

Microbial communities responsible for CO₂ fixation and bioconversion revealed by
using *mcrA*, *cbbM*, *cbbL*, *fthfs*, *feh*-hydrogenase genes as molecular
biomarkers in petroleum reservoirs **with** different **temperatures**
physiochemical properties

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Abstract

Sequestration of CO₂ in oil reservoir is one of the feasible options for mitigating atmospheric CO₂ building up. The *in situ* bioconversion of sequestered CO₂ to methane by microorganisms inhabiting oil reservoirs is feasible. To ~~evaluate~~acquire the ~~potential~~of knowledge of these specific functional microorganisms involved in *in situ* microbial fixation and conversion of CO₂ into CH₄ in oil reservoirs, a ~~comprehensive~~specific molecular survey was performed to reveal microbial communities inhabiting four different oil reservoirs ~~with different temperatures~~ by analysis of functional genes involved in the biochemical pathways of CO₂ fixation and CH₄ 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 ~~*Methanomicrobiales*~~*Methanomicrobiales*, *Methanobacteriales* and *Methanosarcinales* (*mcrA*). The predominant methanogens were all identified to be hydrogenotrophic CO₂-reducing physiological types. These results showed that functional microbial communities capable of ~~microbial~~—fixation and ~~bioconversion~~conversion of CO₂ into ~~methane~~CH₄ inhabit ~~widely~~extensively in the oil reservoirs investigated, which is helpful to microbial recycling of sequestered CO₂ to further new energy in oil reservoirs.

Keywords: Microbial community, CO₂ fixation, Bioconversion, Methanogenesis, Functional gene, Oil reservoir

1 Introduction

Storage of CO₂ in deep geological formations, such as oil reservoirs, is one of the feasible solutions to reduce CO₂ emissions and further build up in the atmosphere. It is of great scientific interest and significance to understand the fate of CO₂ 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 CO₂ into CH₄ are highly feasible.

As to the microbial involvement in CO₂ fixation, six autotrophic CO₂ fixation biochemical pathways have been documented, of which the Calvin–Benson–Bassham (CBB) cycle plays an important role in autotrophic CO₂ 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 CO₂ 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; ~~Vornholt~~ Vorholt 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; Li et al., 2011; Mbadinga et al., 2012) and they are expected to use effective means for CO₂ fixation and bioconversion.~~

~~To investigate whether oil~~Petroleum reservoirs ~~have the potential capability of~~ 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; Li et al., 2011; Mbadinga et al., 2012)
and they are expected to play an important role in CO₂ fixation and bioconversion. To acquire
a specific and systematic knowledge on microorganisms involved in CO₂ fixation and
conversion of CO₂ into CH₄, ~~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 CO₂ fixation and conversion into CH₄ 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 ~~of~~ ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I and II,
~~respectively~~, of the CBB cycle for CO₂ fixation have been applied to study microbial
communities from hydrothermal vents of the Logatchev field (Hugler et al., 2010). 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 CO₂. 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 anaerobic sludge (Ryan et al., 2008).
Methyl-coenzyme M reductase (*mcr*) is vital for CH₄ formation, and the α -subunit of MCR
(*mcrA* gene) is commonly used for the detection of specific groups of methanogens
(Juottonen et al., 2006). 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 H₂-CO₂ and acetate, which are converted into

methane by hydrogenotrophic and acetoclastic methanogens (Mayumi et al., 2011),
respectively. In addition, ~~H₂ should be supplied in the process of CO₂ bioconversion into CH₄.~~
~~However, H₂ hydrogen-producing bacteria are polyphyletic microorganisms~~ are also important
for CO₂ fixation and conversion. On one hand, those microorganisms use the rTCA cycle for
autotrophic CO₂ fixation (Schauder et al., 1987; Thauer et al., 1989). On the other hand,
Fermentative microbes producing H₂ have been postulated to form trophic links with
H₂-consuming methanogens, acetogens (i.e., organisms capable of using the acetyl-coenzyme
A pathway for acetate synthesis) (Drake et al., 2006). [Fe-Fe]-hydrogenases are known to
catalyze H₂ production in fermentative microorganisms. Thus, gene encoding for
[Fe-Fe]-hydrogenases represent a useful marker gene for the detection of H₂-producing
anaerobes (Schmidt et al., 2010).

Microbial fixation of CO₂ and conversion of CO₂ to CH₄ in oil reservoirs is becoming
increasingly recognized. Although the potential of *in situ* microbial conversion of sequestered
CO₂ into CH₄ by hydrogenotrophic methanogens has been observed in a laboratory test by
Sugai *et al* (2012) and some studies on the effects and feasibility of CO₂ 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 CO₂ into CH₄ in
different oil reservoirs is still very limited. ~~The objective of this~~ This study ~~was to~~
~~evaluate~~ focus on the ~~potential of~~ investigation of microorganisms involved in *in situ* microbial
fixation and conversion of CO₂ into CH₄ 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).~~ water-flooding oilfields in China, namely XinJing 6 and 7 Oilfields (XJ6 and XJ7, respectively), Baolige Oilfield (HB), and Daqing Oilfield (DQ). The DQ sample was collected in YSL block with a depth of about 1500 m and a temperature of 90 °C, which has been water-flooded for over 5 yrs. The density of the degassed crude oil and subsurface oil viscosity from YSL block was 0.8581 g/cm³ and 3.3 mPa s, respectively. The HB sample was collected from Ba51 block of Baolige oilfield with a depth of 1101 m and a temperature of 45 °C, which has been water-flooded since 2001. The density and viscosity of the degassed crude oil from Ba51 block was 0.9104 g/cm³ and 643 mPa s, respectively. The XJ6 and XJ7 samples were collected from Xinjiang 6 and 7 Oilfields, respectively. The Xinjiang 7 block with a depth of 1088 m and a temperature of 32 °C has been water-flooded for over 40 yrs. The Xinjiang 6 block with a depth of 480 m and a temperature of 21 °C has been water-flooded for over 30 yrs. The oil viscosity from Xinjiang 6 and 7 Oilfields were 417 and 44.8 mPa s, respectively. As mentioned above, these four sampled petroleum reservoirs, with a temperature range from 21 °C to 90 °C, salinity and pH of these production waters from 3900 to 15728 mg L⁻¹, and 6.0 to 7.2, respectively, are representative oil reservoirs in China. The characterization of the petroleum reservoirs water sampled are listed in Table 1.

Five liters of production water samples from each production oil well were collected directly from the production valve of the pipeline at 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/water phase was separated from the oil/water mixture, was used to concentrate by heating the samples to 50 °C and then phase separation with sterilized separatory funnels. The microbial cells directly through filtration (biomass in the water fraction was finally concentrated onto membrane filter (0.2-μm-pore-size)).

2.2 DNA extractions

Microbial biomass in the oil/water samples DNA extraction was concentrated onto membrane filters as conducted by a method described above and previously by Wang *et al.* (2012). Total Briefly, 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 & 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 Oliver-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 (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~~^{μg} ml⁻¹). The inserted DNAs were amplified by using M13-47 (5'-CGCCAGGGTTTCCCAGTCACGAC-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~~GenBank database using the BLAST 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 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.

2.6 Statistic analysis

Canonical Correspondence Analysis (CCA) was used to explore the potential relationship of the functional microbial community (based on relative abundance) and environmental variables (temperature, salinity and pH). Calculations were performed using the CCA function of the vegan package in R Statistics Program (<http://www.r-project.org/>) using temperature, salinity and pH data as environmental parameters. Due to the fact that anions and cations correlated with salinity and acetate with pH, these variables were not included in the CCA analysis.

2.7 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 CO₂ fixation

3.1 The diversity of *cbbL* and *cbbM* genes

The *cbbL* gene types were detected positively in all samples from the four oil reservoirs with different temperature as shown in Fig. 1; (phylogenetic tree) and Fig. 6(a) (relative abundance), implying that microorganisms inhabited different oilfieldsoil reservoirs

universally and could fix CO₂ 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~~BetaProteobacteria; the other OTU represented by *cbbL*-XJ6-32 (37.1% of the XJ6 *cbbL* clones) sharing 93% similarity with *Allochrodatum minutissimum*, a member of ~~Gamma-Proteobacteria~~GammaProteobacteria. 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~~BetaProteobacteria. 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~~AlphaProteobacteria, 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~~AlphaProteobacteria. 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~~GammaProteobacteria. One OTU represented by *cbbL*-DQ-34 (7.1%) showed the highest identity with *Rhodobacter capsulatus* of ~~Alpha-Proteobacteria~~AlphaProteobacteria. 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~~of AlphaProteobacteria. 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. ~~2,2~~ (phylogenetic tree) and Fig. 6(b) (relative abundance), 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*~~*AlphaProteobacteria* while 8 clones represented by *cbbM*-XJ6-37 showed highest similarity with *Thiobacillus thiophilus* of ~~*Beta-Proteobacteria*~~*BetaProteobacteria*. 7 clones represented by *cbbM*-XJ6-20 shared 90% similarity with *Acidithiobacillus ferrivorans*, a member of ~~*Gamma-Proteobacteria*~~*GammaProteobacteria*. 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*~~*AlphaProteobacteria*, 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*~~*AlphaProteobacteria* with 90% similarity. The OTU represented by *cbbM*-DQ-22 (20%) were closely related to *Magnetospira thiophila* (91%), ~~*Alpha-Proteobacteria*~~*AlphaProteobacteria*. 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~~Diversity of *fthfs* gene

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. ~~3-3~~3) and the relative abundance of these OTUs were presented in Fig. 6(c). 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~~AlphaProteobacteria. While sequences of other clones screened in XJ7 sample shared low similarity (<80%) 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~~AlphaProteobacteria. The *fthfs*-HB-24, 73.3% of the clones retrieved from HB sample, shared ~~less~~low similarity (<80%) 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 Diversity of [Fe-Fe]-Hydrogenase encoding gene

The [FeFe]-hydrogenase genes were detected in all four samples ~~(as shown in Fig. 4)-~~ (phylogenetic tree) and Fig. 6(d) (relative abundance) 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 OTU *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 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 Diversity of mcrA gene

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). As shown in Fig. 6(e), OTUs represented by *mcrA*-HB-73 (49.0%) and *mcrA*-DQ-92 (46.8%) were all closely related to *Methanolinea tarda*, a CO₂-reducing methanogens. OTU *mcrA*-XJ6-105 (31.1%) shared high identities with *mcrA* sequences from the *Methanoculleus bourgensis* MS2 which could generate CH₄ by reducing CO₂. OTU *mcrA*-XJ6-103 (22.2%) was closely related to *Methanocorpusculum labreanum*, also a member of CO₂-reducing 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 CH₄ through CO₂-reducing biochemical pathway. All these results show that the CO₂-reducing methanogens inhabit predominantly in these four different oil reservoirs with different temperaturesphysiochemical properties.

3.5 The relationship between functional microorganisms and environmental variables

The relationship between relative abundance of functional microorganisms and environmental variables (temperature, salinity and pH) was investigated using a multivariate statistical CCA (Fig. 7). As showed in Fig. 7, the first two CCA axes accounted for more than 80.8% of the explained variance which revealed the significance of temperature, salinity and pH in correlation with the functional microbial communities. On the basis of CCA, the microbial communities of *cbbL* and *FTHFS* were strongly influenced by both temperature and pH, the *cbbM* and *mcrA* communities mainly by salinity, and *[Fe-Fe]* dominantly by pH. Regarding *cbbL* community (Fig.7(a)), *Rhodospirillum* and *Acidithiobacillus* had positive relationships with temperature. *Thiobacillus* and *Allochromatium* had positive relationships with salinity. Fig.7(b) presented clearly the potential correlations between *cbbM* microbial community and environmental variables. *Rhodospirillum*, *Thiobacillus*, and *Acidithiobacillus*

had higher tolerance to high salinity and low temperature, but contrary for *Magnetospirillum*, *Lamprobacter*, *Phaeospirillum*, and *Magnetospira*. It could be seen from Fig.7(c) that *Hyphomonas* was positively related to temperature and negatively to pH. Most of the H_2 -producing microorganisms such as *Alistipes*, *Syntrophus*, *Clostridium*, and *Anaerophaga* were positively related to pH and negatively to salinity and temperature as showed in Fig.7(d). As for the *mcrA* community (Fig.7(e)), among the environmental factors, temperature was highly correlated with Axis 1, which explained 67.09% of the variation in this community. The *Methanosaeta*, *Methanoplanus*, *Methanobacterium* as well as *Methanothermobacter* were all positively correlated to temperature and *Methanospirillum*, *Methanocorpusculum*, and *Methanofollis* positively related to salinity.

4 Discussions

Microbial fixation of CO_2 and further conversion it into CH_4 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 specifically investigated the functional microorganisms for autotrophic CO_2 fixation and bioconversion 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*) and found that functional microbial communities capable of microbial fixation and bioconversion of CO_2 into methane inhabit extensively in the oil reservoirs investigated, which is helpful to microbial recycling of sequestered CO_2 to further new energy in oil reservoirs.

4.1 Distribution of microorganisms ~~based on functional genes~~ involved in CO_2 fixation and conversion

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 CO₂. ~~As shown in Fig. 6(a) with the increase of temperature of the sampling oil reservoirs, the predominant microorganisms with *cbbL* genes changed from *Beta-Proteobacteria* 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* (Fig. 6(a)). 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 CO₂ fixation operates in *Proteobacteria* belonging to the ~~*alpha*, *beta*~~*Alpha*, *Beta*, and ~~*gamma*~~*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 CO₂ 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*~~*Hyphomonas*, *Acetobacterium*, *Blastomonas*, *Arthrobacter* in DQ, XJ6, XJ7 and HB samples, respectively. The ~~acetogens~~*phylogenetically diverse nature of acetogens has been reported by (Drake et al., 2006). Acetogens* use the reductive acetyl-CoA pathway not only for CO₂ 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~~ Our results deduce that microorganisms

inhabiting the herein investigated oil reservoirs ~~have~~possess, from functional point of view, the ability to fix CO₂ as well as convert CO₂ into acetic acid.

~~As for~~Most of the hydrogen ~~production microorganisms, the diversity seems to become lower when the temperature increases from 21 °C and 32 °C in XJ6 and XJ7 sample to 90 °C in DQ samples. Most of these producing~~ 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~~*Alistipes*, *Clostridium* (4%), *Synthrophus* (2%) and *Cloacamonas* (2%) in XJ samples. Those microorganisms. We also found that sequences from microorganisms sharing similarities with those from the *Firmicutes*, *Delta-proteobacteria* and *Bacteroidetes* were also reported to use the rTCA cycle for autotrophic CO₂ fixationthe most encountered in clone libraries established for [FeFe]-hydrogenase gene. Our results are consistent with that obtained by Schmidt *et al* (Schauder *et al.*, 19872010; Thauer *et al.*, 1989)). Fermentative microbes producing H₂ have been postulated to form trophic links with H₂-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*),~~

The archaeal *mcrA* gene clone libraries were mostly related to the order of *Methanobacteriales*, *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~~ It has been proposed that syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis ~~is~~was 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*, a hydrogenotrophic thermophilic methanogen. 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 . This allows us to speculate that hydrogenotrophic methanogens will play an important role in CO_2 fixation and bioconversion of CO_2 into CH_4 in oil reservoir system.

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 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 CO₂ emission and also generation of energy. We explored the potential for autotrophic CO₂ fixation and bioconversion with microbial communities in oil reservoirs by detection of relative functional marker genes such as CO₂ fixation (*cbbM*, *cbbL*), acetogenesis (*ftf*), hydrogen formation (*[FeFe]* hydrogenase) and methanogenesis (*mcrA*).

Microbial fixation and conversion of CO₂ into CH₄ are usually implemented by chemolithoautotrophic microorganisms. Methanogens oxidize molecular hydrogen (H₂) anaerobically by transferring electrons from H₂ to CO₂ to form methane. As shown in Fig. 6(e), *Methanolinea* and *Methanoculleus* 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 hydrogenotrophic methanogens will play an important role in CO₂ fixation and bioconversion of CO₂ into CH₄ 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) CO₂ fixation pathway (Campbell and Cary, 2004). Specifically, in subsurface environments, CO₂ 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. 6 (a) 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 *Rhodopseudomonasin* 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 CO₂ 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 CO₂.~~ **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. 6(c)). 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 CO₂-injected oil reservoirs. As a matter of fact, the ability of acetate production on CO₂+H₂ by *Acetobacterium woodii* and *Moorella* had been systematically studied (Demler and Weuster-Botz, 2011; ~~Ragsdale and Pierce, 2008; 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 *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%.~~

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 H₂-dependent methylotrophic 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; Stupperich and Fuchs, 1984b; Shieh and Whitman, 1988; Stupperich and Fuchs, 1984a). However, to our knowledge, no reports have been found on that the genera *Methanomassiliicoccus* bears acetyl-CoA pathway of acetogens.

Great differences exist in relative abundance among all the five functional gene clone libraries established from ~~high and low temperature~~ these four samples (Fig 6). This phenomenon ~~of~~ was also observed by Wang, who studied the microbial ~~community change with temperature has been reported~~ communities inhabiting in different oil reservoirs (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 CO₂ to CH₄ and hence warrant more attentions.

Analysis of functional genes shows that microbial communities were strongly ~~influenced and related to~~ the ~~diversity generally reduced by the increase of temperature~~ physiochemical properties of the oil reservoir ~~sampled~~ investigated. For example, there were four different genera in XJ sample whereas only one retrieved from HB ~~ample~~ sample 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 CO₂ following injection and making better

strategies to make use of microorganisms in subsurface environments for improving the efficiency of injection and microbial fixation of CO₂, hence ensuring the long-term CO₂ storage in subsurface petroleum reservoirs.

~~The *fhfs*-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 H₂-dependent methylotrophic 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; Stupperich and Fuchs, 1984b; Shieh and Whitman, 1988; Stupperich and Fuchs, 1984a). However, to our knowledge, no reports are available on that the genera *Methanomassiliicoccus* bears acetyl-CoA pathway of acetogens.~~

The present results shows a general picture, to some extent, of the specific microbial community involved in CO₂ fixation and bioconversion in oil reservoirs with quite different physiochemical properties. For further evaluation of the potential of this process, quantitative PCR targeting these different marker genes are necessary to gain insight into the abundance of the different functional genes and of the corresponding microbial populations. Such quantitative data would provide a more suitable data set to estimate which CO₂-fixation pathways might be quantitatively relevant in the reservoirs. Also, due to the limitation of clone numbers, the microbial community might not be fully characterized and presented in this study. Even so, The present results are still of some help in predicting the fate of CO₂ following injection and making better strategies for use of microorganisms in subsurface environments for microbial CO₂ fixation and bioconversion of CO₂ into sustainable energy in subsurface oil reservoirs.

4.3 The ~~potential of methane formation~~ of CH₄ from ~~injected~~ CO₂

The fate of CO₂ in subsurface ecosystem is receiving increasing attentions. To date, the *in situ* conversion of CO₂ into CH₄ by methanogens inhabiting oil reservoirs universally is an area of active research and development. This conversion process ~~has, bearing~~ the potential of not only reducing CO₂ emission but also producing natural gas in reservoirs. ~~Hydrogenotrophie, is complemented mostly by hydrogenotrophic~~ methanogens which need both CO₂ and also H₂ to produce CH₄; ~~therefore, H₂ should be supplied to them in reservoirs for this process. It.~~ Fortunately, it has been reported that there are several kinds of microorganisms capable of producing H₂ by degrading crude oil in reservoir environments. The potential of the microbial conversion of CO₂ into CH₄ by enrichment culture experiments using microorganisms indigenous to oil reservoirs has been ~~studied~~demonstrated (Sugai et al., 2012). Different from that mentioned above, ~~we evaluated our work focus on the potential of this process from a viewpoint of functional genes.~~ specific microbial communities involved in the CO₂ fixation and conversion it into CH₄ in oil reservoir. In ~~our~~this study, both the functional genes of H₂-producing and CH₄-producing were detected in all the four sampled oil reservoirs ~~sampled regardless of the temperatures~~ with different physiochemical properties. Furthermore, some H₂-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 H₂-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 CO₂ into CH₄ in oil reservoirs. These results indicate, from a viewpoint of functional genes, that indigenous microbial conversion process of CO₂ into CH₄ has high potential.

5 Conclusion

Analysis of a suite of functional genes shows that a diverse microbial community with potential for CO₂ fixation and conversion of CO₂ into methane inhabit these oil reservoirs with temperature from 21 to 90 °C. Microorganisms affiliated with members of the genera *Methanocelleus* and ~~*methanolinea*~~*Methanolinea* (hydrogenotrophic CO₂-reducing methanogens), *Acetobacterium* and *Thiobacillus* and *Rhodospirillum* as well as hydrogen producers (*Firmicutes*) present the potential for microbial fixation and conversion of CO₂ 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 CO₂ fixation and bioconversion of CO₂ 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 CO₂ and its subsequent bioconversion into methane.

Author contribution

J.F.L., J.D.G. and B.Z.M made the full design of the experiments. S.X.B, S.M.M., and G.C.Y conducted the microbial analysis and S.Z.Y. performed the chemical analysis. J.F.L. prepared the manuscript with contributions from all co-authors.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 41273084) and the NSFC/RGC Joint Research Fund (No. 41161160560).

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

Parameter	Xinjiang J66	Xinjiang J77	Huabei <u>Baolige</u>	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)	15728.0	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~~Baolige, HB; and Daqing, DQ; ND, Not Detected.

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

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

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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).

Fig. 7: Canonical correspondence analysis(CCA) on relationship between environmental conditions (temperature, salinity and pH) and functional microbial community retrieved by marker genes of *cbbL* (a), *cbbM* (b), *fthfs* (c), *FeFe-Hydrogenase* gene (d) and *mcrA* (e). Straight arrows, the direction of increase of each variable; line lengths, strength on the

780 | microbial communities; Plus, species; Blue circle, sample; Percentages on each axis,
781 | variation in the samples.