 OTUs with more closely phylogenetic relatedness, all of which were closely related to *Hyphomonas neptunium* ATCC 15444 of *Alpha-Proteobacteria*. The *fthfs*-HB-24, 73.3 % of the clones retrieved from HB sample, shared less similarity to the known sequences in GenBank. It is reasonable to presume that they are new members of uncultured microorganism. These results suggested that *fthfs* sequences obtained in oil field samples are closely related to sequences from members of *Firmicutes, Proteobacteria* as well as members of uncultured bacteria.

3.3 Hydrogenase encoding gene

The *[FeFe]*-hydrogenase genes were detected in all four samples (Fig. 4). Phylogenetic analysis of the clone sequences was assembled into 5, 12, 10 and 8 OTUs in XJ6, XJ7, HB and DQ samples, respectively. The majority of these sequences obtained were affiliated to uncultured bacteria with an exception of 13 clones (11 of 70 clones in OTU *FeFe-hdy*_HB-36, 1 of 51 clones in OUT *FeFe-hdy*_XJ6-44, and 1 of 49 clones in *FeFe-hdy*_XJ7-46) sharing high similarity with *Alistipes* sp. within *Bacteroidetes*, the OTU *FeFe-hdy*_XJ7-45 with *Clostridium* sp, OTU *FeFe-hdy*_XJ7-14 with *Syntrophus aciditrophicus* SB (82 %) and *FeFe-hdy*_XJ7-33 with *Cloacamonas acidaminovorans* (85 %). Meanwhile, all the other clones in XJ6, XJ7, HB and DQ samples shared low similarity to the known sequences in GenBank. It is reasonable to assume that they

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are new members of uncultured microorganism. It could be deduced from above data that hydrogen-producing microorganisms inhabited universally in oil reservoir.

3.4 Methanogenesis

By using *mcrA*-targeted specific PCR primers set, 10, 10, 11, and 6 OTUs were obtained in XJ6, XJ7, HB and DQ samples, respectively, which dispersed all over the phylogenetic tree (Fig. 5). OTUs represented by *mcrA*-HB-73 (49.0 %) and *mcrA*-DQ-92 (46.8 %) were all closely related to *Methanolinea tarda*, a CO_2-reducing methanogens. OTU *mcrA*-XJ6-105 (31.1 %) shared high identities with *mcrA* sequences from the *Methanoculleus bourgensis* MS2 which could generate CH_4 by reducing CO_2. OTU *mcrA*-XJ6-103 (22.2 %) was closely related to *Methanocorpusculum labreanum*, also a member of CO_2-rducing methanogens. OTUs represented by *mcrA*-XJ7-104, *mcrA*-XJ7-86 and *mcrA*-XJ7-2 (totally 34.9 %) shared high similarity with *Methanoculleus*. In DQ sample, OTU *mcrA*-DQ-84 (44.7 %) was most similar to *Methanoculleus palmolei*, generating CH_4 through CO_2-reducing biochemical pathway. All these results show that the CO_2-reducing methanogens inhabit predominantly in oil reservoirs with different temperatures.

4 Discussions

4.1 Distribution of microorganisms based on functional genes

The structure of the microbial community inhabiting production water samples from these four oil reservoirs was analyzed by means of PCR amplification and cloning based on a suite of functional genes. Our results indicate that members of the *Proteobacteria* (*Thiobacillus*, *Allochromatium*, *Rhodovulum*, *Caenispirillum*, *Acidithiobacillus Rhodospirillum*) were the predominant ones with the ability to fix CO_2. As shown in Fig. 6a with the increase of temperature of the sampling oil reservoirs, the predominant microorganisms with *cbbL* genes changed from *Beta-Proteobacteria*

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in XJ (96.7 %) to *Alpha-Proteobacteria* in HB (98.6 %) and then DQ (78.6 %). Also, the *Gamma-Proteobacteria* appeared as the main part only in DQ (21.4 %) and HB (37.1 %) samples. The majority of *cbbL* gene types obtained were similar to the microorganisms belonging to *Alpha*-, *Beta*-, and *Gamma-Proteobacteria*. For *cbbM genes*, the predominant cultured bacteria were all the *Alpha-Proteobacteria* in samples both from high and low temperature oil reservoirs. It has been noted that the CBB cycle for CO_2 fixation operates in *Proteobacteria* belonging to the *alpha*, *beta*, and *gamma* subgroups and some members of the *Firmicutes* (Caldwell et al., 2007; Zakharchuk et al., 2003). All above data suggest that microorganisms within *Proteobacteria* mainly use the CBB cycle for CO_2 fixation in the studied oil reservoirs.

The acetogens were mostly affiliated to *Alpha-Proteobacteria* in DQ and XJ7 samples, to *Actinobacter* in HB samples and to *Firmicutes* in XJ6 samples. The predominant cultured bacteria belong to genus *hyphomonas*, *acetobacterium*, *blastomonas*, *Arthrobacter* in DQ, XJ6, XJ7 and HB samples, respectively. The acetogens use the reductive acetyl-CoA pathway not only for CO_2 fixation but also for the production of acetic acid. Acetogenic bacteria are among the most phylogenetically diverse bacteria from functional point of view. To date, approximately hundred homoacetogenic species have been identified and classified into twenty-one phylogenetically different genera (Drake et al., 2006). These results deduce that microorganisms inhabiting the herein investigated oil reservoirs have the ability to fix CO_2 as well as convert CO_2 into acetic acid.

As for the hydrogen production microorganisms, the diversity seems to become lower when the temperature increases from 21 and 32 °C in XJ6 and XJ7 sample to 90 °C in DQ samples. Most of these microorganisms were uncultured except that only small percent of the sequences were affiliated to *alistipes* in XJ6 (6.7 %), XJ7 (2 %) and HB (15.7 %) respectively and to *Clostridium* (4 %), *Synthrophus* (2 %) and *Cloacamonas* (2 %) in XJ samples. Those microorganisms were also reported to use the rTCA cycle for autotrophic CO_2 fixation (Schauder et al., 1987; Thauer et al., 1989). Fermentative microbes producing H_2 have been postulated to form trophic links with

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H_2-consuming methanogens, acetogens (i.e., organisms capable of using the acetyl-coenzyme A pathway for acetate synthesis) (Drake et al., 2006). In the present study, we found that sequences from microorganisms sharing similarities with those from the *Firmicutes*, *Delta-proteobacteria* and *Bacteroidetes* were the most encountered in clone libraries established for *[FeFe]*-hydrogenase gene, and these results are consistent with those of Schmidt et al. (2010).

The archaeal *mcrA* gene clone libraries were mostly related to the order of *Methanobacteriales* (*Methanothermobacter*, *Methanobacterium*), *Methanomicrobiales* (*Methanoculleus*, *Methanocorpusculum*, *Methanospirillum*, *Methanolinea*, *Methanoplanus*) and *Methanosarcinales* (*Methanosarcina*, *Methanolobus*, *Methanosaeta*, *Methanomethylovorans*). *Methanoculleus* is predominant in low temperature oil reservoirs and *Methanoculleus* as well as *Methanolinea* predominant in high temperature oil reservoir such as HB (51 %) and DQ (46.8 %). It is very interesting to note that all these predominant methanogens belonging to hydrogenotrophic methanogens. Methanogenesis is believed to be the terminal process of organic compound degradation and plays a major role in the global carbon cycle (Garrity and Holt, 2001; Liu and Whitman, 2008). The most important precursors for methane production during anaerobic digestion of organic matter are H_2-CO_2 and acetate, which are converted into methane by hydrogenotrophic and acetoclastic methanogens (Mayumi et al., 2011), respectively. Interestingly, it is proposed that syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis is the main methanogenic pathway in high temperature petroleum reservoirs (Mayumi et al., 2011). Analysis based on the *mcrA* genes types indicates twelve OTUs detected share high identity with those of the genus *Methanothermobacter*. Based on all above, we can deduce that the indigenous microorganisms in oil reservoirs have the capability to fix CO_2 and convert CO_2 into CH_4.

To the best of our knowledge, the collection of functional genes described in the present work has not yet been investigated in oil reservoir systems. They have been reported from other geothermal environments. The detection of CO_2 fixation genes as

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well as hydrogenases and *fthfs* genes in production fluids of oil reservoirs with different temperature provides new insights on the diversity and composition of microorganisms involved in the microbial fixation of CO_2 and its subsequent conversion to methane.

4.2 The distinct functional microbial communities for fixation and bioconversion of CO_2

Microbial fixation of CO_2 and further conversion into methane in oil reservoirs by indigenous microorganisms is one of the most promising solutions to the mitigation of CO_2 emission and also generation of energy. We explored the potential for autotrophic CO_2 fixation and bioconversion with microbial communities in oil reservoirs by detection of relative functional marker genes such as CO_2 fixation (*cbbM*, *cbbL*), acetogenesis (*fthfs*), hydrogen formation (*[FeFe]*-hydrogenase) and methanogenesis (*mcrA*).

Microbial fixation and conversion of CO_2 into CH_4 are usually implemented by chemolithoautotrophic microorganisms. Methanogens oxidize molecular hydrogen (H_2) anaerobically by transferring electrons from H_2 to CO_2 to form methane. As shown in Fig. 6e, *Methanolinea* and *Methanocelleus* were predominant methanogens in both high and low temperature oil reservoir samples. Considering that the most abundant genera *Methanoculleus*, *Methanolinea*, and *Methanothermobacter* are all the hydrogenotrophic methanogens, this allows us to speculate that hydrogentropic methanogens will play an important role in CO_2 fixation and bioconversion of CO_2 into CH_4 in oil reservoir system.

Ribulose 1,5-bisphosphate carboxylase (Rubisco, specifically, *cbbL*, *cbbM*) are usually used as a biomarker for the Calvin Benson-Bassham (CBB) CO_2 fixation pathway (Campbell and Cary, 2004). Specifically, in subsurface environments, CO_2 fixation is usually conducted by chemolithotrophs through CBB pathway (Badger and Bek, 2008). The community structure of microorganisms with most similarity to the retrieved amino acid sequences of *cbb* gene was distinct between high temperature samples and low temperature samples (Fig. 6a and b). As for *cbbM*, the genera *Rhodospirillum* (71.7 %), *Magnetospira* (33.3 %) were dominant in low temperature

(XJ6 and XJ7) samples, whereas, *Rhodopseudomonas* (100 %) in HB sample and *Magnetospirillum* (26.7 %), *Rhodopseudomonas* (26.7 %), *Magnetospira* (20 %) in DQ sample with high temperature were dominant. In *cbbL* community, the XJ6 and XJ7 were dominated by *Thiobacillus* and *Allochromatium*, whereas HB and DQ by *Rhodovulum* and *Rhodospirillum*, respectively. Alfreider et al. (2003) also detected *Hydrogenophaga*, *Thiobacillus*, and others related *cbb* sequences in a contaminated aquifer. The abundance and diversity of the detected *cbb* genes suggest that microbial fixation of CO_2 via the Calvin cycle within oil reservoirs possesses a significant potential.

Most acetogens are obligately anaerobic bacteria that use the reductive acetyl-CoA pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and cellular carbon from CO_2. Formyltetrahydrofolate synthetase (*fthfs*) is used to detect acetogenic, fermentative bacteria (Leaphart and Lovell, 2001). In the present work, notable difference was observed in the composition of *fthfs* communities (Fig. 6c). For instance, in XJ6 and XJ7 samples from low temperature oil reservoirs, the community was dominated by microorganisms related to genera *Acetobacterium* (46.2 %) and *Blastomonas* (68.8 %), however, only microorganisms related to genus *Hyphomonas* (100 %) were detected in DQ high temperature oil reservoir sample. This observation implies that *Acetobacterium* and *Hyphomonas* as well as *Blastomonas* are probably more suitable for acetogenesis in CO_2-injected oil reservoirs. As a matter of fact, the ability of acetate production on $CO_2 + H_2$ by *Acetobacterium woodii* and *Moorella* had been systematically studied (Demler and Weuster-Botz, 2011; Ragsdale and Pierce, 2008).

Hydrogen is an alternative energy source for autotrophic microbes in a variety of subsurface environments. When hydrogen and carbon dioxide are available, development of autotrophic microorganisms would be possible. For example, methanogens and acetogens may produce reduced organic materials from hydrogen by means of respiring carbon dioxide. As seen from our study (Fig. 6d), almost all of the *[FeFe]*-hydrogenase clone libraries are related to uncultured bacteria. The genera

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Alistipes was detected in three samples less than 45 °C and the genera *Clostridium*, *Syntrophus*, *Cloacamonas* were only detected in XJ7 sample with a relative abundance less than 5 %.

Great differences exist in relative abundance among all the five functional gene clone libraries established from high and low temperature samples (Fig. 6). This phenomenon of microbial community change with temperature has been reported (Wang et al., 2012). Microorganisms with high abundance implies that they may be better withstanding or adapting to the herein environment. Thus, these microorganisms may be of great importance in geological sequestration and bioconversion of CO_2 to CH_4 and hence warrant more attentions.

Analysis of functional genes shows that microbial communities were strongly influenced and the diversity generally reduced by the increase of temperature of the oil reservoir sampled. For example, there were four different genera in XJ sample whereas only one retrieved from HB ample for *[FeFe]*-hydrogenase library. Also, for *fthfs* library, four different genera were detected in XJ6 sample but only one in DQ sample. Knowledge of surviving and thriving microbial populations may help to predict the fate of CO_2 following injection and making better strategies to make use of microorganisms in subsurface environments for improving the efficiency of injection and microbial fixation of CO_2, hence ensuring the long-term CO_2 storage in subsurface petroleum reservoirs.

The *fthfs*-XJ6-43 was closely related to *Methanomassiliicoccus luminyensis* (Fig. 3), which was able to produce methane by reducing methanol with hydrogen as an electron donor (Gorlas et al., 2012). *M. luminyensis*, obligate H_2-dependentmethylotrophic methanogens, was considered to be putative seventh order of methanogens and occupy environments highly relevant for methane production (Borrel et al., 2013). This implies that *M. luminyensis* may bear close biochemical resemblance to the acetyl-CoA pathway of Acetogens. Some methanogens use metabolic pathways containing acetyl-CoA synthase have been reported (Fuchs and Stupperich, 1980, 1984a, b;

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Shieh and Whitman, 1988). However, to our knowledge, no reports are available on that the genera *Methanomassiliicoccus* bears acetyl-CoA pathway of acetogens.

4.3 The potential of methane formation from injected CO_2

The fate of CO_2 in subsurface ecosystem is receiving increasing attentions. To date, the in situ conversion of CO_2 into CH_4 by methanogens inhabiting oil reservoirs universally is an area of active research and development. This conversion process has the potential of not only reducing CO_2 emission but also producing natural gas in reservoirs. Hydrogenotrophic methanogens need both CO_2 and also H_2 to produce CH_4; therefore, H_2 should be supplied to them in reservoirs for this process. It has been reported that there are several kinds of microorganisms capable of producing H_2 by degrading crude oil in reservoir environments. The potential of the microbial conversion of CO_2 into CH_4 by enrichment culture experiments using microorganisms indigenous to oil reservoirs has been studied (Sugai et al., 2012). Different from that mentioned above, we evaluated the potential of this process from a viewpoint of functional genes. In our study, both the functional genes of H_2-producing and CH_4-producing were detected in all the oil reservoirs sampled regardless of the temperatures. Furthermore, some H_2-producing microorganisms (e.g. *Clostridium*, *Syntrophus* and *Cloacamonas*) and hydrogenotrophic methanogens such as *Methanocelleus* and *Methanolinea* as well as *Methanobacterium* are predominant in these samples. Meanwhile, these H_2-producing bacteria and hydrogenotrophic methanogens were both identified in the 16S rRNA genes cloning libraries (data not presented in this paper). It is assumed that these hydrogenotrophic methanogens live in harmony with hydrogen-producing bacteria and convert CO_2 into CH_4 in oil reservoirs. These results indicate that indigenous microbial conversion process of CO_2 into CH_4 has high potential.

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5 Conclusion

Analysis of a suite of functional genes shows that a diverse microbial community with potential for CO_2 fixation and conversion of CO_2 into methane inhabit these oil reservoirs with temperature from 21 to 90 °C. Microorganisms affiliated with members of the genera *Methanocelleus* and *methanolinea* (hydrogenotrophic CO_2-reducing methanogens), *Acetobacterium* and *Thiobacillus* and *Rhodospirillum* as well as hydrogen producers (*Firmicutes*) present the potential for microbial fixation and conversion of CO_2 into methane in oil reservoirs. The results of present work are helpful in making better strategies to fully utilize microorganisms in subsurface environments for microbial CO_2 fixation and bioconversion of CO_2 into sustainable energy (e.g. in the form of methane) in subsurface petroleum reservoirs. Further attentions should be paid to evaluate the activities of those related microorganisms in subsurface ecosystems with the potential of microbial fixation of CO_2 and its subsequent bioconversion into methane.

Author contributions. J.-F. Liu, J.-D. Gu and B.-Z. Mu made the full design of the experiments. X.-B. Sun, S. M. Mbadinga, and G.-C. Yang conducted the microbial analysis and S.-Z. Yang performed the chemical analysis. J.-F. Liu prepared the manuscript with contributions from all co-authors.

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Table 1. The physicochemical properties of production water samples from the oilfields of this study.

Parameter	Xinjiang J6	Xinjiang J7	Huabei	Daqing
Depth (m)	480.0	1088.0	1101.0	1500.0
Temperature (°C)	21.0	32.0	45.0	90.0
pH	7.0	7.1	7.2	6.0
Salinity ($mg L^{-1}$)	15 728.0	4212.0	4091.0	3900.0
Cl^- ($mg L^{-1}$)	5336.0	2000.0	819.0	1914.0
SO_4^{2-} ($mg L^{-1}$)	124.8	7.7	32.4	731.3
PO_4^{3-} ($mg L^{-1}$)	ND	ND	ND	ND
NO_3^- ($mg L^{-1}$)	0.8	1.4	ND	ND
K^+ ($mg L^{-1}$)	35.1	45.6	22.3	6.3
Na^+ ($mg L^{-1}$)	4196.0	5399.0	1064.0	1110.0
Ca^{2+} ($mg L^{-1}$)	103.3	128.2	53.0	97.9
Mg^{2+} ($mg L^{-1}$)	44.7	64.0	17.6	9.2
Mn^{2+} ($mg L^{-1}$)	0.3	0.4	0.1	ND
formate (mg L-1)	ND	ND	ND	ND
acetate ($mg L^{-1}$)	344.0	7.0	57.9	56.2
Propionate ($mg L^{-1}$)	ND	ND	ND	ND
Butyrate ($mg L^{-1}$)	ND	ND	0.5	ND
Isobutyrate ($mg L^{-1}$)	32.7	ND	ND	4.9

Xinjiang, XJ; Huabei, HB and Daqing, DQ; ND, not detected.

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Table 2. The PCR primer sets used for amplification of relevant functional genes.

Target gene	Primer	Sequences (5'-3')	Annealing temp (°C)	Cycles
cbbL	cbbL(f)	GACTTCACCAAAGACGACGA	55	35
	cbbL(r)	TCGAACTTGATTTCTTTCCA		
cbbM	cbbM(f)	TTCTGGCTGGGBGGHGAYTTYATYAARAAYGACGA	55	35
	cbbM(r)	CCGTGRCCRGCVCGRTGGTARTG		
FTHFS	FTHFS(f)	TTYACWGGHGAYTTCCATGC	60	40
	FTHFS(r)	GTATTGDGTYTTRGCCATACA		
mcrA	ML(f)	GGTGGTGTMGGATTCACACARTAYGCWACAGC	55	40
	ML(r)	TTCATTGCRTAGTTWGGRTAGTT		
Fe-Fe -hydrogenase	H1F	TTIACITSITGYWSYCCIGSHTGG	55	40
	H3R	CAICCIYMIGGRCAISNCAT		

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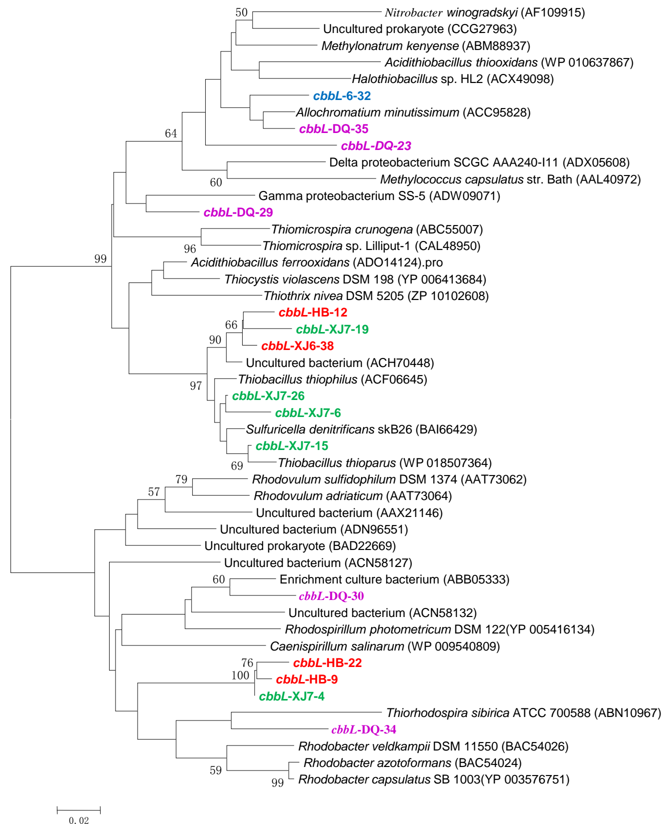

Figure 1. Phylogenetic tree of the *cbbL* gene retrieved from the water samples (shown in colored) and closely related sequences from the GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was generated with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50 % are reported. Scale bar represents 5 % amino acid substitution.

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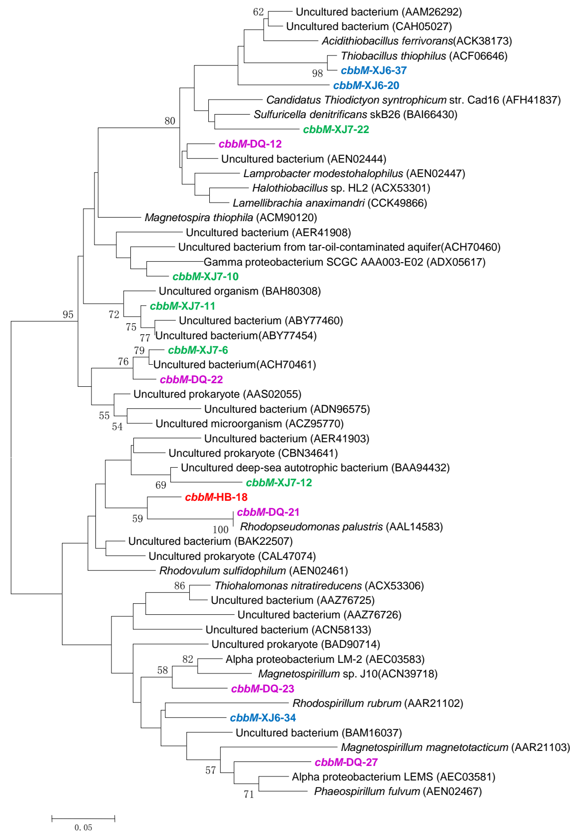

Figure 2. Phylogenetic tree of the *cbbM* gene retrieved from the water samples (colored) and closely related sequences from the GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was generated with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50 % are reported. Scale bar represents 10 % amino acid substitution.

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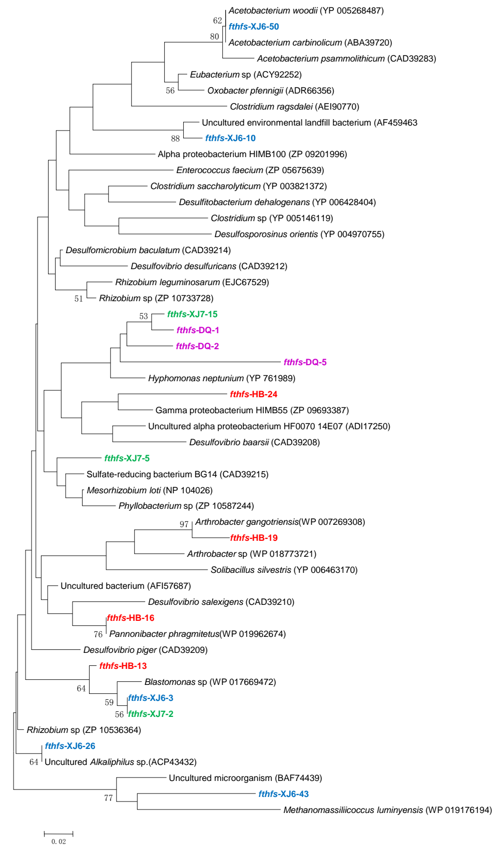

Figure 3. Phylogenetic tree of the *fthfs* gene retrieved from the water samples (colored) and closely related sequences from the GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was generated with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50 % are reported. Scale bar represents 10 % amino acid substitution.

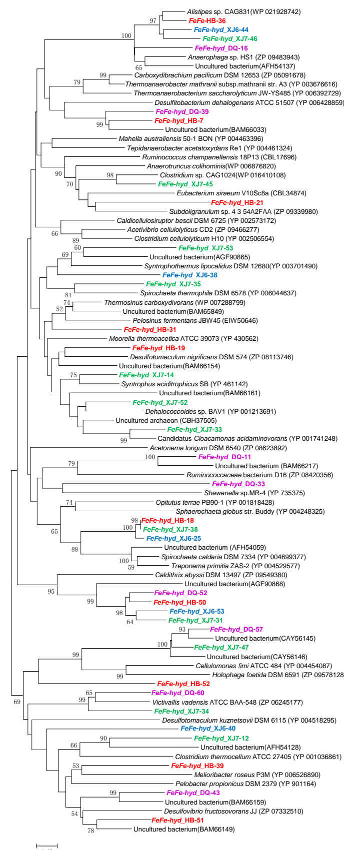

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Figure 4. Phylogenetic tree of the *[FeFe]-Hydrogenase* gene retrieved from the water samples (colored) and closely related sequences from the GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was generated with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50 % are reported. Scale bar represents 10 % amino acid substitution.

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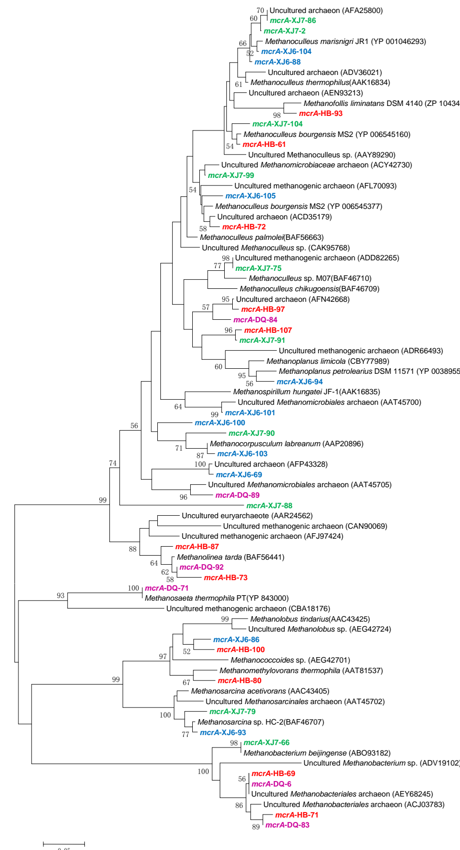

Figure 5. Phylogenetic tree of the *mcrA* gene retrieved from the water samples (colored) and closely related sequences from the GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was generated with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50 % are reported. Scale bar represents 5 % amino acid substitution.

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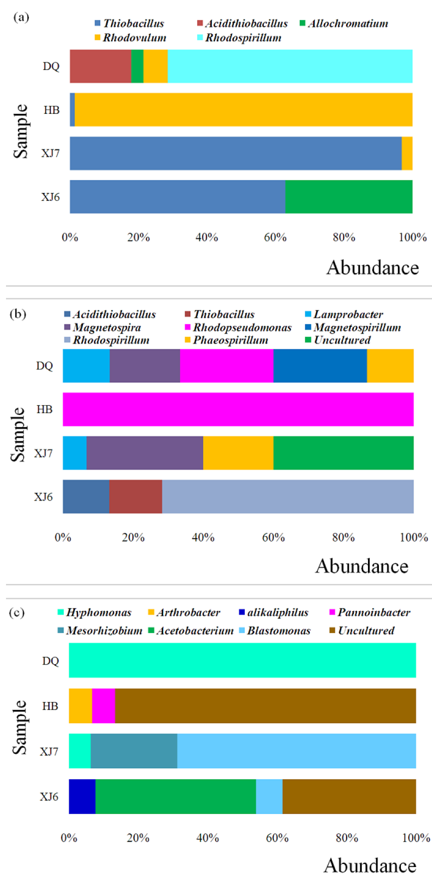

Figure 6. Relative abundance of functional microbes (at the genus level) with respect to the sequences retrieved by functional marker genes of *cbbL* **(a)**, *cbbM* **(b)**, *fthfs* **(c)**, *FeFe-Hydrogenase* gene **(d)** and *mcrA* **(e)**.

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