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NSFC/RGC Joint Research Scheme
Joint Completion Report

*(Please attach a copy of the completion report submitted to the NSFC
by the Mainland researcher)*

Part A: The Project and Investigator(s)

1. Project Title

Biological Methanogenesis of Alkanes: Thermodynamics and Microbial Ecology

2. Investigator(s) and Academic Department/Units Involved

	Hong Kong Team	Mainland Team
Name of Principal Investigator <i>(with title)</i>	Ji-Dong Gu (顧繼東) Associate Professor	Bo-Zhong Mu (牟伯中) Professor
Post		
Unit / Department / Institution	Biological Sciences University of Hong Kong	Applied Chemistry East China University of Science and Technology
Co-investigator(s) <i>(with title)</i>	Jimmy J. Jiao (焦赳赳) Professor Earth Sciences, HKU	Wei-Dong Wang (汪卫东) Chief Research Engineer EOR Institute Shengli Oilfield, SINOPEC, China

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval <i>(must be quoted)</i>
Project Start date	01/01/2012	N/A	
Project Completion date	31/12/2014	N/A	
Duration <i>(in month)</i>	36	N/A	

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. Characterize the microbial community composition of the alkanes-degrading methanogenic enrichment cultures derived from petroleum reservoirs;
2. Determine the initial activation step involved in converting alkanes to methane using a suite of molecular techniques; and
3. Identification of the key biochemical methanogenic pathway in different petroleum reservoirs and optimization of alkanes methanogenesis based on thermodynamics basis.

5.2 Revised Objectives

Date of approval from the RGC: N/A

Reasons for the change: N/A

6. Research Outcome

Major findings and research outcome

(maximum 1 page; please make reference to Part C where necessary)

Petroleum hydrocarbon degradation in oil reservoirs *in situ* plays an essential role in geobiochemical process contributing to oil degradation and methane production, and it has attracted increasing attention from both scientific and industrial communities due to the promising application of this process in energy recovery via bio-conversion of residual crude oil trapped in porous media of depleted oil reservoirs into methane, a clean energy of natural gas. Microbial community of production water of oil fields was analyzed using pyrosequencing technique available to find out the community composition. The diversity of the bacterial community in the aqueous phase was lower than that of the oil phase, a first time report on this evidence. Phylogenetic analysis indicated that the vast majority of them were members of the genus *Arcobacter* spp. within the *Epsilonproteobacteria*, capable of degrading the intermediates of the hydrocarbon degradation such as acetate. The oil phase of reservoirs fluid was dominated by the members of the genus *Pseudomonas* spp. within the *Gammaproteobacteria* and the genus *Sphingomonas* spp. within *Alphaproteobacteria*, which have ability to degrade crude oil through adherence to hydrocarbons both under aerobic and anaerobic conditions. Many known anaerobic crude oil degraders were also found in the oil phase of reservoir fluids mainly in the reservoir with lower water cut, represented by the genus *Desulfovibrio* spp., *Thermodesulfovibrio* spp., *Thermodesulfurhabdus* spp., *Thermotoga* spp. and *Thermoanaerobacterium* spp..

Several biochemical pathways involved in hydrocarbon anaerobic biodegradation and methane production by methanogenic microbial consortia were proposed previously, but the knowledge about the mechanism of hydrocarbon anaerobic biodegradation in oil reservoirs is still limited. This project studied hydrocarbon anaerobic biodegradation pathways by focusing on the initial activation by addition of fumarate to alkanes as one of the mechanisms. Because the potential degradation intermediate chemicals are not available commercially, 5 representative biomarker molecules in alkane anaerobic biodegradation were synthesized chemically in the laboratory and the identification fingerprints were collected for detecting them successfully in 32 production water samples from 3 different oilfields in China. Alkylsuccinates of parent alkanes from C₁ to C₈, 2-benzylsuccinate and naphthoic acid were detected, and the downstream metabolites such as alkylmalonates and 5,6,7,8-tetrahydro-naphthoate was also confirmed in these samples using Gas Chromatography-Mass Spectrometry (GC-MS), which suggested fumarate addition is a primary activation reaction for anaerobic degradation in both alkanes and also aromatics. Meanwhile, the key functional genes for encoding alkylsuccinate synthase (*assA*) and benzylsuccinate synthase (*bssA*) were also detected positive in these samples, which confirmed the presence of an anaerobic biodegradation pathway through the initial activation by addition of fumarate to hydrocarbons. Furthermore, alkane-degrading enrichment cultures from production water amended with C₁₅-C₂₀ alkanes and incubated at 55° under methanogenic condition were established and analyzed. Phylogenetic analysis of 16S rRNA gene sequences retrieved from the enrichment cultures showed that *Firmicutes* (*Gelria* and *Moorella*) and CO₂ reducing methanogens were dominant after incubation at 55° under methanogenic condition; and *assA* and *mcrA* were also detected in the enrichment cultures. In summary, our studies over the last 3-years revealed collectively that oil reservoirs inhabit abundant microbial population capable of hydrocarbon degradation and methane production, and the fumarate addition and methanogenesis were involved in the process with a dominant methanogenesis of CO₂ reducing

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(in particular, at a high temperature oil reservoir) or syntrophic acetate-oxidation route, which is fundamental for the *in situ* bio-conversion of hydrocarbons into natural gas (methane) in petroleum reservoirs.

Potential for further development of the research and the proposed course of action (*maximum half a page*)

Based on the results obtained from this project, a continuation of the research project on petroleum reservoir microbiology can be carried out by focusing more specifically on the degradation mechanisms, biochemical processes, genes and microorganisms involved in anaerobic (sulfate-reducing and methanogenic) degradation of hydrocarbons including selective linear and branched alkanes, aromatics and polyaromatics to further advance the knowledge on this subject. The significance of this research is for recovery of energy from residual oil in reservoirs to meet the energy demand by our society.

The specific information of significance and importance are:

1. Enrichment of microbial consortia for anaerobic degradation of selective hydrocarbons (linear and branch alkanes, aromatics and polyaromatics) to methane;
2. Detection of the degradation intermediates and initial activation biochemical step in degradation of hydrocarbons in the enrichment cultures, production water samples and the inocula chosen;
3. Synthesize the target intermediates in laboratory to establish the fingerprinting of the signature intermediate compounds and detect them in the production water samples, enrichments and also environment samples; and
4. Analysis of the whole community in the enrichment cultures and also the inoculum samples for enrichments, and also quantify the key functional genes involved in the initial activation of hydrocarbon substrates so to establish the relationship between intermediates detected, intermediate products formed, the expression of the functional genes encoding for specific biochemical reaction enzymes, and also the key dominant microorganisms in the community for proper function in producing methane at high rate.

7. The Layman's Summary

(describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Petroleum reservoirs contain at least 30-50% of the initial oil when they are being closed down from production. This fraction of the oil in the forms of hydrocarbons (alkanes, aromatics) has high viscosity and is trapped in the porous sandstone of the oil reservoir system. Since physical and chemical methods are too energy intensive and involve recalcitrant chemicals, biological conversion process may find an acceptable solution to recover this fraction of the energy for our society needs. Because oil reservoirs are underground and anaerobic, the availability of an active microbial population there is the first question to address, followed by their biochemical capability and the specific reaction to activate the oil chemical components. We analyzed the microbial community composition in the production waters, which went down into underground, traveled through the porous reservoirs and then came to above ground, to understand the microbial population in them. There are plenty of microorganisms including those capable of hydrocarbon degradation in the production water samples from different oil fields of China. Such microorganisms can be concentrated in simulated laboratory conditions to produce methane gas from addition of specific hydrocarbons, e.g., alkanes in this study. The key initial reaction in the degradation of hydrocarbons to methane is addition of fumarate to the substrate alkanes and. As a result, both the transformation

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intermediates and also the gene were detected in both production water samples also enrichments, confirming the initial reaction involved fumarate. This information is important because further understanding and manipulation of this reaction may accelerate the initial reaction in transformation and then the methane yield for energy recovery.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project
(Please attach a copy of each publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) <i>(bold the authors belonging to the project teams and denote the corresponding author with an asterisk*)</i>	Title and Journal/Book <i>(with the volume, pages and other necessary publishing details specified)</i>	Submitted to RGC <i>(indicate the year ending of the relevant progress report)</i>	Attached to this report <i>(Yes or No)</i>	Acknowledged the support of this Joint Research Scheme <i>(Yes or No)</i>
Year of publication	Year of Acceptance <i>(For paper accepted but not yet published)</i>	Under Review	Under Preparation <i>(optional)</i>					
2015		√		Bian XY, Mbadinga SM, Liu YF, Yang SZ, Liu JF, Ye RQ, Gu J-D* & Mu BZ*	Insights into the anaerobic biodegradation pathway of n-alkanes in oil reservoirs by detection of signature metabolites. <i>Scientific Reports</i>	N	N	Y
2015				Zhou, Z., J. Chen, H. Cao, P. Han, J.-D. Gu*	Comparison of communities of both methane-producing and metabolizing archaea and bacteria in sediments between the northern South China Sea and coastal Mai Po Nature Reserve revealed by PCR amplification of <i>mcrA</i> and <i>pmoA</i> genes. <i>Frontiers in Microbiology</i> 5: 789. DOI: 10.3389/fmicb.2014.00789	N	Y	Y

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2014				Bian XY, Mbadinga S M, Yang SZ, Gu J-D, Ye RQ & Mu BZ*	Synthesis of anaerobic degradation biomarkers alkyl-, aryl- and cycloalkylsuccinic acids and their mass spectral characteristics, <i>Eur. J. Mass Spectrom</i>	N	N	Y
2014				Wang LY, Sun XB, Liu JF, Gu J-D, Mu BZ*	Comparison of bacterial community in aqueous and oil phases of the water-flooded petroleum reservoir using Pyrosequencing and clone library approaches. <i>Appl Microbiol and Biotech</i>	N	Y	Y
2014				Guan J, Zhang BL, Mbadinga S M, Liu JF, Gu J-D, Mu BZ	Functional genes (dsr) approach reveals similar sulphidogenic prokaryotes diversity but different structure in saline waters from corroding high temperature petroleum reservoirs. <i>Appl Microbiol and Biotech</i>	N	N	Y
2013				Zhou F, Mbadinga SM, Liu JF, Gu J-D, Mu BZ	Evaluation of microbial community composition in thermophilic methane-producing incubation of production water from a high-temperature oil reservoir. <i>Environ Technol</i>	N	N	Y
2012				Wang LY, Duan RY Liu JF Yang SZ, Gu J-D & Mu BZ	Molecular analysis of the microbial community structures in water-flooding petroleum reservoirs with different temperatures. <i>Biogeosciences</i>	N	N	Y

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2012				Li W, Wang LY, Duan RY, Liu JF, Gu J-D & Mu BZ	Microbial community composition in n-alkanes-amended enrichment cultures of nitrate-reducing, sulfate-reducing and methanogenic conditions from production water of a mesophilic petroleum reservoir. <i>Intern Biodet Biodeg</i>	N	N	Y
2012				Zhou L, Li KP, Mbadanga S M, Yang SZ, Gu J-D, Mu BZ	Analyses of n-alkanes-degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques. <i>Ecotoxicology</i>	N	N	Y

9. Recognized International conference(s) in which paper(s) related to this research project was/were delivered (Please attach a copy of each delivered paper)

Month/Year/Place	Title	Conference Name	Submitted to RGC (indicate the year ending of the relevant progress report)	Attached to this report (Yes or No)	Acknowledged the support of this Joint Research Scheme (Yes or No)
10/2014/Heifei, PR China	Role of Microbial Community in Subsurface Petroleum Reservoirs: The Potential Application in Microbial Enhanced Energy Recovery	2014 China-US Joint Annual Symposium, "Water, Energy, and Ecosystem Sustainable Development	N	N	Y
08/2013/Rio Digenero, Brazil	Comparative analysis of microbial communities in water samples from oil fields ever subjected to CO ₂ -flooding and thoroughly water-flooding using 16S rRNA gene	International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS-4)	N	N	Y

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08/2013/Rio Digenero, Brazil	Detection of Signature Biomarkers Implicated in Anaerobic Degradation of Hydrocarbons in Petroleum Reservoirs via Mass Spectral Characteristics of Chemically Synthesized Alkylsuccinates	International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS-4)	N	N	Y
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10. Student(s) trained *(Please attach a copy of the title page of the thesis.)*

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Zhichao Zhou	PhD	09/2012	08/2016

Two post-doctoral fellows (Dr. Li-Ying Wang and Dr. Meng Li) were working on this project.

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

During execution and implementation of this joint research project in the past 3 years, the principle investigators and group members of both parties have made very close research collaboration and exchanges of personnel, laboratory results, research progress and new finding, and a total of 9 exchange visits was made. Members from the Mainland China laboratory had been recruited to the Hong Kong laboratory for further advanced research and such activity is still active and on-going even though the current projection has come to a natural maturation.

This project has also promoted our active involvement in international collaborations with the research institutes including the Institute for Energy and the Environment at the University of Oklahoma in US, and the Biofilm Centre at the University of Duisburg-Essen in Germany. Currently, the 3 parties are working actively in joint research for much higher impact in science.

Over the period of this joint research project for 3-year, in addition to presentations made at international conferences, research papers published in international refereed journals, graduate students and post-doctoral research fellows were trained through the financial support of this project.