Analysis of microbial communities in the oil reservoir subjected to CO₂-flooding by using functional genes as molecular biomarkers for microbial CO₂ sequestration

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Sequestration of CO₂ in oil reservoirs is considered to be one of the feasible options for mitigating atmospheric CO₂ building up and also for the in situ potential bioconversion of stored CO₂ to methane. However, the information on these functional microbial communities and the impact of CO₂ storage on them is hardly available. In this paper a comprehensive molecular survey was performed on microbial communities in production water samples from oil reservoirs experienced CO₂-flooding by analysis of functional genes involved in the process, including cbbM, cbbL, fthfs, [FeFe]-hydrogenase, and mcrA. As a comparison, these functional genes in the production water samples from oil reservoir only experienced water-flooding in areas of the same oil bearing bed were also analyzed. It showed that these functional genes were all of rich diversity in these samples, and the functional microbial communities and their diversity were strongly affected by a long-term exposure to injected CO₂. More interestingly, microorganisms affiliated with members of the genera Methanothemobacter, Acetobacterium, and Halothiobacillus as well as hydrogen producers in CO₂ injected area either increased or remained unchanged in relative abundance compared to that in water-flooded area, which implied that these microorganisms could adapt to CO₂ injection and, if so, demonstrated the potential for microbial fixation and conversion of CO₂ into methane in subsurface oil reservoirs.

Keywords: CO₂ fixation, bioconversion, methane, functional genes, oil reservoir, microbial communities

Introduction

Storage of CO₂ in deep geological formations, such as oil reservoirs, is one of the feasible measures to reducing CO₂ emissions into the atmosphere. Understanding the fate of CO₂ in the subsurface environment is of great scientific interest and significance, and has received increasing attention for more information to assess the feasibility. Due to the fact that abundant microorganisms inhabit in these formations, microbial fixation and conversion of the sequestered CO₂ into CH₄ are becoming an area of active research and development.

After CO₂ injection, characteristics of the formation water may be changed by CO₂ dissolution, including pH, the availability of inorganic and organic components in the brine, microbial
attachment and biofilm formation as well as the microbial activities at in situ oil reservoirs. Generally, as CO₂ is also a potential source of carbon of chemolithoautotrophic microorganisms such as methanogens, the injected CO₂ may activate these microorganisms and notably influence the microbial structure and their activity in situ. Studies have been performed on the physical and chemical changes in the CO₂ storage sites. The first on-shore CO₂ storage site in Europe was established, and the effects and feasibility of CO₂ injection and storage in a 650 m deep saline aquifer was examined (Wandrey et al., 2011). The potential of microbial conversion of CO₂ into CH₄ by hydrogenotrophic methanogens isolated from oil reservoirs has been evaluated based on laboratory experiments by Sugai et al. (2012). As to the microbial involvement, six autotrophic CO₂ fixation pathways were documented, of which the Calvin–Benson–Bassham (CBB) cycle plays an important role in autotrophic CO₂ fixation (Berg, 2011). The CBB biochemical process was reported to occur in Proteobacteria, including some members of Firmicutes, Actinobacteria, and Chloroflexi as well as in plants, algae, and cyanobacteria (Ivanovsky et al., 1999; Zakharuchuk et al., 2003; Berg et al., 2005; Caldwell et al., 2007; Lee et al., 2009). Another important pathway of CO₂ fixation is the reductive acetyl-CoA pathway that has documented to occur in aceticogenic prokaryotes, ammonium-oxidizing Planctomycetes (Strous et al., 2006), sulfidogenic bacteria (Schauder et al., 1988), and autotrophic archaea affiliated with the order Archaeoglobales (Vorholt et al., 1995, 1997). This pathway is also utilized by aceticogenic prokaryotes for energy conservation (Ragsdale and Pierce, 2008; Thauer et al., 2008; Biegl and Muller, 2010).

Petroleum reservoirs are known to harbor diverse microorganisms including bacteria such as Proteobacteria, Firmicutes, Actinobacteria, and Chloroflexi as well as in plants, algae, and cyanobacteria (Ivanovsky et al., 1999; Zakharuchuk et al., 2003; Berg et al., 2005; Caldwell et al., 2007; Lee et al., 2009). Another important pathway of CO₂ fixation is the reductive acetyl-CoA pathway that has documented to occur in aceticogenic prokaryotes, ammonium-oxidizing Planctomycetes (Strous et al., 2006), sulfidogenic bacteria (Schauder et al., 1988), and autotrophic archaea affiliated with the order Archaeoglobales (Vorholt et al., 1995, 1997). This pathway is also utilized by aceticogenic prokaryotes for energy conservation (Ragsdale and Pierce, 2008; Thauer et al., 2008; Biegl and Muller, 2010).

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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 different functional genes (Wang et al., 2012).

**PCD Amplifications**

Amplifications of the cbbL gene fragment (771 bp) and the cbbM gene fragment (328 bp) were carried out under the conditions described by Campbell and Carv (2004). For amplification of a portion (1102 bp) of the flhfs gene, the PCR conditions used were those described previously by Leaphart and Lovell (2001). For amplification of a fragment (620 bp) of [Fe-Fe]-hydrogenase-encoding gene, the PCR 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) with the conditions as reported previously (Galand et al., 2005). Functional genes fragments were all amplified in five parallel PCR reactions in a Peltier thermal cycler (Bio-Rad, USA), which were subsequently pooled for cloning and construction of genes libraries.

**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 µg ml⁻¹). The inserted DNAs were amplified by using M13-47 (5′-CGCCAGGGTTTTCCAGTCAAGAC-3′) and RV-M (5′-GAGCGGATAAACATTTCACAGG-3′) primers targeting the flanking vector sequence, followed by agarose gel electrophoresis with ethidium bromide staining (Guan et al., 2013).

**Sequencing and Phylogenetic Analyses**

Sequencing was carried out 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). DNA sequences with more than 97% similarity were assembled into the same operational taxonomic units (OTUs) using FastGroup II (Yu et al., 2006), and one representative sequence was chosen.
TABLE 1 | Characteristics of the production water samples.

| Parameter       | C     | W     |
|-----------------|-------|-------|
| pH              | 6.4   | 6.0   |
| Salinity (mg L\(^{-1}\)) | 3897  | 3920  |
| Cl\(^{-}\) (mg L\(^{-1}\))    | 1947  | 1872  |
| SO\(_4^{2-}\) (mg L\(^{-1}\))  | 667   | 908   |
| PO\(_4^{3-}\) (mg L\(^{-1}\))  | nd    | nd    |
| NO\(_3^-\) (mg L\(^{-1}\))     | nd    | nd    |
| Na\(^+\) (mg L\(^{-1}\))       | 1110  | 1115  |
| NH\(_4^+\) (mg L\(^{-1}\))     | 24.6  | 25.8  |
| K\(^+\) (mg L\(^{-1}\))        | 6.8   | 6.9   |
| Ca\(^{2+}\) (mg L\(^{-1}\))    | 131.5 | 83.1  |
| Mg\(^{2+}\) (mg L\(^{-1}\))    | 10.0  | 8.9   |
| Mn\(^{2+}\) (mg L\(^{-1}\))    | nd    | nd    |
| Formate (mg L\(^{-1}\))        | nd    | nd    |
| Acetate (mg L\(^{-1}\))        | 109.1 | 7.7   |
| Propionate (mg L\(^{-1}\))     | nd    | nd    |
| Isoleucinate (mg L\(^{-1}\))   | 2.5   | 2.9   |
| Butyrate (mg L\(^{-1}\))       | nd    | nd    |

**pH**, anion, cation were analyzed by **pH** meter, ion chromatography, and **ICP-AES** (Inductively Coupled Plasma-Atomic Emission Spectrometry), respectively; Volatile fatty acids were determined by **GC-MS** after butanol esterification; nd, not detectable.

from each OTU to compare with sequences in the GenBank Database using the BLASTX algorithm to identify nearest related ones (Altschul et al., 1997). Representative OTUs from clone libraries as well as reference sequences from GenBank were translated into corresponding amino acid sequences using EMBOSs Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) with default parameter (Standard Genetic Code) and then aligned using Clustal Omega (Sievers et al., 2011). Phylogenetic trees were generated using MEGAS software (Tamura et al., 2011). The topology of the trees was obtained by the Neighbor-Joining method (Saitou and Nei, 1987) with the Poisson correction method and 1000 bootstrap replicates were applied to estimate the support for the nodes in the tree.

**Nucleotide Sequence Accession Numbers**
Gene sequences data reported here are available in GenBank sequence database under the accession numbers KF111435–KF111455, KF111525–KF111548, KF111456–KF111492, KF111493–KF111501, and KF111502–KF111524 for **cbbM** gene, **cbbL** gene, **mcrA** gene, **fthfs** gene, and gene encoded by [Fe-Fe]-hydrogenase.

**Results**

**Characterization of Clone Libraries**

**cbbL** and **cbbM** Genes
The **cbbL** gene types were positively detected in all two kinds of samples (Figure 2). The **cbbL** gene clone libraries from sample C and W resulted in 11 and 13 OTUs, respectively, and the PCR amplified sequences are spread over the entire tree. Phylogenetic analysis indicates that the **cbbL** gene sequences obtained are related to those of **Alpha-**, **Beta-**, and **Gamma-Proteobacteria**. One OTU (**cbbL-C2-18**) is closely related to **Hydrogenophaga** sp. CL3 affiliated to the family **Comamonadaceae** within **Beta-Proteobacteria** (Garcia-Dominguez et al., 2008).

The sequences of **cbbL-C1-13**, **cbbL-C1-17**, and **cbbL-W4-9** all share high similarity with **Cupriavidus metallidurans** CH34 belonging to the family **Burkholderiaceae** within **Beta-Proteobacteria**. The sequence of **cbbL-W3-24** shares high identity with endosymbiont of **Bathy-modulus azoricus** (Spiridonova et al., 2006), a member of **Gamma-Proteobacteria**. One OTU represented by **cbbL-W4-12** shows highest identity with an uncultured bacterium from iron-rich environment (Kellermann et al., 2012).

Similarly, the **cbbM** gene types were also detected in these two samples and yielded 10 and 11 OTUs in C and W, respectively (Figure 3). The **cbbM** sequences detected are all very similar to those from organisms affiliated with members of **Alpha-**, **Beta-**, and **Gamma-Proteobacteria**. The sequence of **cbbM-C1-3** is related to an uncultured bacterium from cave water of Romania (Chen et al., 2009).

The OTUs represented by **cbbM-W4-22**, **cbbM-W4-32**, **cbbM-W3-12**, and **cbbM-C2-9** are closely related to uncultured bacterium from tar contaminated aquifer and MTBE and ammonium polluted groundwater (Alfreider et al., 2012). Sequences represented by **cbbM-C2-21**, **cbbM-C1-13**, and **cbbM-C1-7** all share similarities with those recovered from the East China Sea and basin water and sediment. Interestingly, these sequences are also closely related to **Halothiobacillus** spp., members of sulfur-oxidizing symbionts belonging to **Gamma-Proteobacteria**. Three OTUs (**cbbM-C2-28**, **cbbM-W3-9**, **cbbM-W4-38**, and **cbbM-C1-16**) are similar to an uncultured organism from iron-rich environmental samples (Kojima et al., 2009). Sequences represented by both **cbbM-W3-14** and **cbbM-C1-21** are closely related to **Rhodopseadomonas palustris**, a member of the order **Rhizobiales** within the **Alpha-Proteobacteria**. OTUs **cbbM-W4-14** and **cbbM-W4-6** representing 29 clones show highest similarities with **Phaoquorum molischianum**, affiliated with the family **Rhodospirillaceae** within **Alpha-Proteobacteria** and with sequences from methane seep sediment. And **cbbM-W3-7**, which appeared to forms its own cluster, is related to **Thauera** spp. within the **Beta-Proteobacteria** and also to an uncultured bacterium from an environmental sample of paddy soil in China (Yuan et al., 2012).

**fthfs** Genes
The **fthfs** gene sequences were also detected in both samples. However, it showed a less abundant diversity as depicted in the phylogenetic tree (Figure 4) with the screened clones divided into 5 and 4 OTUs in sample C and W, respectively. Phylogenetic analysis shows that most of the **fthfs** gene sequences are related to members of the **Firmicutes**. Three OTUs (**FTFHS-C2-9**, **FTFHS-C2-12**, and **FTFHS-C2-19**) of sample C are all most similar to **Acetobacterium psammoliticum**, a member of the order **Clostridiales** within **Firmicutes** while 2 OTUs (**FTFHS-C1-7** and **FTFHS-C1-5**) are obtained in sample C and sharing high similarities with **Firmicutes** members of the genus **Acetobacterium** (Xu et al., 2009). OTUs **FTFHS-W3-24** and **FTFHS-W3-12** are related to sequences...
from genera Moorella, Desulfotobacterium, and Desulfo- sporosinus, also members of the Firmicutes. FTHFS-W3-4 is similar to un cultured Alkaliphilus sp. from anaerobic wastewater of Mesa Northwest Wastewater Reclamation Plant (Parameswaran et al., 2010).

**[FeFe]-Hydrogenase-Encoding Gene**

The [FeFe]-hydrogenase-encoding gene was detected in both C and W samples, and phylogenetic analysis of the sequenced clones were assembled into 9 and 14 OTUs, respectively (Figure 5). The majority of the gene sequences obtained
from the two samples cluster with sequences related to Firmicutes. One OTU represented by FeFe-Hyd_W3-6 shares similarity with *Syntrophothermus lipocalidus* of the Firmicutes. FeFe-Hyd_W4-38 is either related to *Shewanella halifaxensis* HAW-EB4 within the Gamma-Proteobacteria or to *Thermodesulfovibrio yellowstonii* within the Nitrospira (*Figure 5*). FeFe-Hyd_W4-36 is related to *Thermodesulfobium naringense* belonging to the family Thermodesulfobiacae within...
Figure 4 | Phylogenetic tree of the fthfs gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50% are reported. Scale bar represents 10% amino acid substitution.

the Firmicutes. FeFe-Hyd_W4-22 shares high identity with Moorella thermoacetica affiliated to the family Thermotogaceae of Firmicutes. FeFe-Hyd_W4-4, FeFe-Hyd_C2-26, and FeFe-Hyd_W4-32 are all related to Desulfovomaculum kuznetsovii, a member of the order Clostridiales within Firmicutes. FeFe-Hyd_C2-10 and FeFe-Hyd_W4-35 are both similar to Thermotoga lettingae TMO affiliated with the family Thermotogaceae.

mcrA Genes
By using mcrA-targeted specific PCR primers set, 21 and 16 OTUs (37 overall) were obtained in samples C and W, respectively (Figure 6). Phylogenetic analysis shows that 21 OTUs (13 in C and 8 in W) are all closely related to sequences from members affiliated to the Methanobacteriales, an order known to harbor mostly CO2-reducing methanogens. A total of 7 OTUs (3 in C and 4 in W) shared high identities with mcrA sequences from the Methanomicrobiales. And 9 OTUs (5 in C and 4 in W) are closely related to sequences affiliated to methylotrophic and acetoclastic methanogens within the order Methanosarcinales.

Characterization of Functional Microbial Communities
Changes in microbial structure were analyzed by their relative abundance calculated from the number of clones and the results were showed in Figure 7. The community structure of microorganisms with most similarity to the retrieved amino acid sequences of cbbM gene was distinct in W and C samples (Figure 7A). The genera Phaeospirillum (67.4%), Leptothrix (14.0%), Rhodopseudomonas (7.0%), and Thiothrix (7.0%) were dominant in W sample, whereas, Halothioceccus (55.3%) and Leptothrix (36.8%) were dominant in C sample. In the cbbL
FIGURE 5 | Phylogenetic tree of the [FeFe]-Hydrogenase gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50% are reported. Scale bar represents 10% amino acid substitution.
FIGURE 6 | Phylogenetic tree of the mcrA gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values (n = 1000 replicates) greater than 50% are reported. Scale bar represents 5% amino acid substitution.
clones libraries (Figure 7B), the genera *Rhodospirillum* 36.7% and 42.5%, *Hydrogenophaga* 46.7% and 47.5%, *Cupriavidus* 10.0% and 7.5% were dominant in W and C sample, respectively. As for the composition of *fthfs* communities (Figure 7C), in W sample, the community was mainly composed by microorganisms related to genera *Nisaea* (57.1%), *Moorella* (34.3%), and *Alkaliphilus* (8.6%), however, only by microorganisms related to genus *Acetobacterium* (100%) in C sample. It can be seen from Figure 7D that C sample was dominantly composed by microbes related to members of genera *Clostridium* (38.1%), *Desulfotomaculum* (28.6%), and *Thermotoga* (14.3%), while the W sample by *Syntrophothermus* (44.2%) and *Desulfotomaculum* (30.8%). Meanwhile, those related to *Ammonifex*, *Dehalococcoides*, and *Cloacamonas* all rose in relative abundance from undetectable in W sample to 4.8% in C sample. The methanogen community was demonstrated in Figure 7E. As shown in Figure 7E, thermophilic *Methanothermobacter* (55.7% and 57.1% in C and W sample, respectively), *Methanolinca* (11.4% and 7.9% in C and W sample, respectively), and *Methanoaseta* (21.4% and 33.3% in C and W sample, respectively) were the predominant methanogens. *Methanoculleus* (10.0%) were only detected in the C sample.

**Discussion**

**Occurrence of Microorganisms Associated with CO₂ Sequestration in Oil Reservoirs**

The microbial community structure in production water samples in Daqing oilfield of China was analyzed by means of a suite of functional genes as biomarkers. Our results indicate that members of the *Proteobacteria* (Halothiobacillus, *Leptothrix*, *Hydrogenophaga*, and *Rhodospirillum*) were the predominant ones with the ability of fixation of CO₂ in situ oil reservoirs. It has been reported that the CBB cycle for CO₂ fixation operates in *Proteobacteria* belonging to the alpha-, beta-, and gamma-subgroups, and some members of the *Firmicutes* (Zakharchuk et al., 2003; Caldwell et al., 2007). In addition, the acetogens belonging to *Clostridiaceae* within *Firmicutes* can use the reductive acetyl-CoA pathway not only for CO₂ fixation but also for the production of acetic acid, which is substrate for methanogenesis. Other major bacterial sequences in the clone libraries of sample W are related to those of *Hydrogenophilaceae*, and similar microorganisms were reported to use the rTCA cycle for autotrophic CO₂ fixation (Schauder et al., 1987; Thauer et al., 1989). For the archaeal *mcrA* gene clone libraries, the predominance of the genus *Methanothermobacter* belonging to hydrogenotrophic methanogens is notable.

The majority of *cbbL* gene types obtained were very similar to the microorganisms belonging to *Alphaproteobacteria*. And some members of these phyla have been reported in previous studies, but of which *Hydrogenophaga* sp. and *Cupriavidus* sp. were rarely documented (Alfreider et al., 2003). The *cbbM* gene types detected are also related to those of *Alphaproteobacteria*, and this is consistent with the research results of Hugler et al. (2010). All above data suggest that microorganisms within *Proteobacteria* mainly use the CBB cycle for CO₂ fixation in the oil reservoirs studied.
Acetogenic bacteria are among the most phylogenetically diverse bacterial functional groups. To date, approximately hundreds of homoacetogenic species have been identified and phylogenetically classified into 21 different genera. The fthfs gene sequences obtained from CO2-flooded fraction of the reservoir shared high similarities with those from members of the Firmicutes with most of the sequences related to the order Clostridiales, deducing that microorganisms affiliated with Firmicutes inhabiting the herein investigated oil reservoirs have the ability to fix CO2 as well as convert CO2 into acetic acid via the acetyl-CoA pathway.

H2 is necessary to in situ CH4 production by hydrogenotrophic methanogens in oil reservoirs. In the present study, we found that sequences from microorganisms similar with those from the Firmicutes, Gamma-Proteobacteria, and Thermotogae were the most encountered in clone libraries established for [FeFe]-hydrogenase-encoding gene, and these results are consistent with those of Schmidt et al. (2010), who found that members of the order Clostridiales and Thermoanaerobacter sp. were likewise all capable of fermentative production of H2 (Schmidt et al., 2010).

Methanogenesis is the terminal step 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 H2, CO2, and acetate, which are converted into methane by hydrogenotrophic and aceticlastic methanogens (Mayumi et al., 2011), respectively. Interestingly, it is proposed that syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis is an alternative methanogenic pathway in petroleum reservoirs (Mayumi et al., 2011). Analysis based on the mcrA gene types indicates 12 OTUs detected share high identity with those of the genus Methanomethrobacter.

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, although some of them have been reported in geothermal environments. The detection of CO2 fixation genes as well as hydrogenases-encoding and fthfs genes in production fluids of high temperature oil reservoirs provides new insights on the diversity and composition of microorganisms involved in the microbial fixation of CO2 and its subsequent conversion to methane.

Impact of CO2 Injection on Specific Microbial Communities with Respect to Microbial Fixation and Bioconversion of CO2

Microbial fixation and conversion of CO2 into methane in oil reservoir by indigenous microorganisms is one of the most promising solutions to the mitigation of CO2 emission. We explored the potential for autotrophic CO2 fixation and bioconversion with microbial communities in oil reservoir by detection of relative functional biomarker genes such as CO2 fixation (cbbM, cbbL), acetogenesis (fthfs), hydrogen formation ([FeFe]-hydrogenase-encoding gene), and methanogenesis (mcrA). Microbial fixation and conversion of CO2 are usually implemented by chemolithoautotrophic microorganisms, which usually obtain their energy through the oxidation of inorganic compounds and utilization of CO2 as their sole source of carbon. Thus, the CO2 injected as well as the subsequent changes in pH and other geochemical parameters induced by CO2 have an influence on the metabolism of the both heterotrophic and lithoautotrophic microorganisms (Ramos, 2003). Therefore, injection of CO2 may cause some changes in microbial populations as well as their activities, and it is important to characterize these changes with respect to CO2 fixation and bioconversion to methane.

Methanogens use molecular hydrogen (H2) anaerobically by transferring electrons from H2 to CO2 to form methane. As demonstrated in Figure 7E, Thermophilic Methanomethrobacter, Methanolinnea, and Methanoseta were predominant methanogens both in W and C samples. With comparison to W sample, the promotion in relative abundance of Methanolinnea (from 7.9 to 11.4%) and Methanoculleus (from undetectable to 10.0%) as well as the reduction in relative abundance of Methanoseta (from 33.3 to 21.4%) were observed, which implied that the injected CO2 influenced negatively on Methanoseta but positively on Methanoculleus and Methanolinnea. Considering that Methanomethrobacter, Methanolinnea, and Methanoculleus are known to be hydrogenotrophic methanogens, Methanoseta to aceticlastic methanogens, and Methanomethylovorans to methylotrophic methanogens, it is reasonable to conclude that injection of CO2 either increase or maintain the relative abundance of hydrogenotrophic methanogens, but it decreases that of aceticlastic methanogens and methylotrophic methanogens.

More interestingly, Methanoculleus was detected only in C sample. The genus has been found in different habitats including oil reservoir (Berdugo-Clavijo and Gieg, 2014), deep marine sediments (Mikucki et al., 2003), and swine manure storage tank (Barret et al., 2012, 2013). The occurrence of this genus in C sample implies that it may be related to CO2 injection driven high acetate concentration. This assumption is consistent with the fact that Methanoculleus spp. consume acetate while carrying out hydrogenotrophic methanogenesis and the growth of some Methanoculleus members requires acetate even though they do not convert it to methane (Mikucki et al., 2003; Barret et al., 2013, 2015). Also, Berdugo-Clavijo and Gieg found that the relative abundance of Methanoculleus decreased substantially with acetate (Berdugo-Clavijo and Gieg, 2014). In this study, the C-water is highly enriched in acetate relative to W, which one might normally assume favors aceticlastic methanogens. Based on the known properties of Methanoculleus spp., it seems that the acetate is favoring acetate assimilating methanogens.

Ribulose 1, 5-bisphosphate carboxylase (Rubisco, specifically, cbbL, cbbM) are usually used as a biomarker for the CBB CO2 fixation pathway (Campbell and Cary, 2004). Specifically, in subsurface environments, CO2 fixation is usually conducted by chemolithotrophs through the CBB pathway (Kellermann et al., 2012). As Figure 7A showed, the most dominant genus Phaseospirillum (67.4%) in W sample was not detected in C sample and the abundance of Thiobacillus and Rhodopseudomonas in C sample decreased notably while compared to W sample. In addition, the Halothiobacillus (undetected in W sample) appeared to be the most prevalent in C sample. Also, the relative percentage...
of *Leptothrix* in C sample increased compared to that in W sample. In the *cbbL* clones libraries, the abundance of *Rhodospirillum* increased in abundance from 36.7% in W sample to 42.5% in C sample, members of the genus *Hydrogenophaga* increased in abundance slightly in C sample compared to that in W sample, while those affiliated to genus *Cupriavidus* decreased from 10.0% in W sample to 6.5% in C sample (shown in Figure 7B). 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 hint at a significant potential for CO$_2$ fixation via the Calvin cycle within oil reservoir microbial communities.

Most acetogens are obligate anaerobic bacteria that use the reductive acetyl-CoA pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and cell carbon from CO$_2$. Formyltetrahydrofolate synthetase (*fthfs*) is used to detect aceticogenic, fermentative bacteria (Leaphart and Lovell, 2001). In the present work, notable changes were observed in the composition of *fthfs* communities (Figure 7C). The community dominated by microorganisms related to genera *Nisaea*, *Moorella*, and *Alkaliphilus* in W sample was changed completely to be dominated only by microorganisms related to genus *Acetobacterium* in C sample. The mechanism for the change of *Alkaliphilus* from dominance in sample W to undetectable in sample C is not very clear. Generally, this genus is known to be extremely alkaliphilic and thus would not be prone to survive in the acidic conditions caused by the injection of CO$_2$. Although the ability of acetate production on CO$_2$+H$_2$ by *Acetobacterium woodii* and *Moorella* were systematically studied (Ragsdale and Pierce, 2008; Demler and Weuster-Botz, 2011), surprisingly, *Moorella*-like microorganisms were not detected in C sample. This observation implies that *Acetobacterium*-like microbes are probably more suitable for acetogenesis in CO$_2$-injected oil reservoirs.

Hydrogen is an alternative energy source for autotrophic microbes in a variety of subsurface environments. When hydrogen and carbon dioxide are present, development of autotrophic microorganisms would be possible. For example, methanogens and acetogens may produce organic matter from hydrogen by means of respiring carbon dioxide. As it can be seen from our study (Figure 7D), the composition of [FeFe]-hydrogenase-encoding gene clones libraries at the genus level shows interesting differences in relative abundance between W and C samples. The microbes related to *Syntrophothermus* predominated in W sample (44.2%) disappeared in C sample, and *Desulfotomaculum* decreased from 30.8% in W sample to 28.6% in C sample. The relative abundance of members of genera *Clostridium*, *Thermotoga*, and *Ammonifex* increased from 5.8%, 1.9% and undetectable (0.0%) in W sample to 38.1%, 14.3% and 9.5% in C sample, respectively. Interestingly, all the three sulfate reducing bacteria were influenced very markedly as either decreased in relative abundance (*Desulfotomaculum*) or became undetectable in C sample (*Thermodesulfobium* and *Thermodesulfovibrio*). Meanwhile, those related to *Dehalococcoides* and *Cloacamonas* all rose in relative abundance from undetectable in W sample to 4.8% in C sample. Morozova et al. (2010) also showed that CO$_2$ injection caused a decrease in the diversity of microorganisms and revealed temporal out-competition of sulfate-reducing bacteria by methanogenic bacteria. Morozova's experiments showed that after CO$_2$ injection the SRB population declined until it was no longer detected while the archaeal population increased, which indicates that archaea may be able to adapt more readily to the more acidic conditions after CO$_2$ injection. Our results reached the same conclusion. But, Morozova found that after a 5 month period of exposure to CO$_2$, the SRB population returned in numbers greater than that prior to CO$_2$ injection. This phenomenon was not observed in our study at present. The reason for this was not quite clear although it was assumed to be resulted partly from the water–gas alternative injection and long-term exposure of CO$_2$ (about 5 years) in our study which were quite different from that in Morozova's experiments.

We found great differences in relative abundance among all the five functional gene clone libraries established from W and C samples, as showed in Figure 7. This phenomenon of previously undetectable and/or rare members of microbial communities becoming dominant after exposure to CO$_2$ has been reported previously (Gulliver and Gregory, 2011). Microorganisms with increasing abundance implies that they may be better withstanding or adapting to exposure to CO$_2$ and subsequent changes in physical and biochemical conditions resulted by CO$_2$ injection.

Analysis of functional genes shows that microbial communities were strongly influenced and the diversity reduced by CO$_2$ injection. For example, there were eight different genera in W sample whereas only six were retrieved from C sample for [FeFe]-hydrogenase-encoding gene library. Also, for *fthfs* library, three different genera were detected in W sample but only one was found in C sample. Our data agree with Gulliver and Gregory (2011) which showed that different families of bacteria presided with variation in CO$_2$ partial pressure. Knowledge of surviving and thriving microbial populations may help in better understanding of the fate of CO$_2$ following injection and to make better strategy for use of microorganisms in subsurface environments for improving the efficiency of injection and microbial fixation of CO$_2$, and hence ensuring the security for long-term CO$_2$ storage in subsurface petroleum reservoirs.

Primers used for *mcrA* amplification are divided into different groups: MCR, ME, ML, and these primers are able to amplify most methanogens. It has been reported that the ME-related primers are also able to amplify anaerobic methane-oxidizing archaea (ANME) (Nanrihiro and Sekiguchi, 2011). The primers used for *mcrA* amplification to target the methanogenic communities in the samples investigated in the present study were described by Luton et al. (2002) which belonged to the ML group. To the best of our knowledge, the ML group ability to amplify ANME's remains to be demonstrated.

Due to the fact that the CO$_2$ injected had been produced about 1 year before the collection of these samples when the ratio of gas (CO$_2$) to oil was between 22.8 and 145 m$^3$/m$^3$ in production wells, the changes in the relative abundance of five genes relevant to CO$_2$ utilization and methane production by microorganisms can be considered mainly attributed to CO$_2$ injection. The small size of the clone library and the number of clones sequenced would influence, to some extent, on the
analysis of microorganisms with low frequency. Nevertheless, the major functional microorganisms and their changes in relative abundance can still be recognized, even with certain biases, as demonstrated in the present study. The analysis of the changes in microbial community may be influenced by the following factors: (1) The samples were all collected from the sampling valve located at the wellhead of production well and hence, these samples may contain microbes from oil reservoir as well as that survived in oil tubes between the well bottoms to the sampling valve; (2) The sampling water may be produced both from oil-bearing layers or sub-layers with CO$_2$ production (CO$_2$-impacted water) and that with no CO$_2$ production even they received CO$_2$ (non CO$_2$-impacted water); (3) The retention time of CO$_2$ in oil reservoir is relatively short, i.e., while CO$_2$ was injected through injection wells into target oil reservoir, part of them would be produced afterwards from the production well about 250–300 m away from the injector; (4) CO$_2$ was injected into the target oil reservoir with water-CO$_2$ alternative injection manner.

For a more accurate characterization of microbial community and their changes caused by CO$_2$ injection in oil reservoir, the collection of produced water from only the CO$_2$-impacted zones, the qualitative and quantitative analysis of microbial community, the physicochemical changes of subsurface water such as pH, volatile acids over time, as well as the analysis of the origin of volatile acids (by isotopic analysis) and etc. are very important.

**Methane Formation Potential from Injected CO$_2$ in Oil Reservoirs**

Bioconversion of CO$_2$ into CH$_4$ *in situ* oil reservoirs by indigenous methanogens is an area of active research and development. Hydrogenotrophic methanogens need not only CO$_2$ but 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 the viewpoint of functional genes. In our study, both the functional genes of H$_2$-producing and CH$_4$-producing were detected in the CO$_2$-flooding oil reservoirs, and the water-flooding oil reservoirs as well. Furthermore, some H$_2$-producing microorganisms (e.g., Clostridium and Thermotoga) and hydrogenotrophic methanogens such as *Methanotrichobacter* and *Methanolinea* as well as *Methanoculleus* remained or evolving to be predominant after long term exposure to CO$_2$ in CO$_2$-flooding area compared to that in water-flooding area. Meanwhile, these H$_2$-producing bacteria and hydrogenotrophic methanogens were both identified in the 16S rRNA genes cloning libraries (data not shown in this paper). It is assumed that these hydrogenotrophic methanogens live in symbiosis 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.

The detection of CO$_2$ fixation potential is alternative evidence to autotrophic activity *in situ* oil reservoirs. Therefore, attentions should be further paid on the evaluation of the activities of those microorganisms in subsurface ecosystems with the potential of microbial fixation of CO$_2$ and its subsequent bioconversion into methane. Once those microorganisms are activated by means of nutrient injection and etc., taking into consideration of the tremendous capacity of CO$_2$ sequestration in oil and gas reservoir (totally about 9 × 10$^{11}$ tons in the world), it seems more reasonable to believe that the *in situ* fixation and reclamation of CO$_2$ sequestered in oil reservoir will play a notable role in mitigating atmospheric CO$_2$ building up as well as energy shortage.

**Conclusions**

Analysis of a suite of functional genes shows that a diverse microbial community with potential for fixation and conversion of CO$_2$ into methane inhabits oil reservoir. Microorganisms affiliated with members of the genera *Methanothrombacter* (hydrogenotrophic CO$_2$-reducing methanogens), *Acetobacterium* and *Halothiobacillus* as well as hydrogen producers (*Firmicutes*) seem to be more adaptable to CO$_2$ injection and present the potential for microbial fixation and bioconversion of CO$_2$ into methane in subsurface oil reservoirs. Due to the limitation of clone numbers and the co-production nature of CO$_2$-impacted and non-impacted water in the C sampling well, the impact of CO$_2$ injection on microbial community may be not fully characterized and presented in this study. Even so, the present results showing the response, to some extent, of microbial community on the CO$_2$ injection are of some help in predicting the fate of CO$_2$ following injection and making better strategies for use of microorganisms in subsurface environments for microbial CO$_2$ fixation and bioconversion of CO$_2$ into sustainable energy in subsurface oil reservoirs.

**Author Contributions**

This study was designed by JL and BM. XS and GY performed all the laboratory experiments. SM analyzed the functional genes data and constructed the phylogenetic trees of these functional genes. JG provided valuable suggestions in the design of the experiments and the preparation of the manuscript. The manuscript was written by JL, assisted by all co-authors. All authors reviewed the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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